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The relative potencies of heat-killed and acetone-killed vaccines against *Salmonella typhimurium* in mice

By SUJATHA CRONLY-DILLON*
Department of Bacteriology,
University of Edinburgh

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SUMMARY

Mice were immunized against *Salmonella typhimurium* with graded doses of heat-killed (HK) and acetone-killed (AK) vaccines and then challenged by the oral or intraperitoneal routes with two doses of *S. typhimurium*. HK and AK vaccines gave good protection against an intraperitoneal challenge, but failed to protect against an oral challenge which is presumably the natural mode of infection. HK vaccine was as potent as AK vaccine in reducing the mortality rate among mice challenged by the intraperitoneal route but, unlike HK vaccine, AK vaccine was also able to reduce the infectivity rate. With a small intraperitoneal challenge dose it was observed that a gradual increase in vaccine dose is associated with a corresponding fall in mortality rate, but with a larger challenge dose an increase in vaccine dose was associated with a corresponding increase in mortality rates. It was concluded that the protective potency of this type of vaccine may partly depend upon the total amount of antigen in the animal, i.e. including both the vaccine and the challenge organisms, at a critical time after challenge.

INTRODUCTION

'Mouse typhoid' or *Salmonella typhimurium* infection of mice has many interesting parallels in common with enteric fever in man; for example, the best form of protection against the experimental disease in mice appears to be that conferred by recovery from a virulent attack or prior infection with an avirulent strain of the causative organism (Topley, Wilson & Lewis, 1925; Hobson, 1957*a*, *b*). It is extremely difficult to immunize mice effectively against the disease, although many killed vaccines have been reported that have been moderately successful in protecting against an intraperitoneal challenge (Topley, Greenwood & Wilson, 1931; Hobson, 1957*a*). Raistrick & Topley (1934) reported that an acetone-killed vaccine was able to confer better protection against an intraperitoneal challenge than a heat-killed vaccine. MacLeod (1954) demonstrated that a heat-killed vaccine capable of conferring good protection against an intraperitoneal challenge was unable to protect mice against a challenge by the oral or natural route of infection. The present investigation compares the protective potencies of graded doses of heat-killed (HK) and acetone-killed (AK) organisms against challenge by the oral or the intraperitoneal routes.

* Present address: Department of Bacteriology and Virology, University of Manchester.

MATERIALS AND METHODS

Animals

Male Swiss white mice weighing 19–22 g. were used.

Bacterial cultures

Salmonella typhimurium strain 1566, phage type 1*a*/u57, derived from freeze-dried stock was used for preparing vaccines and for challenge.

Preparation of vaccines

Heat-killed phenol-preserved vaccine and acetone-killed freeze-dried vaccine were prepared as described in an earlier paper (Cronly-Dillon, 1972). These vaccines were administered in a volume of 0·1 ml. as a single subcutaneous injection at the root of the tail.

The challenge organisms were suspended in 0·1 M phosphate buffer at pH 8 and the actual dose administered in each case was determined by a viable count done by the method of Miles & Misra (1938). The challenge inoculum was administered in a volume of 0·1 ml. by the intraperitoneal route or by the oral route.

Design of experiments

Mice were prepared for oral challenge by starving overnight to ensure a speedy passage of the inoculum to the intestines. The infecting dose was administered by a narrow polythene cannula (0·5 mm. bore) passed into the stomach of the animal.

The protective potencies of HK and AK vaccines were assessed by challenging groups of immunized mice in parallel by the oral route or the intraperitoneal route. A batch of 280 mice was used for the oral challenge experiments and a similar number for the interaperitoneal challenge work.

In each batch of animals a group of 120 mice were immunized with HK vaccine and a similar number with AK vaccine. The 40 non-immunized mice remained as controls. Among the two vaccinated groups, sets of 40 mice each were given approximately 10^3, 10^5 and 10^7 vaccine organisms as a single subcutaneous injection. All the animals were challenged on the 15th day after vaccination. Twenty mice in each set of 40 animals were challenged with a small dose of *S. typhimurium* strain 1566 and the remaining mice were challenged with a large dose of organisms. For the intraperitoneal challenge, the small dose consisted of 20 organisms and the large dose was 2×10^4 organisms. The doses used for oral challenge were 3×10^4 and 3×10^7 organisms per mouse.

After challenge mice were housed in threes in cages that were cleaned out frequently, and the animals were transferred to freshly sterilized cages during the course of the experiment to reduce the chances of cross and auto-infection.

The animals were examined daily, deaths were recorded and an immediate necropsy was performed. The liver and spleen of each dead animal was cultured to test for the presence of *S. typhimurium*. On the 28th day after challenge, all surviving animals were killed and examined *post mortem* for bacteriological evidence of infection. The percentage mortality, infectivity and mean time to death of those mice that died were then calculated for each group.

Table 1. *Assessment of the protective potencies of heat-killed and acetone-killed vaccines against an intraperitoneal challenge with* S. typhimurium *strain* 1566 *over a period of* 28 *days after challenge*

Subcutaneous immunization						
Vaccine treatment	Dose	Challenge dose	No. of mice	Mortality rate (%)	Infectivity rate (%)	Mean survival time of mice that died (days)
Heat killed	10^3	20	20	20	90	14
		2×10^4	20	53*	100	13
	10^5	20	20	5*	90	15
		2×10^4	20	70*	100	13
	10^7	20	20	0*	100	...
		2×10^4	20	100	100	10
Control		20	20	42	100	15
		2×10^4	20	100	100	9
Acetone killed	10^3	20	20	25	65*	24
		2×10^4	20	50*	100	12
	10^5	20	20	15	90	16
		2×10^4	20	80	100	13
	10^7	20	20	0*	50*	...
		2×10^4	20	40*	100	13

* Indicates that the result was statistically significant when compared with observations on non-immunized controls (χ^2 test of probability).

Statistical analysis

The results were analysed by the χ^2 test of probability incorporating a formula that makes allowance for small numbers.

$$\chi^2 = \frac{\{ad - bc - \frac{1}{2}(a+b+c+d)\}^2(a+b+c+d)}{(a+b)\,(c+d)\,(a+c)\,(b+d)}.$$

When the value for P was 0·05 or less, the result was considered as unlikely to be due to chance variation.

RESULTS

Assessment of protection against intraperitoneal challenge

The results of this study are given in Table 1. When unimmunized control mice were challenged with the test strain of *S. typhimurium* given intraperitoneally in two different doses (20 and 2×10^4 organisms per mouse) the mortality rates were 42 % and 100 % respectively, the mean time to death of the fatal cases was 15 and 9 days, and the infectivity rate was 100 % in each group.

Heat-killed vaccine

Mice that had been immunized with certain doses of HK vaccine showed a significantly reduced mortality rate, but the infectivity rates were not greatly reduced. The mean survival time did not appear to be related to the mortality rate. With a low challenge of 20 organisms per mouse statistically significant

Table 2. *The relationship between heat-killed vaccine dose, intraperitoneal challenge dose, and observed mortality in groups of Swiss white mice observed for* 28 *days after challenge*

Intraperitoneal challenge dose (no. of *S. typhimurium* organisms)	Vaccine dose (no. of HK organisms per mouse)	Mortality rate (%)
20	10^3	20
	10^5	5
	10^7	0
2×10^4	10^3	53
	10^5	70
	10^7	100

protection (as measured by a reduction in mortality) was observed in those mice vaccinated with 10^5 and 10^7 HK vaccine organisms. When a larger intraperitoneal challenge of 2×10^4 *S. typhimurium* per mouse was given, statistically significant protection was observed in animals vaccinated with 10^3 and 10^5 HK vaccine organisms.

It is of interest that, with a low challenge dose of 20 *S. typhimurium* per mouse, a graded increase of the vaccine dose from 10^3 to 10^5 to 10^7 organisms was associated with a corresponding fall in the values for percentage mortality (see Table 2) whereas, with a large intraperitoneal challenge dose of 10^4 *S. typhimurium* per mouse, a gradual increase in vaccine dose is related to a corresponding increase in mortality.

Acetone-killed vaccine

Vaccination with certain doses of AK vaccine conferred significant protection as measured in terms of reduced mortality and infectivity. Although mean survival times were prolonged in all the vaccinated animals they did not provide a good index of the degree of protection. With a small intraperitoneal challenge of 20 *S. typhimurium* it was seen that mice vaccinated with 10^7 AK vaccine organisms showed a statistically significant reduction in mortality and infectivity rates. Vaccination with 10^3 AK vaccine organisms significantly reduced the infectivity rate, and prolonged the mean survival time of those mice that died, but the mortality rate was not reduced to a significant degree. When the intraperitoneal challenge dose was 2×10^4 *S. typhimurium*, those mice that had been vaccinated with 10^3 or 10^7 AK vaccine organisms showed a statistically significant reduction in mortality rates.

Here again, it was seen that in those mice challenged intraperitoneally with 20 *S. typhimurium*, a graded increase in vaccine dose from 10^3 to 10^5 to 10^7 was associated with a graded fall in mortality rates. When the mice were challenged with an intraperitoneal dose of 10^4 organisms an increase in vaccine dose from 10^3 to 10^5 was associated with an increase in mortality from 50 to 80 %, but a vaccine dose of 10^7 organisms was associated with a subsequent mortality of only 40 % (see Table 3).

Table 3. *The relationship between acetone-killed vaccine dose, intraperitoneal challenge dose, and observed mortality in groups of Swiss white mice observed for a period of* 28 *days after challenge*

Intraperitoneal challenge dose (no. of *S. typhimurium* organisms)	Vaccine dose (no. of AK organisms per mouse)	Mortality rate (%)
20	10^3	25
	10^5	15
	10^7	0
2×10^4	10^3	50
	10^5	80
	10^7	40

Table 4. *Assessment of the protective potencies of heat-killed and acetone-killed vaccines against an oral challenge with* S. typhimurium *strain* 1566 *over a period of* 28 *days after challenge*

Subcutaneous immunization						
Vaccine treatment	Dose	Challenge dose	No. of mice	Mortality rate (%)	Infectivity rate (%)	Mean survival time of mice that died (days)
Heat killed	10^3	3×10^4	20	20	35	20
		3×10^7	20	35	95	18
	10^5	3×10^4	20	40	80	18
		3×10^7	20	40	85	16
	10^7	3×10^4	20	16	63	15
		3×10^7	20	50	100	16
Control		3×10^4	20	21	32	13
		3×10^7	20	45	100	15
Acetone killed	10^3	3×10^4	20	17	50	15
		3×10^7	20	42	95	19
	10^5	3×10^4	20	28	44	19
		3×10^7	20	28	83	15
	10^7	3×10^4	20	7	20	23
		3×10^7	20	24	71	14

Assessment of protection against oral challenge

The results of these studies are summarized in Table 4. In unvaccinated control mice, oral challenge with two doses of *S. typhimurium* (3×10^4 and 3×10^7 organisms per mouse) produced mortality rates of 21 and 45 % respectively, infectivity rates of 32 and 100 %, and the mean times to death for those mice that died were 13 and 15 days respectively.

The results of oral challenges (with doses of 3×10^4 and 3×10^7 organisms per mouse) in groups of mice immunized with different doses of HK or AK vaccine show that no significant protection (measured in terms of reduced mortality or infectivity) was conferred. It was again evident that survival times did not correspond with lowered mortality rates.

DISCUSSION

The results of the present study confirm the earlier observation (MacLeod, 1954) that a vaccine which is potent in reducing the mortality of mice against an intraperitoneal challenge may fail to confer any protection against an oral challenge. Mice vaccinated with whole heat-killed or acetone-killed vaccines and challenged intraperitoneally showed significant protection as measured in terms of reduced mortality, as well as prolonged survival time of those mice that died. However, when the challenge was given by the oral route no protection was observed. Resistance to infection by an artificial route is thus not a reliable measure of resistance to infection by the natural route. This observation has already been reported in an earlier paper (Cronly-Dillon, 1972), which also described the preparation of a potent vaccine against oral challenge, viz. Mickle disintegrated HK organisms.

It is interesting that graded doses of whole HK and AK vaccines followed by a small intraperitoneal challenge (20 *S. typhimurium*) produced a gradual fall in mortality rate – i.e. the protection afforded improved gradually with increasing vaccine doses. It appears, in this case, that up to a certain extent an increase in the vaccine dose improved the degree of protection conferred. However, when a larger intraperitoneal challenge dose was used (10^4 *S. typhimurium*) the mortality rates rose in a step-like manner as the vaccine dose was increased gradually from 10^3 to 10^5 to 10^7 organisms per mouse – i.e. the protection afforded decreased with an increasing vaccine dose. With both challenge doses there was a very obvious gradient in the response observed. In the case of AK vaccine, however, mice that received a large dose of vaccine (10^7 AK organisms) and a large intraperitoneal challenge dose (10^4 *S. typhimurium*) showed a significantly lowered mortality rate. This result did not correspond with that obtained with the HK vaccine. The salient feature was the paradoxical result that a small vaccine dose of 10^3 HK or AK organisms per mouse failed to protect the group of mice challenged intraperitoneally with a small dose of *S. typhimurium* (20 organisms per mouse) but offered potent protection against a larger intraperitoneal dose (10^4 organisms per mouse). It is possible that the important factor in protection here is the total amount of antigenic material in the animal, i.e. including both the vaccine and the challenge organisms; it may be that the protective mechanisms are triggered when the critical level of antigen is reached. It is reasonable to assume that beyond a certain critical level there is depression of the response, since 10^7 HK vaccine organisms failed to protect against an intraperitoneal challenge with 10^4 organisms. It would be interesting to study the effects of some higher doses of vaccine organisms since it is possible that there may be a further phase of stimulation following the depression. The effect of 10^7 AK vaccine organisms against 10^4 intraperitoneal challenge organisms may very well point towards this explanation.

It is well known that endotoxin acts on the reticuloendothelial system causing first a depression in activity followed by a phase of increased activity associated with enhanced non-specific resistance to infection. However, in the mouse this phase of immunity is only observed when the challenge organism is one to which

the host is partially resistant or when the organism is not a facultative intracellular parasite of the host phagocytes (Rowley, 1963). Hence, it would appear that endotoxin-induced immunity is unlikely to play any major part in the response of vaccinated mice challenged with *S. typhimurium* which is a facultative intracellular parasite for this animal.

Since both whole HK and AK vaccines produced fairly similar significant reductions in mortality rate against intraperitoneal challenge, it is not possible to say which was superior in this respect. However, AK vaccine reduced the infectivity rates significantly when the animals were challenged intraperitoneally, and the mean time to death of those mice that died was also prolonged in this group. This may explain to some extent the earlier report of Raistrick & Topley (1934) that AK vaccine was superior to the heat-killed form against challenge with virulent *S. typhimurium*.

No protection was conferred against oral challenge by vaccinating mice with whole HK or AK vaccines. Although survival times were prolonged in some groups this was not considered to be a reliable index of protection unless associated with a reduction in mortality or infectivity rates, or both. It should be mentioned that the author has been able to prepare a vaccine, by Mickle disintegration of HK organisms, that was able to confer statistically significant protection by reducing the mortality rate against an oral challenge.

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Colonization resistance of the digestive tract of mice during systemic antibiotic treatment

By D. van der Waaij, J. M. Berghuis and J. E. C. Lekkerkerk
Radiobiological Institute TNO, Lange Kleiweg 151, *Rijswijk (Z.H.)*
The Netherlands

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SUMMARY

During systemic treatment of mice with ampicillin or streptomycin, oral contaminations with exogenous bacterial species resulted in an abnormal colonization pattern. The contaminants persisted much longer and in much higher concentrations in the caecum of systemically treated mice than in control animals. Spread of the contaminant into the mesenteric lymph nodes and the spleen was found much more often in the antibiotic treated group. This, however, was only seen when the contaminant was 'resistant' to the antibiotic injected. The experiments suggest that the 'CR-inducing species' of the microflora live in close contact with the mucosa and therefore could be identical with the anaerobic tapered rods described by Savage & Dubos (1968).

INTRODUCTION

Previously, the occurrence of colonization after oral contamination with various doses of three different Gram-negative bacterial species in mice was investigated (Van der Waaij, De Vries & Lekkerkerk, 1971). Much higher oral contamination doses were required to achieve colonization for 2 weeks or longer in animals with a conventional microflora than in those whose microflora had been altered by prolonged oral antibiotic treatment. This phenomenon, called colonization resistance (CR), was found to depend largely on an anaerobic part of the intestinal microflora. The CR of the digestive tract was expressed as the log of that oral dose which resulted in a 'take' persisting for 2 weeks in 50 % of the animals.

An increased concentration of the contaminant in the intestines was found during the first days after contamination (Van der Waaij, De Vries & Lekkerkerk, 1972). This occurred only when the contamination dose had been at or above the CR-determining dose. During this initial colonization phase (ICP) spread of the contaminant into the mesenteric lymph nodes was found in a certain percentage of the mice. In orally antibiotic-treated animals, the effects found during the ICP in conventional animals after high oral contamination doses, persisted throughout the observation period of 2 weeks.

In the present report, the influence of systemic antibiotic treatment on the CR and therefore on the pattern of the ICP following oral contamination, was investigated in mice. This effect was studied with an *Escherichia coli* strain and with a strain of enterococcus. Two broad spectrum antibiotics belonging to different

groups (penicillins and oligosaccharides) were selected for this study: Ampicillin was to some extent excreted with the bile into the intestines and streptomycin was largely excreted by the kidneys.

MATERIALS AND METHODS

Mice

Conventional random bred ND2 female mice of 9–12 weeks of age and 25–35 g. weight were used.

Housing conditions

The animals were caged separately in polycarbonate autoclavable cages. They were maintained on wire mesh above filter paper to minimize coprophagy. Acidified water (pH = 3) and sterilized food pellets were supplied *ad libitum*.

Oral contamination

Doses of 10^8 and 10^{10} bacteria of the same streptomycin resistant (S.R.)-*E. coli* strain (CR = 7) or with the same levels of a SR-enterococcus strain (CR = 8), were administered in the manner described in a previous paper (Van der Waaij *et al.* 1971). The SR-*E. coli* strain was not only resistant to streptomycin but also to ampicillin. The SR-enterococci were sensitive to 10 μg./ml. ampicillin.

Collection of samples

During the first 4 days after contamination in mice which received 10^8 organisms and at days 6, 8 and 10 in those which received 10^{10} organisms, five mice were killed in each of the three experimental groups (see later). The mesenteric lymph nodes, the spleen and the caecum contents were collected under aseptic conditions and processed in the same way, as previously described (Van der Waaij *et al.* 1972).

Antibiotic treatment

Before antibiotic treatment, 90 mice were randomly distributed over three groups. The first group was treated twice a day with 5 mg of streptomycin intraperitoneally (i.p.), the second was injected twice daily with 10 mg of ampicillin i.p. and the third group was injected with saline. Antibiotic treatment was started 3 days before contamination. The experiment was performed 4 times.

RESULTS

In the present study, a strong negative influence of systemic treatment with streptomycin and to some extent also of systemic treatment with ampicillin was observed on the CR (Fig. 1). After marginal effective oral doses of 10^8 SR-*E. coli* cells and 10^8 SR-enterococci (just above the CR-determining dose), the control animals showed the same type of colonization in the ICP as was seen in previous experiments (Van der Waaij *et al.* 1972) in conventional mice. In the antibiotic-treated mice, however, the contaminant was recovered from the caecal contents in increased concentrations. This effect was stronger in the streptomycin-treated animals than in the ampicillin-treated mice. The effect was seen after contamination

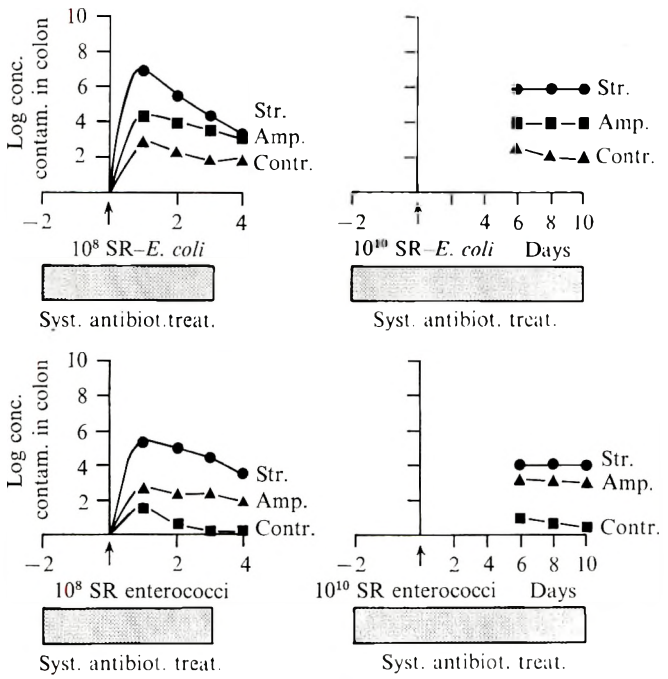

Fig. 1. Colonization pattern during systemic treatment with ampicillin and streptomycin.

Table 1. *Spread of S.R.*–E. coli *into mesenteric lymph nodes* during systemic antibiotic treatment*

	Days after contamination					Days after contamination			
Treatment	1	2	3	4	Mean	6	8	10	Mean
Streptomycin	3	5	1	2	2·7	4	0	2	2
Ampicillin	6	4	4	3	4·2	6	4	5	5
Controls	0	0	0	0	0	1	0	0	0·3

* Figures in the body indicate the number of positive cultures per 20 mice (total of four experiments with five animals per group).

with both *E. coli* and enterococci. Increased concentrations of the contaminants were not confined to the ICP, but appeared to persist thereafter since they were also observed at days 6, 8 and 10, after the higher dose of 10^{10} bacteria (Fig. 1).

The spread of the contaminant into the mesenteric lymph nodes and spleen was also clearly influenced by systemic antibiotic treatment. The two contaminating organisms showed different effects (Tables 1 and 2). Although the CR was found to be decreased for both bacterial strains used for contamination in the ampicillin-treated animals as well as in the streptomycin-treated mice, all cultures of the mesenteric lymph nodes and the spleen remained sterile in the ampicillin-treated enterococci-contaminated animals (Table 2). In the *E. coli*-contaminated animals spread of ampicillin-resistant SR–*E. coli* was obviously not inhibited by ampicillin; on the contrary positive cultures were seen in more animals than in the strepto-

Table 2. *Spread of S.R.-enterococci into mesenteric lymph nodes* during systemic antibiotic treatment*

	Days after contamination					Days after contamination			
Treatment	1	2	3	4	Mean	6	8	10	Mean
Streptomycin	2	6	2	2	3·0	1	1	1	1
Ampicillin	0	0	0	0	0	0	0	0	0
Controls	1	1	3	0	1·2	1	0	0	0·3

* Figures in the body indicate the number of positive cultures per 20 mice (total of four experiments with five animals per group).

mycin-treated group following contamination with *E. coli* (Table 1). This is presumably due to the fact that the enterococci were sensitive to ampicillin and the *E. coli* strain was not. Spread into the lymphatic organs is usually confined to the first 4–6 days after contamination (Van der Waaij *et al.* 1972). This was also seen in the controls. In the antibiotic-treated mice, it evidently persisted longer since it was also found on days 8 and 10 after contamination.

DISCUSSION

The dubious effect that systemic antibiotic treatment can exert on the intestinal microflora has been described by various authors – predominantly clinicians. Changes have been reported in the microflora of the nose (Aly, Maibach, Strauss & Shinefield, 1970), the oropharynx (Louria & Kaminski, 1962) and the faecal flora (Altemeier, Culbertson & Hummel, 1970; Gaya, Admitt & Turner, 1970). In most cases the changes imply colonization with antibiotic-resistant bacterial strains for longer periods and, most often, in usually high concentrations (Louria & Kaminsky, 1962). Colonization with *Salmonella typhimurium* persisted longer in patients treated with antibiotics than in an untreated group (Dixon, 1965; Aserkoff & Bennett, 1969).

Studies in human volunteers contaminated with *Pseudomonas aeruginosa* in various doses indicated that long-lasting colonization occurred only when ampicillin had been administered (Buck & Cooke, 1969; Stoodley & Thom, 1970).

Since we found a similar colonization pattern after experimental oral contamination with SR–*E. coli*, SR–*P. aeruginosa*, and SR–enterococci in conventional monkeys (Van der Waaij, De Vries & Lekkerkerk, 1970) and in conventional mice (Van der Waaij *et al.* 1971) it was decided to use the mouse as a model subject to study the influence of parenteral antibiotic treatment on the CR.

In the present study an obviously negative influence of high dose systemic antibiotic treatment on the CR was found. The concentration of both bacterial strains used for contamination recovered from the caecum was clearly increased during the ICP. Thereafter, a significant difference between antibiotic-treated and control animals was only found after the high dose of 10^{10} cells.

Evidence of spread into the mesenteric lymph nodes was seen much more often in the antibiotic-treated animals than in the controls. Only the SR–enterococci

(which were sensitive to ampicillin) were not recovered from the mesenteric lymph nodes during ampicillin treatment. During treatment with streptomycin (to which the enterococci were resistant), positive mesenteric lymph nodes were seen more often than in the control groups. The *E. coli* strain used in this experiment, being resistant to both the antibiotics used, also invaded more often in the antibiotic-treated groups than in the controls.

The decrease of the CR, as reflected in intestinal concentration, was greater during streptomycin treatment than during ampicillin treatment.

Our observations indicate that, under the conditions described, systemic treatment with antibiotics such as ampicillin and streptomycin enhances the possibility of colonization by exogenous bacterial species. This seems not to be confined to strains resistant to the antibiotics injected such as were found during oral antibiotic treatment (Van der Waaij *et al.* 1972). Spread from the intestines into the lymphatic tissues, on the other hand, appears to be confined to strains which are insensitive to the antibiotic supplied systemically as was to be expected. Although ampicillin is known to be excreted with the bile, in our experiments it evidently has not reached high enough concentrations in the colon to influence growth of the 'sensitive' enterococci used for contamination. Nevertheless, the CR-inducing part of the intestinal microflora appeared to be negatively influenced by the antibiotics supplied. Streptomycin, which is presumably not excreted with the bile, had a stronger 'negative' effect on the CR than ampicillin. This may indicate that these antibiotics influence the 'CR-responsible micro-organisms' via another route; possibly directly through the intestinal mucosa. If this is the case, it could be concluded that the 'CR-inducing' anaerobes are among those species that are described as living in close contact with the mucosa (Savage & Dubos, 1968).

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The mechanism of cross-protection afforded by dengue virus against West Nile virus in hamsters*

By WINSTON H. PRICE and INDERJIT S. THIND

Kenneth F. Maxcy Laboratories, Department of Epidemiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland 21205

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SUMMARY

The protection afforded by similar concentrations of different dengue virus serotypes against a subsequent challenge of West Nile virus was studied in hamsters. The New Guinea C strain of dengue 2 virus gave the best protection. It was found that the anamnestic neutralizing antibody response induced by the challenge West Nile virus against West Nile virus in hamsters, previously immunized with dengue 2 virus, might play a major role in the cross-protection observed in this system.

INTRODUCTION

Studies were recently reported on the protection afforded by a previous injection of dengue virus against a subsequent challenge of West Nile virus in hamsters and on the failure of dengue immune monkeys to develop viremia after challenge with St Louis encephalitis virus (Sather & Hammon, 1968, 1970).

In a recent paper from this laboratory the mechanism of the cross-protection afforded by dengue virus against a subsequent challenge of West Nile virus was studied in hamsters and it was shown that this protective effect was not mediated by interferon or serum protective factor; furthermore, the cross-protection was not due to the mutual exclusion principle (Price & Thind, 1972). The relationship of the protective effect to the *in vivo* production of serum neutralizing antibody was not definitively established. It is the purpose of this paper to report further experiments on the ability of different dengue serotypes to protect hamsters against a subsequent challenge of West Nile virus. Furthermore, the relationship of *in vivo* produced serum neutralizing antibody induced by the challenge virus to the cross-protection was studied.

MATERIALS AND METHODS

Viruses

All virus types used were described in detail in the previous publication (Price & Thind, 1972). All virus pools, as whole infected mouse brains, were stored at −70° C. Freshly prepared homogenized brain suspensions in 0·75 % bovine plasma albumin in phosphate buffered saline, pH 7·4, clarified by centrifugation at 500 *g* for 10 min., were used for vaccination and challenge of animals. All virus

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pools were titrated intracerebrally (i.c.) in 1 to 2-day-old mice. West Nile virus was also titrated subcutaneously (s.c.) in 10- to 11-week-old hamsters when necessary. Unless otherwise stated, LD 50 refer to (s.c.) hamster LD 50.

Cyclosphosphamide

Cyclophosphamide (Mead, Johnson, Evansville, Indiana) was given by the intraperitoneal (i.p.) route in a dose of 60 mg./kg. body weight 24 hr. before and after the challenge virus. There was no mortality associated with this dosage.

Immunization and challenge

Three to 4-week-old hamsters (each weighing 40–60 g.) were immunized by the i.p. route with $10^{7\cdot5}$ newborn mouse i.c. LD 50 of the New Guinea C strain of dengue 2 virus unless otherwise specified. Six weeks later the hamsters were challenged subcutaneously with $10^{7\cdot5}$ (s.c.) hamster LD 50 of West Nile virus in 0·3 ml. unless otherwise specified. Control hamsters were given 0·3 ml. of a 10% clarified suspension of normal newborn mouse brain. All hamsters were observed for 21 days after challenge.

Serum neutralizing antibody

Neutralization tests for West Nile virus antibody were carried out as described previously using the plaque reduction method (Price & Thind, 1972).

To determine the neutralizing antibody for dengue viruses the same method was used as described previously (Price & Thind, 1972). Twenty-four 1 to 2-day-old mice were inoculated intracerebrally with each virus dilution.

RESULTS

Influence of dengue virus type on protection against subsequent West Nile virus challenge

Previous results (Sather & Hammon, 1968, 1970) indicated that of the four dengue serotypes tested dengue 2 New Guinea C (NGC) isolate gave the best protection in hamsters against a subsequent challenge of West Nile virus. Similar results were later reported from this laboratory (Price & Thind, 1972). However, in our previous experiments the concentration of the various dengue serotypes varied over a tenfold range. Since there was little multiplication of dengue virus under the experimental conditions, the concentration of virus that was inoculated was of critical importance and unfortunately dengue 3 virus and dengue 4 virus were inoculated in a lower concentration than dengue 2 virus. Therefore, this time, very similar concentrations of the various dengue serotypes were used for vaccination. From Table 1 it can be seen that under these conditions dengue 2 New Guinea C isolate still gave the best protection against a subsequent challenge of West Nile virus. Next in order came the other two types of dengue 2 virus and type 1 dengue virus. Types 3 and 4 dengue virus gave the lowest protection.

Table 1. *Protection in hamsters immunized with various types of dengue virus and challenged 6 weeks later with West Nile virus*

Immunizing type	Dose of immunizing type (suckling mouse i.c. LD 50)	Neutralization index to dengue viruses†	Survival*
Dengue 1	$10^{6\cdot5}$	2·1	22/50
Dengue 2 NGC	$10^{6\cdot4}$	2·7	42/50
Dengue 2 NGB	$10^{6\cdot7}$	2·2	27/50
Dengue 2 Trinidad	$10^{6\cdot0}$	1·9	28/50
Dengue 3	$10^{6\cdot2}$	1·7	10/50
Dengue 4	$10^{6\cdot5}$	1·6	7/50
Normal mouse brain	—	0	0/50

* Numerator represents number of hamsters that survived and denominator represents number inoculated. All hamsters were challenged s.c. 6 weeks after immunization with $10^{7\cdot5}$ LD 50 (s.c.) West Nile virus. In all tables when hamsters are inoculated with West Nile virus, the LD 50 refers to hamster LD 50 in 0·3 ml. of inoculum.

† Neutralization index is to homologous virus.

Table 2. *Protection and neutralizing antibody titres in hamsters immunized with dengue 2 NGC virus or dengue 4 virus and challenged 6 weeks later with a large concentration of West Nile virus*

Expt. no.	Immunizing inoculum*	Serum dilution which neutralized 50 % of West Nile virus plaques. Days after challenge			Survival in observation group†
		0	4	6	
1	Dengue 2 NGC	0	1/4	1/512	8/10
	Dengue 4	0	1/4	1/128	7/50
	Normal mouse brain	0	1/2	1/128	0/10
2	Dengue 2 NGC	0	1/8	1/1024	9/10
	Normal mouse brain	0	0	1/128	0/10
3	Dengue 2 NGC	0	1/4	1/512	7/10
	Normal mouse brain	0	1/2	1/128	0/10

* $10^{7\cdot5}$ to $10^{7\cdot1}$ suckling mouse i.c. LD 50 of dengue 2 NGC in a volume of 0·3 ml was given i.p. to each immunized hamster. 0·3 ml. of 10% normal mouse brain was given i.p. to each control hamster. All hamsters were challenged s.c. with $10^{7\cdot5}$ LD 50 (s.c.) West Nile virus 6 weeks after immunizing inoculum.

† Numerator shows number of hamsters surviving and denominator shows number inoculated.

Protection and serum neutralizing antibody titres

Three separate experiments were carried out to determine the neutralizing antibody titres to West Nile virus after challenging with the large dose of West Nile virus. Samples were stopped after 6 days because many of the control hamsters started to die on the 7th day after challenge. Table 2 shows that in all three experiments the hamsters that were previously immunized with the dengue 2 NGC isolate formed fourfold to eightfold more neutralizing antibody against the challenge virus than the hamsters immunized with normal mouse brain. It will also be noted from Table 2 that very few hamsters immunized with dengue 4 virus survived the challenge with West Nile virus. There is no anamnestic neutralizing

Table 3. *Protection and neutralizing antibody responses of Dengue 2 NGC immunized hamsters* challenged with large and small concentrations of West nile virus*

Expt. no.	Initial treatment	Dose of West Nile challenge virus LD 50 (sc)	Serum dilution which neutralized 50 % of West Nile virus plaques. Days after challenge 0	Days after challenge 6	Survival in observation groups†
1	D_2NGC	$10^{7.5}$	0	1/512	8/10
	NMB‡	$10^{7.5}$	0	1/128	0/10
	D_2NGC	$10^{2.5}$	0	1/128	0/10
	NMB	$10^{2.5}$	0	1/64	1/10
2	D_2NGC	$10^{7.5}$	0	1/512	8/10
	NMB	$10^{7.5}$	0	1/128	0/10
	D_2NGC	$10^{2.5}$	0	1/128	1/10
	NMB	$10^{2.5}$	0	1/32	0/10

* All specified hamsters immunized with dengue 2 NGC strain as described in Table 2. Challenged with varying doses of West Nile viruses 6 weeks after immunization.

† Same as Table 2.

‡ Normal mouse brain suspension.

antibody response against the challenge virus under these latter conditions. Since dengue-immunized hamsters inoculated with $10^{2.5}$ LD 50 (s.c.) of West Nile virus do not survive (Price & Thind, 1972), neutralizing antibody was determined in dengue-immunized hamsters challenged with either $10^{7.5}$ or $10^{2.5}$ LD 50 (s.c.) West Nile virus. Two such experiments were carried out. In both instances the hamsters inoculated with $10^{7.5}$ LD 50 (s.c.) of West Nile virus formed four-fold more neutralizing antibody than the hamsters challenged with $10^{2.5}$ LD 50 (s.c.) of West Nile virus (Table 3). It will be noted that the dengue-immunized hamsters inoculated with the low concentration of West Nile virus formed the same amount of antibody as hamsters immunized with normal mouse brain and challenged with the large dose of West Nile Virus as shown in Table 2, and there was the same survival rate in these both groups.

Effect of cyclophosphamide on cross-protection and neutralizing antibody response

In view of the results shown in Tables 2 and 3 it was thought of interest not only to see the effect of cyclophosphamide on the cross-protection, but to see the effect of the immuno-suppressant drug on the neutralizing antibody response. To do this a group of hamsters were immunized with dengue 2 NGC isolate. Six weeks later they were divided in half. One half received cyclophosphamide and 24 hr. later all hamsters were challenged with $10^{7.5}$ LD 50 (s.c.) of West Nile virus. Twenty-four hours later the hamsters that received the cyclophosphamide received another injection of cyclophosphamide. Table 4 shows that cyclophosphamide completely abolished the cross-protection afforded by dengue virus against a subsequent challenge of West Nile virus. Furthermore, it completely inhibited the neutralizing antibody response induced by West Nile virus against West Nile virus.

Table 4. *Effect of cyclophosphamide administration on cross-protection and neutralizing antibody response against West Nile virus**

Expt. no.	Cyclophosphamide administration	Serum dilution which neutralized 50 % of West Nile plaques. Days after challenge 0	Days after challenge 6	Survival in observation group†
1	No	0	1/512	7/10
	Yes	0	0	0/10
2	No	0	1/1024	8/10
	Yes	0	0	0/10

* All hamsters immunized with dengue 2 NGC strain as described in Table 2 and challenged s.c. 6 weeks later with $10^{7\cdot5}$ LD 50 (s.c.) West Nile virus. The cyclophosphamide group received 60 mg. of the drug per kg. 24 hr. before challenge and 24 hr. after challenge.

† Numerator shows number of hamsters surviving while denominator shows number inoculated in the observation groups.

Table 5. *The effect of cyclophosphamide during Dengue immunization on the subsequent anamnestic neutralizing antibody response against the West Nile virus challenge**

Immunizing inoculum	Time of administration of cyclophosphamide	Neutralization index to dengue 2 virus	Serum dilution which neutralized 50 % of West Nile virus plaques. Days of challenge 0	Days of challenge 4	Days of challenge 6	Survival in observation group†
Dengue 2 NGC	None	2·7	0	1/4	1/512	8/10
Dengue 2 NGC	During immunization‡	0	0	1/4	1/512	7/10
Normal mouse	None	0	0	1/2	1/64	0/10

* All animals challenged s.c. with $10^{7\cdot5}$ LD 50 (s.c.) West Nile virus.

† Numerator shows number of hamsters surviving while denominator shows number inoculated in the observation groups.

‡ Cyclophosphamide in a dose of 100 mg./kg. body weight was given 24 hr. before and after immunizing inoculum. Three additional doses of cyclophosphamide (100 mg./kg. body weight) were given i.p. 10 days apart after the immunizing inoculum.

It was thought of interest to determine whether an anamnestic neutralizing antibody response to West Nile virus occurred when dengue neutralizing antibody had been suppressed by the use of cyclophosphamide during the immunization period. These experiments were carried out exactly as described previously (Price & Thind, 1972) except that the anamnestic neutralizing antibody response was measured to West Nile virus as well as the cross-protection. Table 5 shows that there is a similar anamnestic response whether cyclophosphamide is administered during the course of immunization or not.

Effect of passive immunization of hamsters on West Nile virus challenge

Since the titres of neutralizing antibody due to the anamnestic response produced by large concentrations of West Nile virus in hamsters sensitized by NGC

Table 6. *Effect of different amounts of passively administered neutralizing antibody on challenge with West Nile virus*

Group	Level* of passively administered neutralizing antibody at time of challenge	Survivors† Challenged with $10^{7.5}$ LD 50 (s.c.) West Nile virus	Survivors† Challenged with $10^{2.5}$ LD 50 (s.c.) West Nile virus
1	1/512	8/10	9/10
2	1/128	2/10	2/10
3	1/64	0/10	1/10
4	0 (no passive antibody given)	0/10	0/10

* Serum dilution which neutralized 50 % of West Nile virus plaques

† Numerators show number of hamsters surviving and denominators show number challenged with West Nile virus

dengue 2 virus might be related to the protection observed in these hamsters, it was thought of interest to study the effect of different amounts of passively administered neutralizing antibody on West Nile virus challenge. Three groups of hamsters were passively immunized with different amounts of antisera prepared in hamsters. From Table 6 it can be seen that good protection was observed in hamsters having a dilution of 1/512 of neutralizing antibody against West Nile virus challenged with high or low doses of West Nile virus while a fourfold lower dilution of passively administered antibody resulted in little protection. These results would fit in with the hypothesis that the anamnestic neutralizing antibody produced by large concentrations of West Nile virus in previously sensitized dengue 2 NGC hamsters accounts for the protection observed in these animals.

DISCUSSION

The data reported in this paper show that when similar concentrations of different dengue virus serotypes are studied in hamsters, the New Guinea C strain of dengue 2 virus gave the best protection against a subsequent challenge of West Nile virus. This confirms earlier observations (Sather & Hammon, 1970).

The results reported here suggest that in hamsters, in the dengue virus–West Nile virus system, the anamnestic neutralizing antibody response to West Nile virus produced in a hamster previously sensitized with dengue virus and challenged with large doses of West Nile virus plays a key role in the cross-protection described. This hypothesis brings to mind a similar hypothesis made previously concerning cross-protection in other group B arbovirus systems (Price *et al.* 1963) and for cross-protection among group A viruses and between viruses in the Bunyamwera group (Casals, 1963). These experiments may also be related to those which showed that when 'lightly' immunized mice were challenged intracerebrally with varying doses of western equine encephalomyelitis virus death occurred following small but not large virus inocula (Scheslinger, 1949). It was also suggested earlier

that the booster response in a partially immunized animal was associated with resistance to the related virus challenge using various group B arbovirus combinations (Imam & Hammon, 1957). However, it may well be that increased resistance and the higher titres of neutralizing antibody are not directly related but are the concurrent manifestations of an altered immune state.

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Diphtheria in Iran

By IRAJ ZAMIRI and M. G. McENTEGART
Department of Medical Microbiology, University of Sheffield, Sheffield S10 2TN
and ALICE SARAGEA
Institute Dr I. Cantacuzino, Bucharest, Romania

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SUMMARY

A study of diphtheria in Iran was undertaken during the summer of 1969. Diphtheria was found to be more common among the younger people and to reach its peak 3 months after the start of the school term, during the coldest period of the year. Owing to the liberal use of antibiotics a number of modified clinical cases were observed. Of the strains isolated, 89·7 % were gravis, 1·3 % intermedius and 9 % mitis. Phage typing of these strains showed that the mitis and intermedius strains could only be typed by adapted phages and the majority of gravis strains were phage type XIV, which is the epidemic type seen in other countries.

As the mass immunization campaign reduces the incidence of diphtheria in Iran, the epidemiological pattern of the disease will change as seen in the U.K. and U.S.A. It is, therefore, suggested that in the future further studies of diphtheria in Iran be undertaken in order to provide us with information about the changing epidemiological pattern of the disease.

INTRODUCTION

Although the epidemiological pattern of diphtheria in the world is rapidly changing, the disease still remains a significant health problem in many countries (Editorial, 1970). Better standards of living, mass immunization, improved diagnosis, prompt treatment, and more effective carrier tracing have within the last decade reduced the incidence of diphtheria in the remaining European foci. South American, African and Asiatic countries are now taking similar steps.

While the number of doctors in Great Britain who have had experience of diphtheria in either its clinical or laboratory aspects continues to dwindle, the risk of the disease being imported into the U.K. and occurring in classical or modified form remains (Zamiri, 1970). With these problems in mind, an investigation of diphtheria in Iran was undertaken and some first-hand experience of the disease obtained. Over 200 Iranian strains of *C. diphtheriae* were brought back so that their biotypes and phage types could be studied.

The programme included a study of clinical diphtheria in Iran during July, August and September 1969, followed by the examination of the strains of *C. diphtheriae* in Sheffield. During the latter stage of the investigation Dr A.

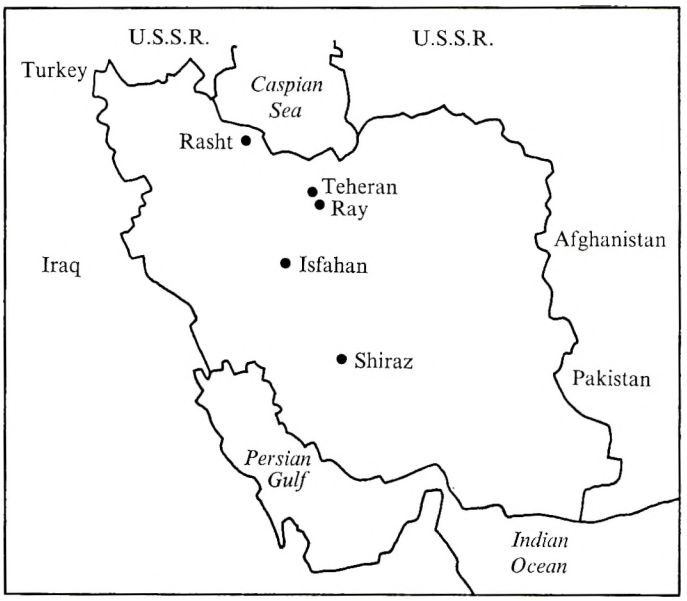

Fig. 1. Map of Iran, showing areas visited during this study.

Saragea came to Sheffield in order to demonstrate and organize the phage-typing methods which had been developed in her laboratory in Romania.

Area studied

The map of Iran and neighbouring countries (Fig. 1) shows the cities (Teheran, Ray and Rasht) where the clinical cases were seen and those (Teheran, Ray, Isfahan and Rasht) from which strains of *C. diphtheriae* had been isolated in earlier surveys.

The incidence of diphtheria in Iran

Examination of the W.H.O. statistical reports (1960–4) on diphtheria in Iran showed, in contrast to most other countries, a rising incidence of the disease (Fig. 2). Further investigation of this rise, which had occurred despite an increased immunization rate, showed some contradictions (as, for example, a relatively low death rate – 2·2 %) compared with another report from Iran (Tahernia & Motamed, 1969) suggesting that the figures given to W.H.O. exaggerate the incidence of the disease, or immunization was too late to prevent this epidemic. Another influencing factor is the rapidly growing population, a large proportion of whom were not protected (Fig. 2). Considering the rapid rate of population increase, the falling incidence of diphtheria in Iran is more dramatic than it may appear (Fig. 2).

General medical progress in Iran led to a greater awareness of the problem of diphtheria. As the rate of development of laboratory facilities to confirm the diagnosis had not kept pace, the use of clinical criteria alone was likely to lead to an exaggeration of the incidence. The mass immunization campaign which started in 1965 is showing good results (Fig. 2). In Teheran, with a population of nearly 3 million, the annual incidence of diphtheria, formerly about 2500 cases, has been

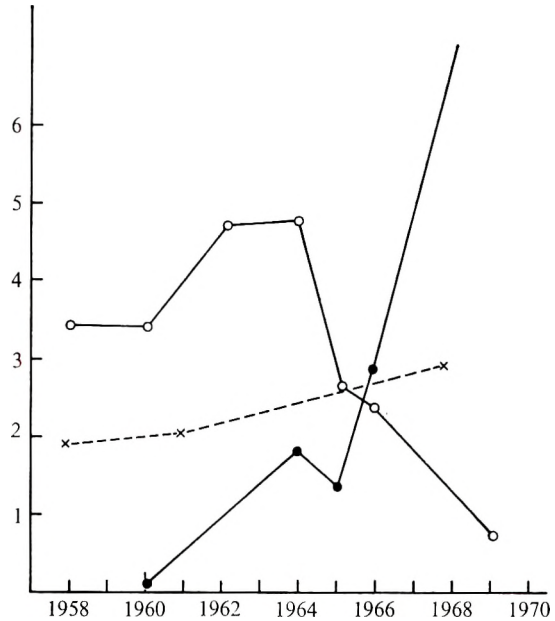

Fig. 2. Annual incidence of diphtheria, immunization rate, and population increase in Iran (1958–69). ○—○, Incidence of diphtheria, in thousands; ●—●, immunization rate, in millions; ×– – –×, population, in 10 millions.

reduced to a fraction of that number (H. Mirchamsy, pers. comm.) From 1965 to 1968, over 7 million people, especially in rural areas, were immunized (Iranian Health Corps Report, 1969). The effect of immunization is clearly seen in this report, which shows that in 1969 the incidence in the peak months of January/February was one-seventh of that for the same months before the mass immunization campaign (1964).

The seasonal incidence of diphtheria

The variation in the seasonal incidence in Iran, with the peak in the winter (Fig. 3) is very similar to that seen in diphtheria epidemics in other countries. This seasonal variation becomes less marked as the epidemic incidence of the disease gives place to the endemic incidence. The start of the school year in September may also contribute to the rising incidence of carriers and cases in the winter season (Fig. 3).

Carrier rate

There is only one report on the incidence of diphtheria bacilli in routine throat and nose swabs in Iran. Esterabady, Taslimi & Nategh (1963) reported that 200 (33 %) of 600 nose and throat swabs sent to the Razi and Pasteur Institutes in Teheran were positive for *C. diphtheriae*. Unfortunately, they do not comment on the selection of their patients, but it is probable that they were all suspected cases of diphtheria. If this is so, the figure of 33 % does not represent the true incidence of carriers.

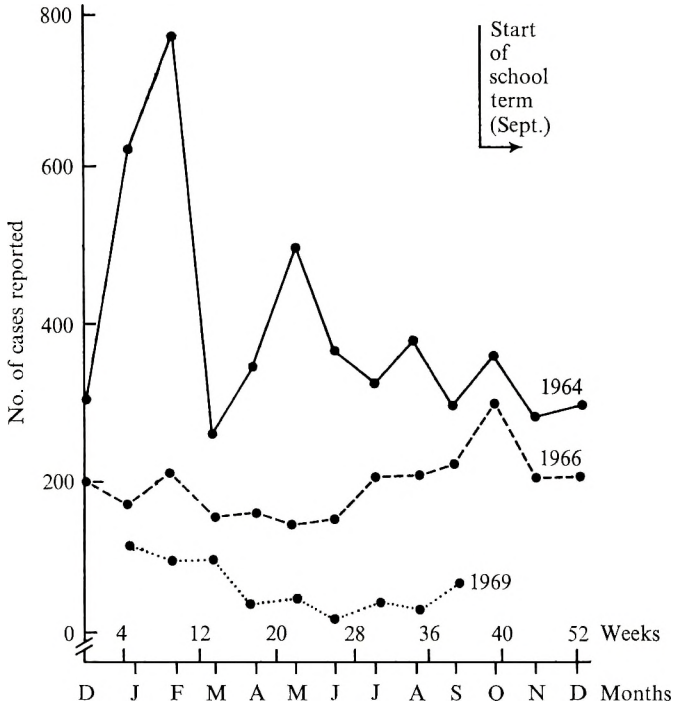

Fig. 3. Monthly incidence of diphtheria in Iran during 1964, 1966 and 1969.

Age incidence

Throughout the world classical diphtheria is still mainly a disease of children. Tahernia & Motamed (1969) confirmed this for Shiraz, where 62 % of the 95 cases studied occurred in children below the age of 6. However, during the field study which was carried out in the north, only five (20 %) of the 25 cases seen were in this age group. This difference may be explained by the school holiday accentuating the season of minimal incidence and possibly by variation due to areas studied. It does seem likely that school children carry the infection to the pre-school age siblings. In many under-developed countries children live in crowded conditions, and during the colder season, sleep with their elders. Such close contacts among the members of one family, often undernourished and suffering from respiratory infections, must contribute to the rising incidence.

MATERIALS AND METHODS

Nine of the strains of *C. diphtheriae* studied in this survey were isolated from the 25 clinical cases seen during the field study in Iran. The low isolation rate may be due to the early use of antibiotics. The remaining strains, most of which were lyophilized, were provided through the kindness of colleagues in Teheran and Isfahan. All were isolated from cases of clinical diphtheria. The total number of strains examined was 232.

All strains were typed according to the criteria of McLeod (Anderson, Happold, McLeod & Thompson, 1931; Anderson, Cooper, Happold & McLeod, 1933). The

Table 1. *Biotypes of* 232 *strains of* C. diphtheriae *isolated in Iran*

	Gravis		Intermedius		Mitis	
Total no.	No.	%	No.	%	No.	%
232	208	89·7	3	1·3	21	9

toxigenicity of the first 20, and all subsequent strains giving equivocal results by *in vitro* tests, were confirmed by their lethal effect on guinea-pigs. Each strain was phage typed by the method of Saragea & Maximescu (1966). A few minor changes in their method were made in order to make use of commercial media available in the United Kingdom. The strain for phage typing was grown overnight on a Loeffler slope and the whole of the growth from this culture was used to inoculate a 3 ml. volume of Lab-Lemco broth containing $CaCl_2$ and cystine. This was broth incubated at 35° C for $1\frac{1}{2}$ hr. and was then used to seed the entire surface of a heart brain agar plate (heart brain agar was prepared by incorporating 1 % Oxoid agar in heart brain broth (Difco)). Seeded plates were dried in the incubator for 40 min. The basic set of 24 diphtheria phages (prepared by Drs Saragea and Maximescu in Bucharest) were used at concentrations of 100 RTD. The phage types were determined according to the lytic 'patterns' described in the original phage-typing scheme in which all degrees of lytic reactions were taken into account.

The phage samples were applied initially by Pasteur pipettes, but later use was made of a modified P.H.L.S. phage applicator (Lidwell, 1959).

Strains which were, at this point, untypable were tested by a series of 'additional' phages which consisted of a further set of 33 adapted phages from the Romanian Diphtheria Laboratory. A few remaining strains, which were still untypable, were further investigated by adapting the lysogenic phage, 951, to one of them and then testing this adapted phage (951/9) against the other untypable strains. Thus it was possible to show that the untypable strains behaved in the same way when tested with the adapted phage 951/9.

RESULTS

Biotyping

Of the 232 strains examined, 89·7 % were gravis, 9·0 % mitis and 1·3 % intermedius (Table 1). Three of the gravis and one of the mitis varieties were non-toxigenic.

Phage typing

All but eight of the strains were phage typable. About 95 % of the gravis toxigenic strains fell into eight phage types (Table 2); among these 53 % were phage type XIV, 36 % were phage type XVI. The remainder were distributed amongst phage types XIV 'a' and 'b', VI, XVII, XVIII and XIX.

The intermedius and mitis strains were typable, with the adapted phages 951/9 and 951/553. Some Albanian and French strains have been found to be of this phage type.

The biotypes were in general agreement with the phage types apart from two

Table 2. *Phage types of* 232 *strains of* C. diphtheriae *isolated in Iran*

Biotype	'Phage type	No. of strains
Gravis	XIV	107
	XIVa	9
	XIVb	4
	XVI	75
	XVII	2
	XVIII	2
	XIX	1
	VI	5
Mitis, Intermedius	Adapted phages	18

strains which behaved atypically in that they were morphologically and biochemically mitis, but were typed by gravis phages (type XIV).

Lysogenicity of some of the strains was also studied. Eleven strains tested proved to be lysogenic and, as expected, all these were toxigenic.

DISCUSSION

In Iran, improved health services plus the massive immunization programme which began in 1965, have brought about a rapid fall in the incidence of diphtheria. As might have been predicted, the major fall has been during the peak period, but this fall has also been very significant in other seasons.

As the disease becomes less common, it is likely that the pattern of diphtheria may soon be similar to that seen in more developed countries, a much lower annual incidence with a proportionately higher death-rate. The higher death-rates in the western countries and in U.S.A. may be due to the modification by antibiotics of the classical picture of diphtheria, plus a general unfamiliarity with the disease.

The change in the age incidence of diphtheria in children as a result of the immunization campaign, social betterment, etc., was seen as a relative increase of cases in the 5- to 10-year-old group and a decrease in the 0- to 5-year-old group. This change was the same as that seen in the U.K. between 1900 and 1935.

Among the 232 strains studied, two toxigenic gravis strains *each* produced two distinctive types of colony on tellurite medium, which although biochemically indistinguishable proved to be of different phage types. This may represent a double infection. The culture from one person showed both mitis and gravis biotypes, which were also different in phage typing. The mitis variety was typable by the adapted phage I (951/9), but the gravis variant was morphologically and biochemically a toxigenic gravis and phage typed as group VI. Once more, this raises the question of a mixed infection (or mixed cultures).

The most common phage type was type XIV. This type has been found in most of the European countries, in Australia and U.S.A. and it also played a major part in the 1956–64 epidemic in Romania, when it was the commonest epidemic type. Type XVI, the next most common type, has also been found in Europe, Australia, Canada, U.S.A., etc. Four of the five examples of phage type VI, more

commonly seen among the carriers, were isolated in Isfahan, whereas only 20% (48) of the strains examined were from that region. The two strains isolated in Rasht were of the same phage type (XIV) and biotype (gravis).

The biotypes and phage types of the 232 Iranian strains of *C. diphtheriae* which were isolated from centres investigated were those associated with epidemic outbreaks of the disease in Europe.

A preponderance of toxigenic gravis strains is characteristic of epidemic diphtheria. The phage typing and biotyping of strains from Iran followed this expected pattern and was in general agreement with the Australian study described by Gibson, Cooper, Saragea & Maximescu (1970). As has been shown in most countries studied, the climate, social conditions and state of immunity play an important part in the epidemiology of the disease, especially as the main prevalence is in children. Recent antidiphtheria measures in Iran are showing results. There has already been a rapid fall in the incidence of diphtheria in the larger towns and a similar fall in rural areas should follow. These developments provide an ideal situation for the epidemiological study of diphtheria.

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Studies on respiratory immunization with tetanus toxoid: the role of adjuvants

By H. C. BARTLEMA, RIENTSJE BRAUNIUS and LILY HÖLSCHER
Medical Biological Laboratory TNO, 139 Lange Kleiweg, Rijswijk 2100, The Netherlands

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SUMMARY

Aerosol vaccination of mice with purified plain tetanus toxoid does not induce an immune response unless a suitable adjuvant is added.

Aluminium phosphate is without effect by aerosol treatment. Killed cells of *Klebsiella pneumoniae*, although effective, are unsatisfactory owing to the long inhalation period needed.

Killed *Bordetella perussis* cells were found to be an excellent adjuvant. A single aerosol treatment with a toxoid–*B. pertussis* mixture during a moderate exposure period evoked a considerable immune response. With repeated aerosol treatment of primed mice the addition of adjuvant is not required; booster treatment with plain toxoid is at least as effective.

Extracts from *B. pertussis* cells exert as good an adjuvant effect as the whole-cell vaccine. The remaining cell-wall debris also appears to be an active adjuvant.

In combination with constant doses of adjuvant (10^8 *B. pertussis* cells), the 50% protective doses (ED50) of toxoid were determined by inhalation and by s.c. injection and were found to be 0·1875 and 0·0625 LFU respectively. This would imply that, as a result of the adjuvant action, the s.c. ED50 is reduced by approximately a factor of 20; whereas the respiratory ED50 is decreased by at least a factor of 100.

It is suggested that the much more pronounced adjuvant activity in aerosol immunization is associated with the induction of strong cell-mediated hypersensitivity in the respiratory tract.

INTRODUCTION

In recent years there has been increasing interest in immunization by the respiratory route. This is partly because mass vaccination could be more easily performed in this way (Fontanges, 1966) and partly in the hope that a local immunity might be established in the respiratory tract by secretory immunoglobulins (IgA type antibodies), thus producing a protective barrier against micro-organisms penetrating by this route. The idea has been supported by studies on virus vaccines (Waldman *et al.* 1968; Waldman, Mann & Small, 1969; Perkins *et al.* 1969). Most studies so far reported concern the use of killed or attenuated bacterial and viral vaccines, either in a dry or in a liquid state. Only a few investigations have dealt with toxoid. Yamashiroya, Ehrlich & Magis (1966) demon-

strated that in guinea-pigs aerosol vaccination with tetanus toxoid induced a distinct primary response which, however, was much weaker than that induced by subcutaneous (s.c.) vaccination. On the other hand, aerosol revaccination after a primary s.c. vaccination was as effective as a s.c. booster. Likewise, Wigley, Wood & Waldman (1969) found that in volunteers soluble tetanus toxoid given by aerosol as a booster treatment induced increases in serum antibody titres comparable with those resulting from a s.c. booster. An excellent booster effect of aerosol treatment with lyophilized diphtheria toxoid was observed also by Fontanges in monkeys first vaccinated by s.c. injection. Unlike the guinea-pigs studied by Yamashiroya *et al.* (1966) the monkeys also showed a very satisfactory primary response to aerosol vaccination, the calculated inhaled dose being less than that needed for a s.c. primary dose (Fontanges *et al.* 1970). Similar results were reported by Fournier in primary aerosol vaccination of monkeys with lyophilized tetanus and diphtheria toxoid (Fournier, 1969).

To obtain insight into the fundamental processes involved in respiratory immunization, studies were undertaken in our institute using tetanus toxoid as a simple antigen and mice as test animals. Syngeneic mice can be used easily in large numbers and so permit better statistical analysis of results. In addition, the cellular processes underlying the immune response to an antigen introduced by the respiratory route can, in mice, be studied at a more fundamental level since the costs involved in sacrificing large numbers of these animals are generally acceptable.

The present article describes the results of aerosol immunization with purified toxoid and the effect of aluminium phosphate ($AlPO_4$) and preparations of *Klebsiella pneumoniae* and *Bordetella pertussis* as adjuvants.

MATERIALS AND METHODS

Test animals

D 57 mice (F_1 hybrids from DBA males and C 57 Black females) of either sex weighing 20–25 g were used.

Vaccines and reagents

Highly purified concentrated solutions of tetanus toxoid containing 7000–10,000 Limit of Floculation Units (LFU) per ml., lyophilized toxin, standard antitoxic serum, and *B. pertussis* preparations were obtained from the National Institute of Public Health (N.I.P.H.), Bilthoven, The Netherlands. Apart from a whole-cell vaccine with a concentration of 128×10^9 cells/ml., two other *B. pertussis* preparations were used. One was a soluble extract which was prepared in the N.I.P.H. by a procedure for manufacturing a non-toxic pertussis vaccine; the other was a suspension, mainly consisting of the cell-wall debris which remains after extraction of the bacterial cells (van Hemert, van Wezel & Cohen, 1964).

Whole-cell *B. pertussis* vaccine was mixed with plain toxoid solution to give a final concentration of approximately 7000 LFU of toxoid and $3{\cdot}8 \times 10^{10}$ bacterial cells per ml. of spraying liquid. The other *B. pertussis* preparations were mixed with the toxoid solutions in ratios equivalent to that of the whole-cell–toxoid mixture.

'Tetanus phosphate toxoid' (TPT) was prepared by mixing a purified toxoid solution of 3200 LFU/ml. and an $AlPO_4$ suspension of suitable dispersion containing 25 mg./ml. in various ratios. The mixture showing an optimum adsorption ratio was selected for use as an aerosol. It contained approximately 2000 LFU toxoid and 10 mg. $AlPO_4$ per ml.

An 18 hr. culture of *K. pneumoniae* in tryptose broth containing $5–6 \times 10^9$ cells/ml. was centrifuged, the cells were killed by treatment with 0·5 % formalin for 18 hr. at 4° C., repeatedly washed and resuspended to the original volume in a plain toxoid solution containing 5400 LFU/ml. The toxoid was not adsorbed to the bacterial cells as it was to $AlPO_4$; after 24 hr. agitating at 37° C. and pH 6 nearly all the toxoid could be recovered in the supernatant of the mixture.

Aerosol vaccination

Groups of 20 mice were exposed in a modified Henderson apparatus (Henderson, 1952) to aerosols sprayed with a Collison type nebulizer. By measuring the droplet size distribution of the cloud produced (May, 1945) the 'count median diameter' (CMD) and the 'mass median diameter' (MMD) were found to be 2·5 and 5 μm. respectively (Wolf *et al.* 1959). Therefore, almost all of the resulting droplet nuclei will penetrate easily into the lower respiratory tract of the test animals.

The 'absolute spray factor' (ASF), defined by Henderson as the ratio of the measured amount of substance per litre of aerosol to the amount of substance per litre of sprayed liquid, had been found for our aerosol device to have a constant value of 2×10^{-6}. Using this value and assuming a mean respiratory volume for mice of 25 ml./min. (Guyton, 1947) a rough estimate could be made of the exposure period required for any vaccine dose to be inhaled. During treatment cloud samples were taken in all-glass liquid impingers and evaluated by the indirect haemagglutination inhibition (HI) test (Stavitsky, 1954). By means of these assays the doses given were determined.

Immune assay

Immune responses were evaluated 3 weeks after immunization by s.c. challenge with 50 mouse LD 50 of toxin, and by antitoxin titrations in the pooled sera. All sera were titrated *in vitro* by the indirect haemagglutination (HA) technique as described by Stavitsky (1954). In addition, some sera were titrated *in vivo* by the mouse protection test on an 0·001 antitoxin unit (AU) level (J. D. van Ramshorst, personal communication).

RESULTS

Effect of plain toxoid inhalation

To find an adequate aerosol dosage range the 50 % protective dose of plain toxoid by s.c. injection (s.c. ED 50) was determined in preliminary experiments and found to be 1·2 LFU. Studies with ^{131}I-labelled toxoid, not to be reported here, have shown that only 10–20 % of the estimated inhaled doses are primarily retained in the lower respiratory tract (J. L. F. Gerbrandy, to be published). Accordingly, doses in the range of 10–50 LFU were given by exposures varying from 30 min. to

Table 1. *Primary immune response of mice 3 weeks after administration of plain tetanus toxoid by aerosol or by subcutaneous injection*

Route of administration	Dose (LFU)	Survivors 5 days after challenge*	Antitoxic serum titre (haemagglutination)†
Aerosol	10–12	0/20	n.d.
	20–24	1/20	n.d.
	40–48	1/16	< 10
S.c. inj.	0·5	11/20	128
	1·0	20/20	n.d.
	2·0	20/20	320

n.d. = not determined.
* Numbers of survivors/numbers challenged.
† HA titrations of pooled sera of four animals from each group.

Table 2. *Immune response of mice 3 weeks after repeated administration of plain tetanus toxoid by various combinations of inhalation and s.c. injection*

1st dose (LFU)	2nd Dose* (LFU)	Survivors 5 days after challenge†	Antitoxic serum titre (haemagglutination)‡
10–12 (R)	10–12 (R)	1/20	n.d.
0·5 (I)	10–12 (R)	16/16	4000
0·5 (I)	0·5 (I)	16/16	4000

n.d. = not determined. (R) = given by the respiratory route. (I) = given by injection.
* Doses given 3 weeks apart.
† Numbers of survivors/numbers challenged.
‡ HA titrations of pooled sera of four animals from each group.

2 hr. Control animals were given toxoid by the s.c. route in doses of 0·5–2·0 LFU. The results of this first series of experiments are presented in Table 1.

The figures clearly demonstrate that almost no immune response is provoked in mice by inhalation of plain toxoid in doses up to 50 LFU. On the other hand, s.c. injection of as little as 0·5 LFU already induces partial protection and a measurable amount of antitoxin in the serum.

Results obtained with aerosol treatment in two doses given 3 weeks apart are given in Table 2. In the same test two groups of control mice were given a first dose of toxoid by injection. One of these groups was revaccinated by the same route, the other group was given a booster treatment by aerosol.

It can be seen that inhalation of plain toxoid on two occasions did not induce a better immune response than did a single inhalation. However, when given as a booster to mice already first vaccinated by injection, the inhaled toxoid provoked a marked anamnestic reaction similar to that induced by a booster injection.

To exclude the possibility that the poor results of plain toxoid inhalation were due to a denaturation and loss of immunogenicity by the process of aerosolization mice were vaccinated with aerosol sampling fluid by s.c. injection. No change in s.c. ED 50 as compared with the original toxoid solution was noted.

Addition of aluminium phosphate

The strong adjuvant activity of $AlPO_4$ in s.c. tetanus vaccination is well known. To investigate the possibility that TPT given by aerosol might also induce a good immune response 20 mice were exposed for a period of 90 min. to an aerosol of this preparation.

The presence of $AlPO_4$ did not interfere with the toxoid titrations by indirect HI. Determination of the ASF by toxoid titrations and by phosphate analysis (Dr Oosterbaan, Biochemical Department, Med. Biol. Lab.) yielded similar values. This indicates that phosphate and toxoid are sprayed from the mixture with equal efficiency. However, the ASF was less than for plain toxoid and diminished further during the production of aerosol. The inhaled dose of toxoid was calculated to be approximately 3 LFU. The 50 % protective dose by s.c. injection of TPT in our test mice was found to lie between 1/2 and 1/6 LFU. If the antigenic activity of the inhaled TPT had proved equal to that of the injected TPT some protective effect might have been expected. However, the results were disappointing since none of the test animals survived the challenge. An explanation for this negative result was found in a second test by measuring the droplet size distribution of TPT at the site of its emerging from the nebulizer and after it had passed through the system. The droplet size distribution in the emerging TPT aerosol did not deviate from that in plain toxoid aerosol; the CMD and the MMD being 2·9 and 4·4 μm. respectively. Pl. 1, fig. 1 is a photomicrograph showing a few TPT aerosol droplets trapped on a greased slide which was placed a few cm. from the mouth of the nebulizer. The $AlPO_4$ particles which are distinctly visible in these droplets were supposed to remain dispersed in the aerosol as droplet nuclei. However, microscopical examination of aerosol particles which were trapped at the end of the animal exposure tube revealed a picture as shown by Pl. 1, fig. 2. Hardly any single droplet nuclei were found. Instead, larger or smaller conglomerates of particles could be observed, the majority of which had grown to such a size that they could neither be inhaled nor penetrate very far into the bronchial tree. Conglomeration of $AlPO_4$ droplet nuclei following evaporation of the surrounding water may be due to electrostatic forces resulting from the charges on the micro-crystals. The mice in this second test were killed immediately after exposure. The lungs were removed *in toto* and pooled. Lungs of 20 untreated mice were collected as a control and the materials were assayed for aluminum in the 'Analytical Institute TNO' by means of activation analysis. No aluminum could be detected in the lungs of either group.

Addition of bacterial adjuvants

The addition of bacterial cells as a possible adjuvant was based on the finding in routine vaccination that when using combined bacterial and toxoid vaccine the presence of the first often increases the immune response to the latter. The particularly strong potentiating effect of *B. pertussis* on the immunogenicity of injected protein antigens has been extensively studied (Munoz, 1964). However, little is known about the effect of bacterial adjuvants in respiratory immunization.

At first, killed *K. pneumoniae*, which is a respiratory pathogen for mice, was tested. The animals were exposed to an aerosol of the toxoid–*Klebsiella* mixture for a period of 2 hr., since it was expected that the spraying efficiency of this slightly viscous fluid would be lower than that of a plain solution. From cloud sample titrations the inhalation dose was estimated to be approximately 20 LFU. Three groups of 10 mice each were vaccinated with the same mixture by s.c. injection in doses of 0·6, 1·2, and 2·4 LFU respectively.

Twelve of the 20 aerosol-treated mice (60 %) survived challenge, whereas all the injected animals survived. It is evident that *K. pneumoniae* cells had a significant effect on the immunogenicity of inhaled toxoid since most of the animals were protected. However, to afford this partial protection a long inhalation period was needed, and since the animals would hardly endure a more prolonged exposure in the Henderson apparatus a higher rate of protection might not be easily attainable.

In view of its great reputation as a bacterial adjuvant *B. pertussis* was used in subsequent tests. The ASF of the mixed toxoid–*B. pertussis* preparation was found to be the same as that for plain toxoid solution (2×10^{-6}). Three groups of mice were exposed to an aerosol for a period of 40 min. Inhalation doses were estimated to be 16 LFU of toxoid and approximately 10^8 cells. Two of the groups were given a second treatment of equal toxoid dosage 3 weeks later, one group with and one without *B. pertussis* cells. Control groups received plain toxoid treatment only. One of them was given a second treatment also. In comparative tests three groups of mice were vaccinated with the toxoid–*B. pertussis* mixture by s.c. injection of 0·5 LFU toxoid and 3×10^6 *B. pertussis* cells. Two groups had a booster injection, one with and one without adjuvant. From each group four animals were killed at the time the others were challenged, and their sera were pooled for antibody titration. Table 3 presents the results of this test series.

It is evident from the table that tetanus toxoid given by aerosol, together with *B. pertussis* cells, confers a significant degree of immunity in contrast to plain toxoid. As would be expected a booster treatment with plain toxoid provoked at least as strong a secondary immune reaction as a booster treatment with the mixture. The figures suggest also that at the given doses aerosol immunization with the toxoid-adjuvant mixture compares favourably with s.c. vaccination. The primary and secondary immune responses were at least as good as those following injection.

It has been shown by others that the adjuvant effect of *B. pertussis* extracts on the antibody response to injected protein antigens can be as potent as that of the intact bacterial cells (Farthing & Holt, 1962). In the next series of experiments the effect of two preparations of *B. pertussis* on the immunogenicity of inhaled toxoid was compared with that of the whole-cell vaccine. According to data provided by the Laboratory for Vaccine Production of the N.I.P.H. one of these preparations, the soluble extract, exhibited nearly all the biological activity contained in the original cell suspensions in terms of immunizing potency and histamine-sensitizing factor. In the other preparation almost no biological activity could be discovered.

As can be seen from the data summarized in table 4 the extract has indeed an adjuvant activity similar to that of the whole-cell vaccine. However, the cell-wall debris also appears to be an active adjuvant.

Table 3. *Immune response of mice 3 weeks after aerosol or s.c. vaccination with tetanus toxoid combined with* B. pertussis *cells*

Route of administration	1st dose (LFU)	2nd dose* (LFU)	Survivors 5 days† after challenge	Antitoxic serum titre‡	
				Indirect HA	Mouse test
Aerosol	16 (+*B. pert.*)	—	15/16	128	n.d.
	16 (+*B. pert.*)	16 (+*B. pert.*)	16/16	16,000	1·15 AU/ml.
	16 (+*B. pert.*)	16	16/16	n.d.	2·56 AU/ml.
Aerosol	16	—	0/16	n.d.	< 0·001 AU/ml.
	16	16	1/16	< 2	n.d.
S.c. injection	0·5 (+*B. pert.*)	—	13/16	32	n.d.
	0·5 (+*B. pert.*)	0·5 (+*B. pert.*)	16/16	8000	0·80 AU/ml.
	0·5 (+*B. pert.*)	0·5	6/6	n.d.	0·45 AU/ml.

* Doses given 3 weeks apart. † Numbers of survivors/numbers challenged.
‡ Titrations of pooled sera of four animals from each group. n.d. = not determined.

Table 4. *Immune response of mice 3 weeks after single or repeated aerosol administration of mixtures of tetanus toxoid and* B. pertussis *preparations*

Mixture	1st dose (LFU)	2nd dose* (LFU)	Survivors 5 days† after challenge	Antitoxic serum titre‡	
				Indirect HA	Mouse test
T+C	8	—	20/20	n.d.	n.d.
T+E	8	—	20/20	n.d.	n.d.
T+D	8	—	16/20	n.d.	n.d.
T+C	8	8	10/10	320,000	32 AU/ml
T+E	8	8	10/10	160,000	32 AU/ml
T+D	8	8	10/10	80,000	8 AU/ml

T+C: toxoid+intact cells. T+E: toxoid+extract. T+D: toxoid+cell-wall debris.
* Doses given 3 weeks apart.
† Numbers of survivors/numbers challenged.
‡ Titrations of pooled sera of 10 animals from each group; n.d. = not determined.

To make a more accurate comparison between the adjuvant effects in vaccination by aerosol and by injection, groups of mice were treated with varying doses of toxoid in combination with a constant dose (10^8 whole-cell equivalents) of *B. pertussis* extract. In the aerosol treatments the adjuvant was given immediately before the toxoid; the dosage of the latter was adjusted by varying the concentration of the spray solution and the period of inhalation. For s.c. injections the required doses of toxoid and adjuvant were mixed and injected in 0·5 ml volumes.

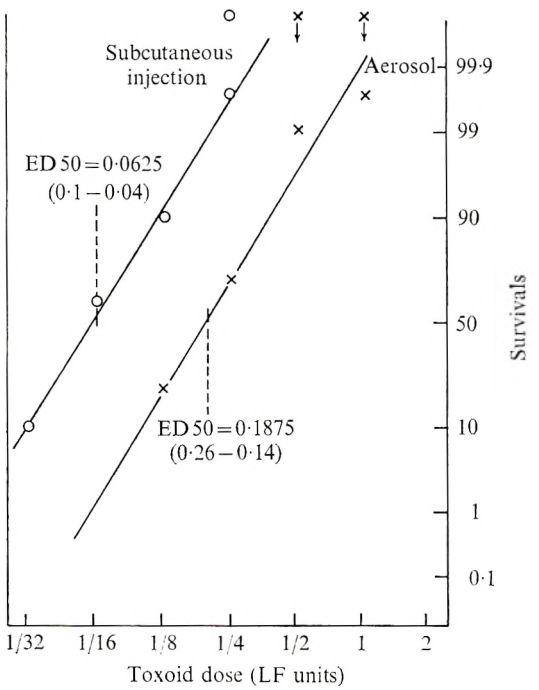
From the challenge survivals given in Table 5 the 50 % protective dose for both the s.c. and respiratory route was calculated according to Litchfield & Wilcoxon (1949). These values were for the aerosol route 0·1875 with 95 % confidence limits of 0·26 and 0·14 and for the s.c. route 0·0625 with 95 % confidence limits of 0·1 and 0·04. Figure 1 shows the log. dose–response lines, both of which have a b-(slope-) value of 4·5.

The s.c. ED50 of plain toxoid was determined in previous studies at 1·2 LFU.

Table 5. *Survival rates of mice challenged* 3 *weeks after immunization with varying doses of tetanus toxoid in combination with a constant dose of* B. pertussis *extract, by aerosol or s.c. injection*

Aerosol		s.c. injection	
Toxoid dose (LFU)	Survivors* 5 days after challenge	Toxoid dose (LFU)	Survivors* 5 days after challenge
0·125	2/10	0·031	1/10
0·25	7/10	0·062	6/10
0·5	10/10	0·125	9/10
1·0	10/10	0·25	10/10

* Numbers of survivors/numbers challenged.



Text-fig. 1. Log. dose–response relationships in s.c. and aerosol vaccination with tetanus toxoid combined with *B. pertussis*.

It appears that this value can be lowered by a factor of the order of 20, if sufficient adjuvant is added. The respiratory ED 50 of plain toxoid could not be evaluated by aerosol treatment but, according to the data already presented, it is likely to be considerably higher than 20 LFU. It would follow that the addition of adjuvant here results in a decrease of the ED 50 value by at least a factor of 100. The indication given already by the preceding experiments (Table 3) that the adjuvant effect is much stronger in immunization by inhalation than by injection is thus fully confirmed.

DISCUSSION

The present study clearly shows that in our experimental model single or repeated inhalation of purified tetanus toxoid in doses up to 50 LFU does not provoke an appreciable immune response in mice. An explanation for these negative results was found in separate studies which demonstrated that plain toxoid given to mice by the respiratory route induces partial unresponsiveness to subsequent injection of the same antigen (Bartlema & Braunius, 1969). It is suggested that this development of tolerance is associated with a gradual and prolonged resorption of the soluble antigen which is distributed over the large surface area of the pulmonary epithelium. The local concentration of the resorbed toxoid would then be too low for it to be effectively phagocytosed and concentrated by macrophages, thus enhancing a tolerogenic effect (Mitchell & Nossal, 1966; Cohn, 1969). It is also conceivable that the inhaled toxoid is subject to a process of 'biological filtration' (Frei, Benacerraf & Thorbecke, 1965) by lung macrophages, resulting in the removal of that part of the antigen which is responsible for its 'intrinsic adjuvanticity' (Dresser, 1968) and, consequently, in a shift of the balance between immunogenic and tolerogenic activity in the resorbed moiety.

To explain the discrepancy between our results and those of others two factors should be considered: (1) the type of test animal, and (2) the purity of the antigen.

The first factor may account for Yamashiroya's observation of a distinct, although weak, primary immune response in guinea-pigs after aerosol treatment with purified tetanus toxoid (Yamashiroya *et al.* 1966). On the other hand, Leclercq (1971), in a recent study on aerosol vaccination of mice with purified toxoid, found results which were very similar to ours in that no antibody production was seen unless a suitable adjuvant was added.

Both factors may be involved in the excellent results of respiratory immunization of monkeys with crude toxoid preparations reported by French authors (Fournier, 1969; Fontanges *et al.* 1970). Here the impurity of the vaccine probably constituted a high degree of 'extrinsic adjuvanticity' (Dresser, 1968).

In our experiments the addition of an adequate adjuvant to the inhaled toxoid changed the type of immune response from tolerance to antibody formation. This has been described for protein antigens like bovine serum albumin – a 'weak immunogen' – when given by intraperitoneal injection in a well-defined dosage scheme (Mitchison, 1964). For toxoid the phenomenon may be strictly associated with aerosol administration.

The failure of $AlPO_4$ to bring about this switch in immune response may be due entirely to the method of administration, i.e. by aerosol. Of the bacterial adjuvants tested the *B. pertussis* preparations had a remarkable effect which could be roughly estimated in terms of reducing the respiratory ED 50 of plain toxoid by at least a factor of 100. This approximate evaluation was confirmed in later experiments where ED 50 values were more precisely determined by intratracheal instillation (J. L. F. Gerbrandy, to be published).

Studies reported by others have yielded evidence that bacterial adjuvants such as Freund's complete adjuvant or *B. pertussis* exert their activity by stimulation

of thymus-derived lymphocytes which would imply that both antibody production and cell-mediated hypersensitivity to the thymus-dependent protein antigen are promoted (Allison & Davies, 1971). This might provide an explanation for the strong adjuvant effect of *B. pertussis* by aerosol treatment. M. P. C. Karelse (to be published) has observed the appearance of excessive cellular infiltrations in the lung after *B. pertussis* administration. These would favour an effective recruitment and stimulation of T-lymphocytes which in turn would promote a state of cell-mediated hypersensitivity but also, through cooperation with B-lymphocytes, would strongly potentiate the antibody response (Roitt *et al.* 1969).

That respiratory vaccination with toxoid combined with *B. pertussis* may provoke a high degree of cell-mediated hypersensitivity to the protein antigen was strongly suggested by our finding that mice, vaccinated in this way and boosted with a small dose of toxoid only, displayed a marked cellular immunity, manifested by their increased resistance against *L. monocytogenes* infection (cf. Dodd, 1970). Ten mice were vaccinated (by intratracheal instillation) with 2 LFU of toxoid and 6×10^8 *B. pertussis* cells. After 3 weeks a second dose of 1 LFU was given by the same route and 2 days later the animals were challenged by i.p. injection of 9×10^5 cells (*ca.* 25 LD 50) of virulent *L. monocytogenes*, along with a control group that was vaccinated with comparable doses by s.c. injection. Nine of the intratracheally vaccinated mice survived, whereas all animals in the control group died. This observation of an enhanced cell-mediated reactivity also would imply that primary respiratory immunization with a thymus-dependent protein antigen and a bacterial adjuvant would not be advisable in view of an increased risk of untoward side effects.

Other factors may be involved as well in the remarkable adjuvant activity displayed by *B. pertussis* in our experimental model. The adjuvant may stimulate the phagocytic or antigen-processing potency of lung macrophages or may enhance the transport of antigen to the draining lymph nodes. A more conclusive explanation of the phenomena observed in the intact animals requires insight into the cellular processes involved in the reactions to the introduction of antigens and adjuvants by the respiratory route. Histological and immunological studies both *in vivo* and *in vitro* were undertaken to explore these processes and will be reported in subsequent articles.

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EXPLANATION OF PLATE

Fig. 1. $AlPO_4$-toxoid aerosol droplets emerging from the nebulizer. $AlPO_4$ particles are visible within the droplets.

Fig. 2. Conglomerates of droplet nuclei formed during passage through the Henderson apparatus.

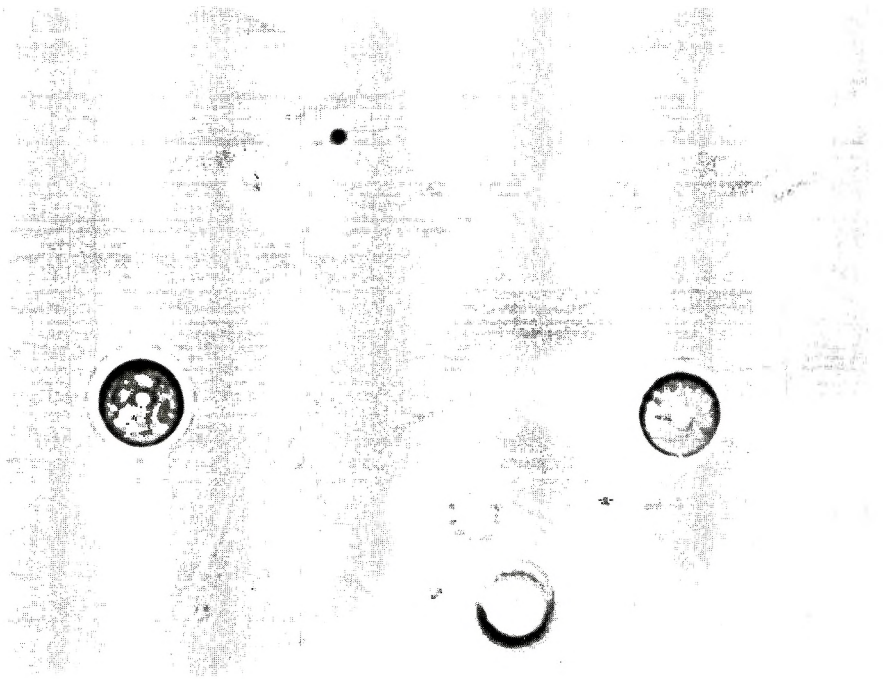

Fig. 1

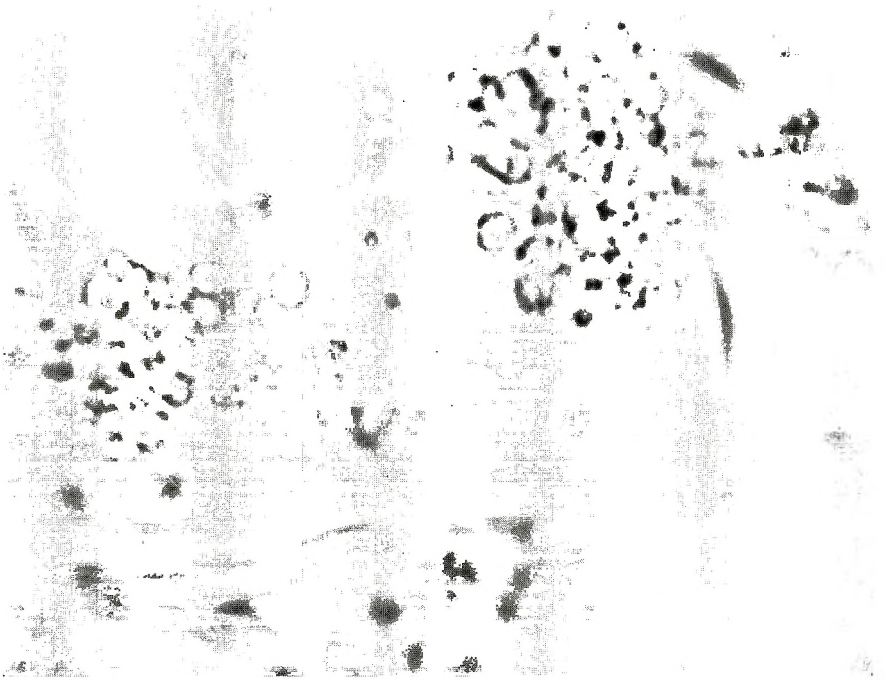

Fig. 2

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Biotyping of Enterobacteriaceae as a test for the evaluation of isolation systems

By D. van der WAAIJ and T. M. SPELTIE
Radiobiological Institute TNO, Lange Kleiweg 151,
Rijswijk (Z.H.), The Netherlands

and J. M. VOSSEN
Isolation Pavilion of J. A. Cohen Institute for Radiopathology and Radioprotection, University Hospital, Leiden, The Netherlands

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SUMMARY

Arguments in favour of biotyping of Enterobacteriaceae excreted in the faeces of isolated patients, as a method of investigating the efficiency of the isolation procedures, are presented as well as a technical outline of the procedure. The study included three kidney transplantation patients, five acute myeloid leukaemia patients and four healthy persons as controls.

The results show, apart from new colonizations during isolation, a difference in the mean number of contaminations and colonizations with different Enterobacteriaceae biotypes. It is concluded from these results, that the isolation procedures were not completely effective and that the AML patients studied had a decreased colonization resistance of their digestive tract. This was less evident in the kidney transplant group.

INTRODUCTION

With the growing interest in the application of reverse isolation systems for patients, the necessity increases for an adequate test-system to evaluate the efficacy of the isolation procedures. In the literature, two approaches have been described: the use of germ-free mice (Barnes, Tuffery & Cook, 1968; Meindersma & Van de Waaij, 1968) and the typing of *Staphylococcus aureus* and *Pseudomonas aeruginosa* obtained from swabs or other samples from isolated patients (Jameson, Gamble, Lynch & Kay, 1971; Lidwell & Towers, 1972). The maintenance of germ-free mice in an isolator is, in fact, only a test of the quality of the isolator and not necessarily of the isolation system. The latter involves, apart from an isolator, all precautions taken to prevent contamination of the inside of the unit. Typing of staphylococci isolated from the patients seems much more adequate. Isolation of staphylococci of a different phage type than was found at admittance, may indicate the occurrence of contaminations in an isolated patient. In a properly functioning isolation system, such contaminations should not occur. Schneider *et al.* (1969) express the efficacy of isolation by comparing the incidence of infections in isolated patients with that in similar patients undergoing similar immuno-

suppressive treatment without isolation. We feel that this is a questionable criterion since it is influenced by many factors apart from protective isolation.

A great majority of the infections seen in patients with a decreased resistance against infections, however, are caused by Gram-negative rods (Hersh, Bodey, Nies & Freireich, 1965). These bacteria, *Pseudomonas aeruginosa* and Enterobacteriaceae species, have a different epidemiology than staphylococci. It is, for example, possible to select the human contacts of a patient on the basis of 'carriage of *Staphylococcus aureus*'. Nurses are excluded for some time from helping isolated patients should they carry *Staph. aureus*. This holds also for *Ps. aeruginosa* (Schneider *et al.* 1969). This species is more frequently involved in dangerous infectious complications in patients with decreased immunocompetence than is *Staph. aureus* (Hersh *et al.* 1965). Selection of the medical personnel or other human contacts for an isolation unit on the basis of 'carriage of Enterobacteriaceae species' is impossible, since everyone is colonized by one or more of these species.

These considerations have also been found to be applicable to bone marrow transplantation experiments in monkeys. About 3 years ago, a typing procedure was sought for the typing of Enterobacteriaceae species in isolated *Macaca mulatta*. Serological typing was discarded, since (except for salmonellas and shigellas) it is mainly confined to two Enterobacteriaceae species: *Escherichia coli* (Kauffmann, 1947) and representatives of the family of *Klebsiella* (Ørskov, 1952, 1954). Secondly, it is a very laborious technique. The possibility of 'biotyping' was therefore investigated. With this technique, the isolation precautions taken in the treatment of lethally irradiated monkeys could be investigated and improved (Van der Waaij, unpublished data).

Recently, this approach was also applied to patients treated in the Unit for protective isolation at the University Hospital of Leiden. In two groups of patients and in four healthy controls an inventory was made at the onset of the study. Subsequently, the occurrence of contaminations (if a biotype was only isolated once) as well as of colonization (if a biotype was found in several subsequent samples from the same patient) with Enterobacteriaceae species, was investigated. To this end, at least twice a week, faecal samples were processed for pure culturing of at least 20 Enterobacteriaceae colonies per sample. These were then biotyped. One group of five patients consisted of acute myeloid leukemia (AML) patients. The other consisted of three patients treated with immunosuppressive drugs after kidney transplantation. The term 'contamination' will be used in this report to describe the situation where the patient acquires such a number of bacteria of a certain biotype that a 'take' occurs. This implies that for a short period of 1 or 2 days, a concentration of at least 10^4 cells/g. is reached in the faeces of that patient. The term 'colonization' will be used in those cases where a biotype persists in the patient for a time interval longer than 4 days.

MATERIALS AND METHODS

Sampling

The investigation period in the eight patients involved in this study depended on the duration of their stay in the isolation ward, but was at least 2 weeks. The

Table 1. *Tests used in biotyping Enterobacteriaceae*

Serial code number	Test	Serial code number	Test
1	Xylose	11	Dulcitol
2	Maltose	12	Ornithine decarboxyl.
3	Rhamnose	13	Lysine decarboxyl.
4	Mannitol	14	H_2S
5	Arabinose	15	Urease
6	Sorbitol	16	Citrate
7	Lactose	17	Inositol
8	Indol	18	Adonitol
9	Sucrose	19	Raffinose
10	Salicine		

four healthy controls were followed for 6 weeks. Faeces samples were collected twice a week from the patients as well as from the controls. In most cases, faecal samples of 1–2 g. were either directly processed, or stored for some time in liquid nitrogen. Fresh faecal material, as well as material that had been stored in liquid nitrogen, was suspended (1/10) in brain heart infusion (BHI) broth (DIFCO). Directly, and after incubation for 4 hr. at 37° C., subinoculation onto Endo-agar (DIFCO) was performed. The faeces were also streaked directly onto Endo-agar after suspending in saline in a dilution of 1/10.

Isolation of pure cultures

After 24 hr. incubation of the Endo plates, at least 20 (where possible) morphologically different colonies (per faecal sample) were picked from the Endo-agar and subinoculated back onto fresh Endo-agar. Each colony received its own serial number (1 to 20) which was not changed until the typing had been completed. Pure culturing was continued after incubation of the second series of Endo-agars on Kligler slants (DIFCO). On this medium the 'purity' of the cultures was carefully checked. In addition, the Kligler slants gave the results of two biochemical reactions: fermentation of lactose and formation of H_2S.

Fermentation reactions

Most of the fermentation reactions (Table 1) were performed in plastic trays with 64 cups of 2 ml. volume. For this purpose the technique and the media described by Guinée, van Leeuwen & Jansen (1972) were used. Tests for decarboxylase and urease production were performed in the same plastic trays. These cups, however, were sealed after inoculation. Inoculation of cups was performed with two drops of a saline suspension consisting of approximately 10^8 bacteria/ml. Such suspensions were prepared from each pure culture on the Kligler slants. To prevent contamination and dehydration of the media during incubation, the trays were incubated inside well closed and disinfected plastic boxes.

Reading of the tests

The tests were read after 24 hr. of incubation at 37° C. Reading after longer time intervals gave different and less constant results. It should be mentioned here

that, in several cases, the classical routine bacteriological results were not obtained. 'Slow lactose fermenters', for example, are listed as 'positive' in routine bacteriology. Twenty-four hours incubation, however, has mostly been too short in our typing system for these strains to demonstrate this capacity. For typing purposes, we were not interested in strain identification, but in constancy of the results. Each strain, when tested repeatedly, should give identical results in the 19 reactions selected. Use of this system in monkeys had indicated that, with a few exceptions, these biotype characteristics are stable properties of Enterobacteriaceae.

Coding of the results

The results were coded by use of the binary system. The strains were thus identified by a number generated by the formula

$$\sum_{i=1}^{20} a_i \times 2^{(20-i)},$$

in which i = code number of the test (Table 1), $a_i = 1$ if the ith test is positive and $a_i = 0$ if the ith test is negative. For example: if the tests with the serial numbers 1, 2, 12, 13 and 16 were positive, the biotype of this particular strain would be

$$2^{20-1} + 2^{20-2} + 2^{20-12} + 2^{20-13} + 2^{20-16} = 786{,}832.$$

Decoding and 'translation' of the biotypes back to the standard bacteriological nomenclature is obviously possible at any time, but this is unnecessary in epidemiological studies.

Method of determining whether as many as possible different Enterobacteriaceae biotypes had been isolated per sample

Twenty Enterobacteriaceae colonies per sample were typed as a minimum. Whether or not a sufficient number of morphologically different colonies had been typed to allow a good inventory of an individual was investigated by giving the isolates a serial number. The number of different biotypes found in the first group of four colonies was then plotted at the 4th place of the abscissus. The number of biotypes differing from the first four and from each other in the second group of four colonies typed is plotted at the 8th place on the abscissus, etc. (Fig. 1). A good insight into the adequacy of the number of colonies typed was then obtained by drawing a curve through these points. If this curve reached the abscissus in the 5th group of four (numbers 17, 18, 19 and 20) it was assumed that a sufficient number of colonies had been typed. If the curve did not reach the abscissus in the 5th group, more colonies were isolated from the original Endo plates that had been stored in the refrigerator. The number of subsequent isolates depended on the slope of the curve found with the first 20 colonies (Fig. 1).

Test for minimum detectable concentration of Enterobacteriaceae biotypes in faeces

For this purpose, suspensions of streptomycin-resistant strains of *E. coli*, *Proteus mirabilis* and *Klebsiella pneumoniae* were mixed in different concentrations with human faeces free of streptomycin-resistant Enterobacteriaceae species. The biotypes of these antibiotic resistant strains were known, the resistance to streptomycin gave the strains an additional marker. The concentrations obtained in the

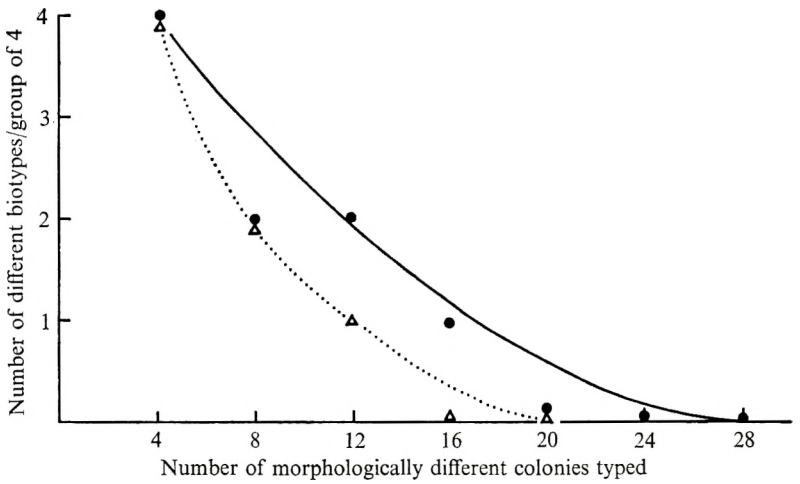

Fig. 1. Number of colonies required for a good inventory of the number of different Enterobacteriaceae biotypes in a faecal sample. The curves are shown of two representative cases: in one 20 colonies were sufficient, in the other eight more were required.

faeces varied between 10^3 and 10^7 bacterial cells per g. Isolation of the 'test strains' from these mixtures was performed in two ways: (1) the method described above; and (2) the faecal material was streaked onto Endo-streptomycin agar (2 mg/ml).

Isolation system for patients

The isolation facilities and the precautions used have been described elsewhere in detail (Vossen & Van der Waaij, 1972). The patients are maintained in single-bed rooms provided with a personnel ante-room as well as an entry lock for materials. The rooms are mechanically ventilated with filtered air (unipack AAF) providing nine changes/hr. The rooms are well disinfected before use. All items, except food and beverages, are double wrapped and sterilized by steam or ethylene oxide. Food and beverages are prepared in the kitchen of the Unit. Each room has a sink and is provided with a toilet that can be reached via the personnel ante-room.

Antibiotics

The antibiotics used for the therapy of infections in the isolated patients were:

cephaloridin (parenteral)	ampicilin (parenteral)
gentamycin (parenteral)	orbenin (oral)
rifampicin (parenteral)	acipen (oral)
celbenin (parenteral)	

The sensitivity pattern of 'colonizing' biotypes was investigated on BHI-agar with antibiotic disks consisting of the following antibiotics: streptomycin, kanamycin, ampicillin, carbenicillin, terramycin, chloramphenicol.

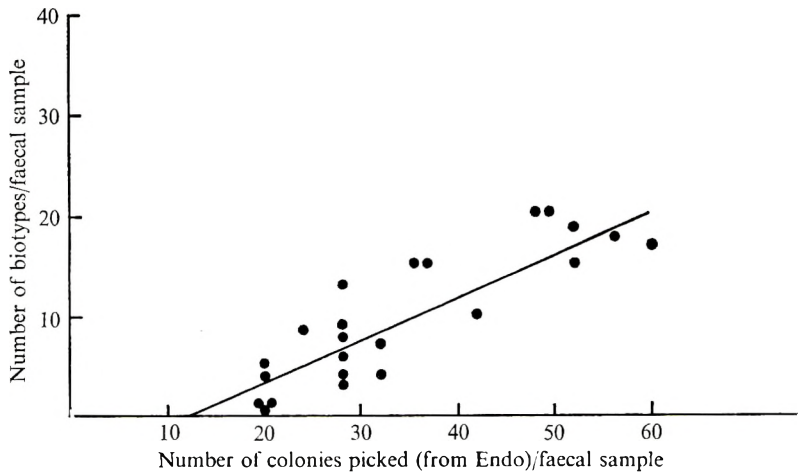

Fig. 2. Correlation between the number of different biotypes and the number of colonies picked from an Endo plate inoculated with faecal material.

RESULTS

Technical

The minumum detectable concentration of Enterobacteriaceae species in the faeces appeared to be about 10^4 bacteria/g. Both by biotyping and on the Endo-streptomycin agar, the Enterobacteriaceae species mixed with the faeces were recovered from six out of nine samples containing 10^4 cells/g. and from all higher concentrations. Since the sample size for biotyping was approximately 1 mg., it is to be expected that samples containing 10^3 cells/g. or less, would not yield sufficient colonies for our purposes.

The number of colonies to be picked from an Endo according to the curve (Fig. 1) was plotted against the number of different biotypes found per faecal sample (Fig. 2). The correlation between the number of isolates and the number of different biotypes appeared to be rather constant (S.E. = 0·060).

Patients

During their stay in the Unit, five patients had infections and were treated with antibiotics. Both the oral and the parenteral route of administration was used. The first turned out to have an interesting influence on the contamination and colonization (C and C) pattern. In Fig. 3, the 'C and C-pattern' of a representative healthy control is depicted, whereas Figs. 4 and 5 show the C and C-patterns of a representative kidney transplantation patient and an AML patient respectively.

The mean number of 'contaminations' seen in the unisolated healthy controls was found to be lower (mean number less than 2) than in the isolated AML-patients (mean number between 3 and 6) (Fig. 6). The isolated kidney transplanted patients were more of the control type in this respect. It is also seen that the mean number of contaminations that resulted in colonization for some time, was somewhat higher in the AML patients than in both other groups (Fig. 6). The influence of oral and systemic antibiotic treatment on the 'C and C pattern' is given in Table 2. Systemic antibiotic treatment appeared to have no significant influence. Oral

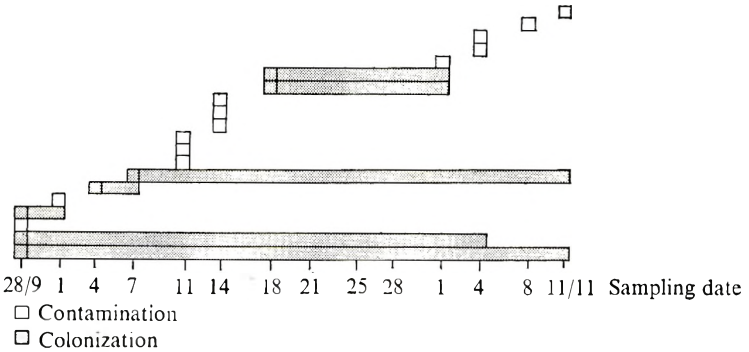

Fig. 3. Results of biotyping of Enterobacteriaceae species of faecal samples from a healthy control (not isolated).

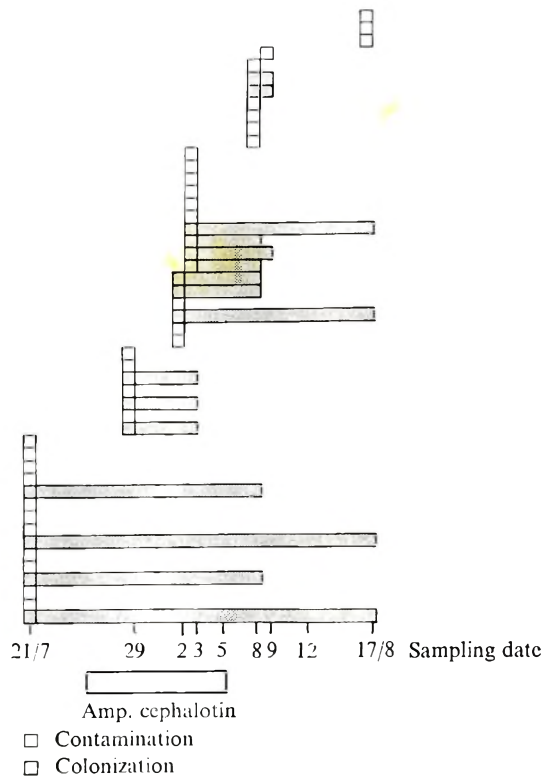

Fig. 4. Results of biotyping of Enterobacteriaceae species of faecal samples from a kidney transplantation patient (isolated).

treatment for which only penicillins like acipen and orbenin were used, strongly increased the number of contaminations.

Similar results have been obtained in monkeys (van der Waaij, unpublished data). An increase in the mean number of contaminations and in some degree of colonization was seen in all nine animals studied, during the period of decreased immunecompetence. This was the more evident, the less the precautions taken to prevent

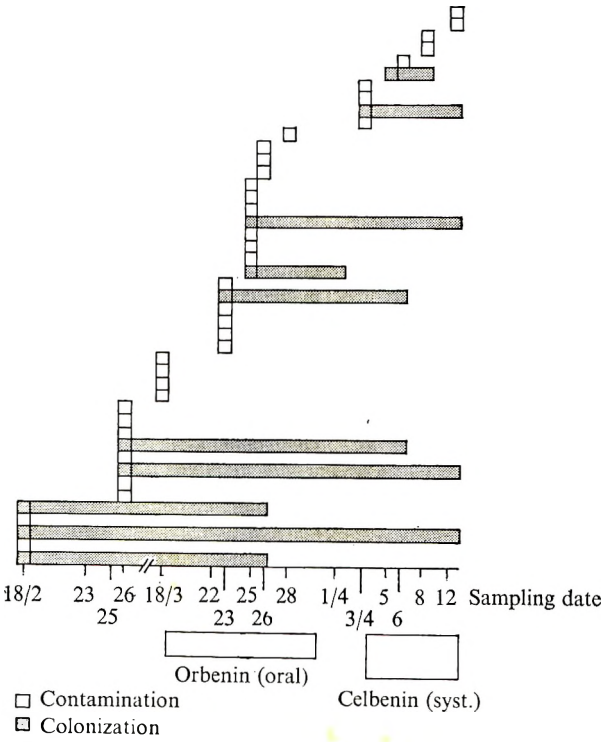

Fig. 5. Results of biotyping of Enterobacteriaceae species of faecal samples from an AML patient (isolated).

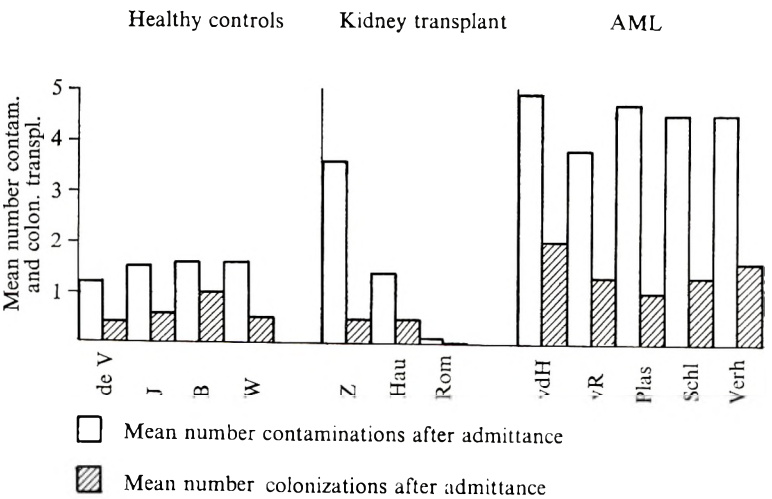

Fig. 6. Mean number of contaminations and colonizations after admission in the three groups studied.

contaminations. The influence of antibiotic treatment has also been clearly demonstrated in monkeys.

The occurrence of cross-contaminations with Enterobacteriaceae species between patients was our next concern. During the period of March, April and part of May

Table 2. *Mean number of contaminations and colonizations in six isolated acute myeloid leukaemia patients during antibiotic treatment*

Treatment	Mean number of contaminations	Mean number of colonizations
Oral antibiotic	5·1	0·9
Systemic antibiotic	3·4	1·3
No antibiotic treatment	3·3	0·9

Table 3. 173 *contaminations in five patients during* 135 *isolation days in the period March–April* 1971

146	occurring in one patient at a time
23	occurring in two patients at a time
2	occurring in three patients at a time
2	occurring in four patients at a time

1971, five patients involved in this study were isolated, either with an overlap or simultaneously. In these patients, identical biotypes had been observed on several occasions (Table 3). In about 50 % of the cases, a certain biotype was isolated from two patients in the same week. This may indicate that both had been contaminated by the same primary (food?) source. In the other 50 %, however, cross-contamination between patients could not be excluded because of an interval of at least 1 week between the isolation of the same biotype from two different patients. As in observations described by Selden *et al.* (1971), and Winterbauer, Turck & Petersdorf (1967), only 'colonizing biotypes' appeared to be involved in infectious processes in the patients. In the three kidney transplantation patients, only biotypes were involved that were also found at admission. This was different in the AML-patients. In this group, in two out of four cases, biotypes that colonized the patient for the first time during isolation were involved.

Colonizing biotypes were also submitted to an antibiotic sensitivity test. All 16 strains thus tested were found to have a normal sensitivity pattern. This may indicate that the 'leak' found in the barrier of the isolation system was not one from the general hospital outside, since many resistant Enterobacteriaceae species are known to circulate there.

DISCUSSION

The results of this study have shown that the biotyping technique is applicable for testing the efficiency of isolation systems. Incidental airborne contaminations of the tray in the laboratory may have influenced the result of biotyping in that it 'changed' a biotype in making one of the fermentation reactions falsely positive. We feel however, that, provided the test is performed very carefully and precisely, *particularly with regard to pure culturing*, biotyping of Enterobacteriaceae gives a good insight into the efficiency of the various precautions taken to prevent exogenous infections. Even if one should now and then falsely report a new biotype, which is due to a false positive test, this will not influence the conclusion which is based on the mean number of contaminations and colonizations. With regard to

isolation, it should be mentioned that only colonizing strains are potentially dangerous with respect to causing infections (Kessner & Lepper, 1967). Secondly, only an increase in the mean number of contaminations recorded in a period of at least two weeks, should be considered as an indication that the Colonization Resistance (CR) (Van der Waaij, Berghuis-de Vries & Lekkerkerk-van der Wees, 1971) of the digestive tract of a patient has decreased.

Patients suffering from AML, being remarkably susceptible to infections, all showed an increased mean number of contaminations with Enterobacteriaceae biotypes. This may indicate that they also had a decreased CR, which may have enhanced the risk of colonization by an infectious agent. Colonization by Enterobacteriaceae species, however, was apparently limited to biotypes that were introduced into the isolation ward from sources other than the general ward.

In the AML patients more contaminations and colonizations were observed than in healthy controls. The Contamination–Colonization incidence in the kidney transplantation patients under immunosuppressive treatment however, was more of the type of the healthy controls, than of the AML group of patients. Immunosuppression performed in both groups of patients, under conditions provided at this Isolation Unit, apparently does not necessarily adversely influence the Colonization Resistance. Oral treatment with penicillins, however, significantly increased the number of colonizations during isolation. Possibly, the factors controlling the occurrence of contaminations are more strongly influenced by oral penicillin treatment than are those controlling colonizations. A more likely explanation, however, seems to be one that takes into account an asymmetric distribution of the numbers of bacteria of the various biotypes involved in contamination and colonization. Because of all precautions taken to prevent contamination, in the Isolation Pavilion, the peak occurrence of contamination will have been in the very low dose range. Assuming that, as in mice, higher numbers of bacteria are required for colonization than for contamination (Van der Waaij *et al.* 1971), the large majority of contaminations may have gone unnoticed in isolated AML patients. When, however, the CR was significantly decreased by the oral penicillin treatment, several of these 'low dose contaminations' may have taken for a few days, reaching the minimally detectable concentration in the faeces of 10^4 cells/g. In the biotyping procedure they were then recorded as a contamination. If the dose distribution had indeed been asymmetrical only a few biotypes in the higher dose contaminations may have moved into the colonization category (which requires relatively higher doses).

Isolation of patients with a strongly decreased defence capacity in single-bed rooms under isolation conditions appeared to be more complicated than was expected. The relatively high incidence of contaminations and colonizations seen in the isolated patients indicates a potential chance of contamination with more pathogenic and more resistant Gram-negative rods. Most of the contaminating Enterobacteriaceae species isolated were, presumably, of low pathogenicity. All colonizing exogenous strains had a good sensitivity pattern. This may explain the fact that, in the patients studied, most of the exogenous colonizing biotypes did not cause infections.

Others have described similar experiences with comparable isolation systems. Jameson *et al.* (1971) mentioned the occurrence of a flaw in kitchen procedure as the source of contaminations with a biochemically atypical coliform. They also describe an outbreak of *Pseudomonas* colonizations which could be traced to a nurse carrying this phage type of *Pseudomonas*. In our system, we also have indications that both the food and the staff may have been the main contamination sources. The personnel did not wear gloves during handling of the patients or in preparing the food. Solberg *et al.* (1971) described a very successful isolation of a patient in a laminar flow room. The latter will have only prevented airborne contamination. All materials entering the room, including food, were double wrapped and sterilized. The staff were well shielded wearing sterilized hoods, coats, calf-length boots, face masks, and gloves. Applying the same type of isolation, Vossen & Van der Waaij (1972) also reported similar successful isolation of several patients.

After this investigation was completed, the ventilation system of the isolation rooms was improved. Preparation of sterile food and beverages is now performed under strict aseptic conditions, while handling of the patients is performed with sterile gloves. Use of the toilet by the patient is no longer permitted. Instead, sterilized disposable bed-pans are employed. The sink has been modified so as to make effective daily disinfection possible. Under these isolation conditions, the study will be repeated.

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Use of tartaric acid isomers and citric acid in the biotyping of *Salmonella typhimurium*

By G. A. ALFREDSSON, RUTH M. BARKER, D. C. OLD and J. P. DUGUID

Bacteriology Department, University of Dundee

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SUMMARY

The colour-change and lead acetate tests for fermentation of *d*-, *l*- and *m*-tartaric acids and citric acid used in the Kristensen scheme for biotyping *Salmonella typhimurium* were found to be unreliable because, whatever the conditions of culture, they gave different results in replicate tests of the same strains. Many genotypically non-fermenting strains gave inconsistent reactions due to the emergence of fermenting mutant bacilli in some of their test cultures. No reliable test was found for the fermentation of citric acid.

A 'turbidity' test was found to give consistent and reliable results with the three tartaric acid isomers. It demonstrated fermentation by the significantly greater amount of growth obtained in a 24 hr. culture in Oxoid peptone water with added isomer than in a control culture without isomer. Lewis & Stocker's (1971) plate-inhibition test for fermentation of *m*-tartrate, which identifies *m*-tartrate-negative strains because *m*-tartrate inhibits their growth on citrate- or glycerol-containing minimal medium, was found to be as reliable as, and easier to read than, the turbidity test.

Use of the turbidity test for *d*- and *l*-tartrates and the plate-inhibition test for *m*-tartrate in biotyping 1435 strains of *S. typhimurium* showed that many strains had previously been mistyped by the lead acetate test and distinguished 16 new biotypes in addition to the 22 biotypes already recognized.

INTRODUCTION

Type differentiation within a pathogenic species such as *Salmonella typhimurium* is valuable for epidemiological studies and may be done with different kinds of discriminating tests. Bacteriophage typing gives reliable results. A system devised by Felix & Callow (1943, 1951) and extended by Callow (1959) distinguished 34 phage-types of *S. typhimurium*, and many further 'provisional' types in this scheme have been identified at the Enteric Reference Laboratory of the Public Health Laboratory Service, London (E. S. Anderson, unpublished). Another phage-typing scheme, that of Lilleengen (1948), subdivides *S. typhimurium* into 24 types.

Biochemical methods have been used to differentiate strains of *S. typhimurium* into epidemiologically significant 'fermentation types', or 'biotypes'. With tests on nine substrates, Kristensen, Bojlen & Faarup (1937) differentiated 361 strains

Table 1. *The Kristensen scheme for biotyping* Salmonella typhimurium *as used by Kallings & Laurell* (1957)

Biotype	Acid produced within 24 hr. in peptone water from: Xylose	Rham-nose	Inositol	Stern's glycerol reaction in 48 hr.	Lead acetate test for fermentation in peptone water: *d*-Tartrate (24 hr.)	*l*-Tartrate (24 hr.)	*m*-Tartrate (5 days)	Citrate (3 days)	Growth on glucose ammonium agar in 48 hr.	Acid in Bitter-xylose medium in 20 hr.
1	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	−
2 M	−	+	+	+	+	+	+	+	+	−
3	+	+	+	+	+	+	+	+	−	−
4	+	+	+	+	+	+	−	+	+	−
5	+	+	+	+	+	−	+	+	+	−
6	+	+	+	+	−	+	+	+	+	+
7	+	+	+	−	+	+	+	+	+	+
8	+	+	+	−	+	+	+	+	+	−
9	+	+	−	+	+	+	+	+	+	+
10	+	+	−	+	+	+	+	+	+	−
11	+	+	−	+	+	+	+	+	−	−
12	+	+	−	+	+	+	−	+	+	−
13	+	+	−	−	+	+	+	+	+	−
14	+	−	+	+	−	+	+	−	+	−
15	+	−	−	+	+	+	+	+	+	−
16	+	−	−	+	+	+	−	+	+	−
17	+	−	−	+	−	+	+	−	+	−
18	+	−	−	−	−	+	+	−	+	−
19	+	−	+	+	+	+	+	+	+	−
20	+	−	+	+	−	+	+	−	−	−

of *S. typhimurium* into 18 biotypes and their scheme was extended by the addition of type 20 by Hansen (1942) and types 2M and 19 by Harhoff (1948). The extended scheme is shown in Table 1. Kallings & Laurell (1957), Kallings, Laurell & Zetterberg (1959), Rische & Kretzschmar (1962) and Lewis & Stocker (1971) later showed that strains of the same phage-type can be differentiated into a number of different biotypes, so that the combination of biotyping with phage-typing gives greater precision in defining relationships between strains.

The successful application of biotyping depends on the use of *reliable* tests, i.e. tests in which each strain gives the same result on different occasions of testing and in which the result is not unduly influenced by minor or uncontrollable variations in the method. In a study of 1435 strains of *S. typhimurium* we have found that many strains are inconstant in their reactions with the four organic acids used in the scheme of Kristensen *et al.* (1937) when the tests are performed by the methods of these and later workers. The organic acids are the *dextro* (*d*), *laevo* (*l*), and *meso* (*m*) isomers of tartaric acid, and citric acid. The methods of testing for their fermentation were originally developed by Brown, Duncan & Henry (1924). Bacteria are cultured at 37° C. in Difco Bacto–Peptone water containing the organic acid and a positive reaction is read by the observation of (i) an increased amount of growth, (ii) a reduced production of precipitate on the

addition of lead acetate, or (iii) a change of colour in bromothymol blue indicator to yellow (acid). Different authors have used different periods of incubation from 24 hr. to 14 days and different methods of observation for the definitive readings, and there is no agreement on what methods and criteria are optimal for each organic acid.

In our preliminary work we found that many strains of *S. typhimurium* gave different results in replicate test cultures observed after the same period of incubation and that the proportion of positive reactions in sets of replicate tests increased with the period of incubation from 1 to 14 days. These 'variable' strains appeared to be genotypically non-fermenting but to give late fermentation by the emergence of fermenting mutants on prolonged culture, as has been shown for *d*-tartrate by Kristensen *et al.* (1937) and Kristensen (1944). We therefore investigated different methods of performing the tests in an attempt to determine a method of testing and a duration of incubation that would give the same results in all replicate tests of the same strain and would reliably distinguish genotypically fermenting ('positive') strains from genotypically non-fermenting ('negative') strains that are capable of giving late, mutative fermentation. We included among the methods to be evaluated the recently described plate-inhibition test of Lewis & Stocker (1971) for fermentation of *m*-tartrate.

MATERIALS AND METHODS

Bacteria

Strains (1435) of *Salmonella typhimurium* were mainly from a collection previously biotyped and examined for fimbriae by Duguid, Anderson & Campbell (1966). They were from a wide range of sources and places of origin and included representatives of all 21 biotypes, except type 14, in the extended Kristensen scheme and biotype 19Xd, which resembles type 6 except that it is rhamnose-negative (Morgenroth & Duguid, 1968).

We describe these strains and their biotypes as being 'positive' or 'negative' for the organic acid under test according to whether or not they utilized the acid for growth under either aerobic or anaerobic conditions. The biotypes of strains are given as ultimately determined by the tests recommended in this paper and in some cases differ from those determined by the methods of previous authors. Biotypes resembling previously recognized types, but differing from them in giving a negative reaction with one or more of the tartaric acid isomers, are designated by the number of the recognized type followed by the symbols *d*T−, *l*T− or *m*T−, e.g. 2*d*T−, 10*d*T−*l*T−.

Organic acids

Sodium citrate ($Na_3C_6H_5O_7.2H_2O$) and the three isomers of tartaric acid, namely *d*(+), $[(CH(OH).COOH)]_2$, *l*(−), $[(CH(OH).COOH)]_2$, and *m* (inactive), $[(CH(OH).COOH)]_2.H_2O$, were from British Drug Houses Ltd. *d*-Tartrate was used in some experiments as potassium (+) tartrate. The acids were prepared as 10% solutions in deionized water and neutralized with 5N-NaOH; the solutions were autoclaved and their pH was then 7·2.

Culture media

Nutrient broth was Oxoid Nutrient Broth no. 2, pH 7·5. Nutrient agar was Oxoid Nutrient Agar, pH 7·4; it was poured in *ca.* 20 ml. amounts in plastic Petri dishes, 8·5 cm. diameter.

Media for colour-change and lead acetate tests were 1 % Difco 'certified' Bacto-Peptone with 1 % *d*-tartrate (potassium salt), 0·5 % *l*-tartaric acid, 0·5 % *m*-tartaric acid or 1 % sodium citrate (Kauffmann, 1966). The acids were neutralized with NaOH and the pH of the completed medium was 7·2. For colour-change tests, 12 ml. of 0·2 % aqueous solution of bromothymol blue was added per litre. The medium was dispensed in 5 ml. amounts in screw-capped bijou bottles, capacity 6 ml.; the caps were closed tightly and the bottles autoclaved at 121° C. for 15 min.

Medium for turbidity tests was 1 % Oxoid peptone water (pH 7·2), i.e. 0·67 % Oxoid L37 peptone and 0·33 % sodium chloride in deionized water; it was autoclaved at 121° C. for 15 min. A portion of each batch of peptone water without the addition of organic acid was kept for use for control cultures. The four organic acids were added as sterile (autoclaved) 10 % solutions to four other portions of the peptone water, the *d*-, *l*- and *m*-tartrates to give final concentrations of 10 g./l. and the sodium citrate to give 2·5 g./l. The completed media (pH 7·2) were dispensed in 10 ml. amounts in cotton-wool stoppered test-tubes (1·5 × 15 cm.) and, for shaken cultures, in 20 ml. amounts in wide-mouthed glass jars, capacity 200 ml., with aluminium screw caps. Comparative tests were made with similar media in which 1 % Difco Bacto-Peptone solution was used as the basic peptone water.

Minimal medium was that of Davis & Mingioli (1950). It contained per litre of deionized water, 7 g. K_2HPO_4, 3 g. KH_2PO_4, 1 g. $(NH_4)_2SO_4$ and 0·1 g. $MgSO_4 . 7H_2O$, pH 7·0. Minimal agar medium contained also 12 g./l. Oxoid Ionagar no. 2. Carbon sources were added in the following amounts (g./l.): sodium citrate 2·5; glycerol 5·0; *d*-, *l*- or *m*-tartrate 5·0; or sodium citrate 2·5 plus *m*-tartrate 5·0. The media were poured in amounts of 20 ml. in dishes 8·5 cm. diameter.

Preparation of inocula for tests

Cultures of *S. typhimurium* were maintained on Dorset's egg slants at ambient temperature. The test strain was plated on nutrient agar and after incubation for 20–24 hr. at 37° C. the bacterial inoculum was prepared by one of the following methods, which gave nearly similar results: (i) a single colony was inoculated into nutrient broth and incubated aerobically and statically for 24 hr., or (ii) several colonies were suspended in saline (0·85 % NaCl) solution to a density of about 10^9 bacteria per ml. Test media were given inocula of *ca.* 0·05 ml. of the broth culture or saline suspension per 10 ml.

Colour change and lead acetate tests

In the definitive tests the inoculated bijou bottles were incubated for 24 hr. at 37° C. under aerobic and static conditions with their caps lying *loosely* on their tops. In comparative tests incubation was prolonged to 2 or more days, or the

bottles were tightly capped. The colour of the incubated test was noted, yellow being read as positive and blue or green as probably negative. A saturated aqueous solution of lead acetate was then added to the culture, or to a 1 ml. sample of it taken into a small tube, in the proportion of 0·1 ml. lead acetate per 1 ml. culture. After mixing and standing for 18–24 hr. to allow the precipitate to settle, the height of the precipitate in the bottle or tube was observed. If the precipitate occupied less than a half the height of the medium (generally less than a fifth) the test was read as positive and if more than a half (generally about three-quarters) it was read as negative.

Turbidity test for stimulation of growth

Cultures were inoculated and incubated in parallel in Oxoid peptone water containing the organic acid under test and in Oxoid peptone water without added organic acid. In the definitive tests incubation of tubes of 10 ml. medium was for 24 hr. at 37° C. under aerobic static conditions, i.e. in air, but without movement or artificial aeration, and the tubes were stoppered with cotton-wool. In special tests, 20 ml. cultures in wide jars were incubated aerobically for 24 hr. at 37° C. with shaking at 160 rev./min. in an incubator shaker. The cultures were killed by the addition of formaldehyde to 0·1 % and after uniform mixing the turbidity of the organic acid-containing culture was compared with that in the organic acid-free culture, first with the naked eye and then by measurement with a Spekker photoelectric absorptiometer with a no. 5 filter.

In tests for the utilization of *d*- or *l*-tartrate the result was read as positive if the turbidity in the tartrate-containing medium was more than 100 % greater than the turbidity in the tartrate-free (control) medium and as negative if it was less than 50 % greater than that in the control. The few tests in which the difference was 50–100 % were read as doubtful and repeated. In most positive tests the difference was so great, e.g. 500 %, that reliance could be placed on reading with the naked eye.

In tests for utilization of *m*-tartrate the result was read as positive if the turbidity of the *m*-tartrate-containing culture was more than 35 % *greater* than the turbidity in the control culture and as negative when it was more than 10 % *less* than that in the control, i.e. the growth of the culture had been inhibited by the presence of the *m*-tartrate. Tests in which the difference was intermediate, that is between + 35 % and − 10 %, were read as doubtful and repeated.

Test for growth on minimal agar medium

Saline suspensions of bacteria of twelve strains were inoculated with a twelve-pronged inoculator in spots in equivalent positions, 2 cm. apart, on a series of plates of minimal agar medium, each of which contained one of the organic acids or glycerol as sole carbon source. The plates were enclosed in plastic bags to minimize drying and were incubated aerobically at 37° C. They were read for the presence and amount of growth over each spot inoculation after 24 hr., 48 hr. and 7 days.

Plate-inhibition test for m-*tartrate*

Bacteria of twelve strains were cultured in parallel on two plates of minimal agar medium, the first of which contained sodium citrate as sole carbon source and the second, *m*-tartrate and sodium citrate together. The bacteria were inoculated and the plates incubated as in the test for growth on minimal agar. The amount of growth was observed after 24 and 48 hr., the definitive reading being at 48hr. A strain was read as positive with *m*-tartrate if it gave as much growth on the plate with *m*-tartrate and sodium citrate as on that with sodium citrate alone, and as negative if its growth was very much less on the former than on the latter plate, i.e. if it was *inhibited* by the presence of *m*-tartrate.

A few strains did not grow well on sodium citrate as sole carbon source and these strains were re-tested on plates containing glycerol in place of the sodium citrate. Auxotrophic strains were re-tested on sets of plates supplemented with the required growth factor at the concentration recommended by Meynell & Meynell (1965, pp. 36, 37).

RESULTS

Comparison of methods

Each of the 1435 strains of *S. typhimurium* was tested at least twice by the colour-change and lead acetate methods and once by the minimal agar growth method with each of the four organic acids, and at least once by the 24 hr. turbidity method with *d*- and *l*-tartrates and by the plate-inhibition method with *m*-tartrate. In addition, a number of strains selected from the principal biotypes were examined in series of 3–10 replicate tests by the lead acetate and turbidity methods and in tests by various modified methods.

The colour-change test proved to be very unreliable and frequently gave false-negative results. In tests in which the bromothymol blue indicator changed to the acid, yellow colour the positive result was generally confirmed by the lead acetate and turbidity methods, but in many of the tests that remained blue or green, and therefore were read as negative, the addition of lead acetate showed that the organic acid had in fact been utilized.

Lead acetate test for fermentation of d-tartrate

When done on cultures incubated in loosely capped bottles for 24 hr., the lead acetate test gave clear-cut results with the majority of strains, dividing them into two groups: (1) strains, mostly in biotypes 1, 2, 8, 9, 10, 11 and 12, that regularly gave positive results in replicate tests, and (2) strains, mostly in biotypes 6, 17, 18 and 19Xd, that regularly gave negative results in replicate tests. Strains of the latter, negative group gave positive results in a proportion of replicate tests if incubation was prolonged beyond 24 hr.; a few tests were positive at 2 days and an increasing proportion became positive after longer periods up to 14 days.

Unfortunately there was a minority of strains that did not give regular results in replicate tests made at 24 hr. Some strains of biotypes 15 and 16, which generally gave a positive result at 24 hr., and which turbidity tests at 24 hr. also

showed to be *d*-tartrate-positive, occasionally gave a negative lead acetate reaction at 24 hr. Conversely, some strains of biotypes 2*d*T−, 10*d*T− and 16*d*T−, which generally gave a negative lead acetate reaction at 24 hr., and which turbidity tests also showed to be *d*-tartrate-negative, occasionally gave a positive lead acetate reaction in replicate tests at 24 hr. Thus a single 24 hr. lead acetate test sometimes wrongly determined the *d*-tartrate genotype of a strain in these minority groups.

Mutative fermentation

The irregular production of positive results after periods of incubation greater than 24 hr., i.e. 'late' fermentation, appeared to be due to the emergence of *d*-tartrate-positive mutants in cultures of strains of a *d*-tartrate-negative genotype. This explanation was confirmed in the case of a few strains by plating out the late-fermented culture and growing separate subcultures from several of the colonies. A proportion of these subcultures were found to behave as typical *d*-tartrate-positive strains, regularly giving positive reactions in tests incubated for 24 hr.

There were marked differences in the frequency with which strains in the different *d*-tartrate-negative biotypes gave mutative fermentation in tests in loosely capped bottles. Strains of types 2*d*T−, 10*d*T− and 16*d*T− occasionally gave mutative fermentation within 1 day and generally did so within 2–3 days; strains of types 6 and 19Xd gave mutative fermentation sometime between 2 and 14 days in a large proportion of replicate tests, whilst strains of types 17 and 18 gave mutative fermentation in only a small proportion of 14-day tests.

The frequency of mutative fermentation was also influenced by the conditions of culture, particularly by the degree of aeration. Thus, *d*-tartrate-negative strains gave 'late' positive reactions in a larger proportion of replicate tests and after shorter periods of incubation when the culture bottles were incubated with their caps screwed on tightly to prevent access of air than when they were incubated with the caps applied loosely. For example, in several replicate series of ten tests of a strain of biotype 19Xd, all of ten tests in tightly capped bottles fermented on the sixth day, whereas only one of ten tests in loosely capped bottles fermented within 14 days. In a further series of tests incubated for 14 days all of 31 strains in biotypes 6, 17, 18 and 19Xd gave fermentation in each of two tightly capped bottles, but only four of the 31 strains gave fermentation in either of two loosely capped bottles.

Turbidity test for fermentation of d-*tartrate*

These tests were done by growing each strain for 24 hr. in two tubes of Oxoid peptone water, with and without 1 % *d*-tartrate. The amount of growth of strains in the *d*-tartrate-positive biotypes was much greater in the medium with *d*-tartrate than in that without it; the difference in turbidity between the two media was generally very obvious to the naked eye and measurements with the absorptiometer showed that the degree of stimulation of growth by *d*-tartrate varied between +100 and +600 %. Strains of *d*-tartrate-negative biotypes did not give noticeably greater growth in the medium containing *d*-tartrate than in that without it and

Table 2. *Results of representative turbidity tests showing the effect of* d-*tartrate on the amount of growth of strains of* Salmonella typhimurium *from* d-*tartrate positive (above) and* d-*tartrate negative (below) biotypes in two different liquid peptone media incubated for* 24 *hr. at* 37° *C. under aerobic static conditions*

		Results in Oxoid peptone medium			Results in Difco Bacto–Peptone medium		
		Amount of growth (turbidity) in medium		% stimulation of growth by *d*-tartrate	Amount of growth (turbidity) in medium		% stimulation of growth by *d*-tartrate
Strain	Biotype	Without *d*-tartrate	With 1 % *d*-tartrate		Without *d*-tartrate	With 1 % *d*-tartrate	
S2644	1*m*T−	0·14	1·00	+615	0·21	0·80	+280
S1827	10	0·15	1·02	+580	0·25	0·85	+240
S1464	12	0·21	0·90	+330	0·20	0·77	+285
S2294	16	0·19	0·80	+320	0·17	0·62	+264
S2327	16	0·15	0·84	+460	0·19	0·53	+177
S2332	16	0·14	0·53	+280	0·17	0·43	+153
S2633	16	0·15	0·47	+215	0·21	0·47	+121
S2348	6	0·19	0·19	0	0·20	0·27	+35
S1811	17	0·13	0·15	+15	0·15	0·22	+46
S2257	17	0·12	0·13	+8	0·13	0·22	+69
S2314	17	0·12	0·14	+17	0·13	0·23	+77
S2316	17	0·15	0·14	−7	0·17	0·27	+58
S2335	17	0·13	0·11	−15	0·13	0·23	+77
S2581	17	0·12	0·14	+17	0·14	0·19	+36

the turbidity measurements in the tartrate medium were generally between 20 % less and 50 % more than in the tartrate-free medium. The results for some representative strains are shown in Table 2.

The results of the turbidity tests read at 24 hr. were generally similar to, but more consistently reproducible than those of the 24 hr. lead acetate tests in loosely capped bottles. Thus they were uniform in replicate tests of the exceptional strains of biotypes 2*d*T−, 10*d*T−, 15 and 16, which gave variable results with lead acetate. The only strains giving irregular results by the 24 hr. turbidity method were some of biotype 16*d*T−, which occasionally gave a positive, mutative reaction. These strains might have been mistyped if only a single test had been done on them. When turbidity tests were done after periods of incubation longer than 24 hr., strains of all the *d*-tartrate-negative biotypes gave irregular results due to the occurrence of mutative fermentation.

Turbidity tests made in Difco Bacto-Peptone water gave generally similar results to those made in Oxoid peptone water, but the differences between positive and negative strains were less marked. The *d*-tartrate-positive strains showed rather less stimulation of growth by *d*-tartrate in the Difco than in the Oxoid medium and, surprisingly, the *d*-tartrate-negative strains regularly showed a small stimulation of growth, e.g. +50 %, in the Difco medium (Table 2). The *d*-tartrate-negative strains, moreover, gave mutative fermentation earlier and oftener in the Difco than in the Oxoid medium. Strains of biotype 2*d*T−, for example, gave positive results due to the emergence of *d*-tartrate-positive mutants, confirmed by

plating and testing of colonies, in 3–9 out of 20 replicate 24 hr. turbidity tests in Bacto-Peptone but in none of 20 parallel tests in Oxoid peptone. The reason why the Difco medium favoured the emergence of mutants is unknown, though the amount of growth of the 2*d*T− strains was somewhat greater in the Difco than in the Oxoid medium.

The 24 hr. turbidity test in Oxoid peptone water classified our 1435 strains into 1045 that were *d*-tartrate-positive and 390 that were *d*-tartrate-negative. The latter group included 145, 174, 10, 15 and 1 strains, respectively, in the previously recognized *d*-tartrate-negative biotypes 6, 17, 18, 19Xd and 20, but also 15, 4, 1, 24 and 1 strains, respectively, in biotypes 2*d*T−, 10*d*T−, 10*d*T−*l*T−, 16*d*T− and 16*d*T−*l*T−, not recognized by previous workers.

Absence of utilization of d-*tartrate under fully aerobic conditions*

The conditions of culture in the static liquid media used for the lead acetate and turbidity tests were poorly aerobic, and we found that it was only under such conditions that *d*-tartrate could be utilized. Fourteen strains of the *d*-tartrate-positive biotypes 1, 2, 10, 12, 15 and 16 were cultured for 24 hr. in Oxoid peptone media with and without 1% *d*-tartrate (1) under poorly aerobic conditions in static tubes and (2) under highly aerobic conditions in continuously shaken flasks. In the static cultures the presence of *d*-tartrate stimulated the growth of every strain by more than 400%, whilst in the shaken cultures the difference in turbidity between the tartrate-containing and tartrate-free media was in all cases less than 10%.

The inability of *S. typhimurium* to utilize *d*-tartrate under fully aerobic conditions was also demonstrated in tests for growth on minimal agar medium containing *d*-tartrate as the sole source of carbon and energy. None of the 1045 positive and 390 negative strains gave visible growth within 7 days on this medium, though most of these strains grew well in 2 days on minimal media containing glucose or sodium citrate.

Fermentation of l-*tartrate*

The lead acetate and turbidity tests done at 24 hr. gave concordant results for the fermentation of *l*-tartrate with most strains and the turbidity test gave consistent results in replicate tests of representative strains. In the turbidity test, chosen as standard, the degree of stimulation of growth of the *l*-tartrate-positive strains in the medium containing *l*-tartrate was in all cases so great that the results could be read reliably with the naked eye; the turbidity measurements were mostly between 500 and 800% greater than those in the tartrate-free medium.

Only 34 of the 1435 strains were found to be *l*-tartrate-negative; 10 belonged to biotype 5, the only *l*-tartrate-negative type in the Kristensen scheme, and 24 belonged to the hitherto unrecognized biotypes 1*l*T− (3 strains), 10*l*T− (11 strains), 17*l*T− (2 strains) and 1*l*T−*m*T−, 3*l*T−, 6*l*T−, 8*l*T−, 9*l*T−, 10*d*T−*l*T−, 11*l*T− and 16*d*T−*l*T− (1 strain each). Several of these negative strains gave 'late' positive results in lead acetate and turbidity tests incubated for 3–4 days due to the emergence of *l*-tartrate-fermenting mutants; the remainder gave only negative results up to 14 days.

Table 3. *Results of representative lead acetate and turbidity tests for fermentation of* m-*tartrate by strains of* Salmonella typhimurium *of* m-*tartrate-positive (above) and* m-*tartrate-negative (below) biotypes*

Strain	Biotype	Results at 2 and 14 days in lead acetate test in bottles with caps: Loose 2 days	Loose 14 days	Tight 2 days	Tight 14 days	Amount of growth (turbidity) after 24 hr. in Oxoid peptone water with (+*m*T)* and without (−*m*T) 1 % *m*-tartrate when culture was: Static −*m*T	Static +*m*T	Shaken −*m*T	Shaken +*m*T
S712	1	+	+	+	+	0·21	0·38 (+81)*	0·90	1·95 (+117)*
S1446	2	−	+	+	+	0·16	0·30 (+88)	1·13	2·10 (+86)
S1535	6	+	+	+	+	0·18	0·46 (+155)	1·13	1·68 (+49)
S1444	8	+	+	+	+	0·22	0·43 (+95)	1·13	1·86 (+65)
S1542	9	−	+	−	+	0·17	0·30 (+77)	1·25	1·73 (+38)
S206	10	+	+	+	+	0·19	0·52 (+173)	0·85	1·74 (+105)
S576	15	+	+	+	+	0·13	0·33 (+153)	0·63	1·38 (+118)
S6624	17	+	+	+	+	0·16	0·30 (+87)	1·14	2·15 (+88)
S706	18	+	+	+	+	0·13	0·23 (+77)	1·03	2·15 (+108)
S1180	19Xd	+	+	+	+	0·15	0·34 (+127)	1·08	2·00 (+85)
S527	1*m*T−	−	−	−	+	0·20	0·10 (−50)	1·15	0·71 (−38)
S2317	1*m*T−	−	−	−	−	0·21	0·10 (−52)	1·15	0·71 (−38)
S1573	4	−	−	−	+	0·19	0·13 (−32)	1·08	0·82 (−24)
S2591	4	−	−	−	−	0·13	0·09 (−31)	1·10	0·46 (−58)
S747	12	−	−	−	+	0·24	0·10 (−58)	1·02	0·62 (−39)
S826	12	−	+	−	+	0·17	0·10 (−41)	1·16	0·94 (−19)
S1412	12	−	−	−	−	0·16	0·09 (−44)	1·04	0·76 (−27)
S1423	12	−	−	−	+	0·13	0·10 (−23)	1·11	0·62 (−44)
S2306	16	−	−	−	−	0·12	0·10 (−17)	1·17	0·70 (−40)
S2565	16	−	−	−	−	0·15	0·11 (−27)	1·12	0·76 (−32)

* Figure in parentheses shows percentage difference in amount of growth between *m*-tartrate-containing and *m*-tartrate-free medium, i.e. the degree of stimulation (+) or inhibition (−) of growth by *m*-tartrate.

Most of the strains that fermented *l*-tartrate in peptone water were also able to utilize this tartrate under the more highly aerobic conditions on an agar plate; thus they gave good growth within 7 days on minimal medium containing *l*-tartrate as sole source of carbon and energy.

Lead acetate test for fermentation of m-*tartrate*

The lead acetate test gave very irregular results for the fermentation of *m*-tartrate whatever period of incubation was used and regardless of whether the bottles were loosely or tightly capped. When replicate tests in loosely capped bottles were put up with any *m*-tartrate-positive strain, most of them became positive after 2, 3 or 4 days' incubation, but a few remained negative until 5–7 days. Conversely, when such tests were done with an *m*-tartrate-negative strain, most of them were negative at 4 days, but a few were positive at 3–4 days and an increasing proportion, up to 50 %, became positive after longer incubation up to 14 days. Thus, although readings made at 4 days generally distinguished the

positive from the negative strains, reliance could not be placed on a single 4-day test of a given strain (Table 3). It was necessary to inoculate a series of, say, five replicate tests of the strain and judge its status from the results in the majority of the tests.

In tests made after 2, 3 or 4 days in tightly capped bottles both the *m*-tartrate-positive and *m*-tartrate-negative strains gave a greater number of positive results than in parallel tests in loosely capped bottles, but all strains gave aberrant results in a proportion of replicate tests.

The irregular production of positive results between 2 and 14 days by most strains of all *m*-tartrate-negative biotypes was due to the ability of these strains to give rise to *m*-tartrate-positive mutants. Some of the 'late' positive tests were plated on nutrient agar, subcultures were made from separate colonies and a proportion of the subcultures from each test were shown to have *m*-tartrate-positive characters in the lead acetate, turbidity and plate-inhibition tests.

Turbidity test for fermentation of m-*tartrate*

Selected strains from different biotypes were tested in Oxoid peptone water or Difco Bacto-Peptone water with and without the addition of 1% *m*-tartrate in tubes incubated under aerobic static conditions for 24 or 48 hr. The results did not differ significantly with the type of peptone or the period of incubation, and the method using Oxoid peptone incubated for 24 hr., which was optimal for tests with *d*- and *l*-tartrates, was adopted also as standard for *m*-tartrate. Representative results are shown in Table 3.

The 24 hr. turbidity test drew a clear distinction between *m*-tartrate-positive and *m*-tartrate-negative strains and, with rare exceptions, results were consistent in series of replicate tests on a given strain. The growth of *m*-tartrate-positive strains was stimulated by the presence of *m*-tartrate by amounts ranging in different tests from 35 to 220%. That of the *m*-tartrate-negative strains was never stimulated, but instead was inhibited by the presence of *m*-tartrate and depressed by amounts ranging from 10 to 60% below those in the control, tartrate-free cultures. The differences in amount of growth in these tests were commonly too small for reliable reading with the naked eye and it was necessary to read them with the absorptiometer. In most of the few cases in which a single test of a strain showed neither stimulation greater than +35% nor inhibition greater than −10%, repeated tests showed clearly that the strain was either *m*-tartrate-positive or *m*-tartrate-negative. There were, however, three strains in our series of 1435 that were truly indifferent to *m*-tartrate, neither utilizing for increased growth nor being inhibited by it; the plate tests, described below, showed that these strains were *m*-tartrate-positive.

When turbidity tests were done in peptone water aerated by continuous shaking during incubation for 24 hr., the amounts of growth were five- to tenfold greater than in the static cultures, but positive and negative strains still showed comparable degrees of stimulation and inhibition of growth to those observed under static conditions (Table 3). Highly aerobic conditions, therefore, did not prevent the utilization of *m*-tartrate as they did that of *d*-tartrate.

Minimal medium and plate-inhibition tests with m-*tartrate*

The 1435 strains were tested in parallel cultures on three plates of ammonium-salts agar medium in which the carbon sources were: (1) citrate alone, (2) citrate and *m*-tartrate, and (3) *m*-tartrate alone. Most strains grew well on the medium with only citrate and formed heavy disks of growth within 48 hr. The few that did not were retested on, and gave good growth on, medium containing glycerol instead of citrate.

All the strains found to be *m*-tartrate-positive in the turbidity tests gave heavy growth within 7 days on the minimal medium containing *m*-tartrate as sole carbon source. They also gave good growth within 48 hr. on the medium containing both citrate (or glycerol) and *m*-tartrate. All strains found negative in the turbidity tests failed to give any growth within 7 days on the minimal medium containing *m*-tartrate or any growth within 48 hr. on the medium containing citrate (or glycerol) and *m*-tartrate (Pl. 1, figs. 1 and 2). The *m*-tartrate-negative strains, therefore, could be recognized either by their failure to utilize *m*-tartrate for growth on citrate-free medium or by their susceptibility to inhibition of growth by *m*-tartrate on citrate-containing medium. Since the former observation could be made only after 7 days, but the latter after 48 hr., the test for inhibition was preferred to that for growth. Since, moreover, the plate-inhibition test was as reliable as, and easier to read than, the turbidity test, it was chosen as the standard method for *m*-tartrate. It was applied to auxotrophic strains by using media supplemented with the required growth factor.

The 1435 strains of *S. typhimurium* were classified by the turbidity and plate-inhibition tests into 1181 that were *m*-tartrate-positive and 254 that were *m*-tartrate-negative. Of the latter, 5, 158 and 11, respectively, belonged to the recognized *m*-tartrate-negative types 4, 12 and 16, but 55, 1 and 24 belonged to the previously unrecognized types 1*m*T−, 9*m*T− and 16*d*T−.

Lead acetate test for fermentation of citrate

In the Kristensen scheme only biotypes 14, 17, 18 and 20 fail to ferment citrate. Our series of strains included none of type 14 and only one of type 20, and these two types were also rare in previous series. The main use of tests with citrate is therefore in confirming the distinction made by tests with *d*-tartrate between the inositol-negative rhamnose-negative biotypes that ferment citrate (types 15 and 16) and those that do not (types 17 and 18). We were unable, however, to find any conditions under which tests with citrate would give reliable results. In tests in loosely capped bottles, strains of biotypes 17 and 18 regularly failed to ferment citrate within 14 days, but strains of biotypes 15 and 16, identified by their reactions with *d*- and *m*- tartrates, sometimes fermented and sometimes failed to ferment citrate in this period.

Turbidity test for fermentation of citrate

Forty strains of the citrate-fermenting biotypes 1, 2, 10, 12, 15 and 16 and the non-fermenting biotypes 17 and 18 were grown for 24 hr. in Oxoid peptone media with and without the addition of 0·25 % sodium citrate. The difference of amount

of growth in the citrate-containing compared with the citrate-free medium ranged from −25% to +486% (mean +39%) with strains of the citrate-fermenting biotypes and from −29% to +10% (mean −8·3%) with those of the non-fermenting types. The test, therefore, did not reliably distinguish the fermenting from the non-fermenting types and no better differentiation was obtained by the substitution of Difco Bacto-Peptone for Oxoid peptone, the substitution of liquid ammonium-salts medium for peptone water, an extension of the incubation period to 48 hr., an increase in the concentration of sodium citrate to 1% or the substitution of potassium citrate for the sodium salt.

Growth on citrate minimal agar medium

The ability of a strain to grow on minimal agar medium with citrate as sole carbon source was unrelated to its ability to ferment citrate under the poorly aerobic conditions of static liquid cultures in the lead acetate and turbidity tests. The majority of strains of all biotypes, including the citrate-non-fermenting types 17 and 18, gave good growth within 2 days on citrate minimal medium or, in the case of auxotrophic strains, on this medium supplemented with the required growth factor. Among strains, e.g. in biotype 10, that fermented citrate readily in lead acetate tests, there were a few that could not utilize citrate for growth on minimal agar medium.

DISCUSSION

Our results show that the generally used methods of testing *S. typhimurium* for the fermentation of organic acids are unreliable. These methods gave different results in replicate tests of the same strain, so that an incorrect result must often be obtained in any single test. The method of observing an acid colour change was so unreliable with all four substrates that we abandoned its use entirely. The lead acetate test was highly unreliable for determining the fermentation of citrate and we were unable to develop any other test that was reliable with this substrate. Since reactions with citrate do not distinguish any biotypes in the Kristensen scheme that are not also distinguished by reactions with other substrates, the citrate-fermentation test should be omitted from the biotyping procedure.

The lead acetate test, which is done with cultures grown in Difco Bacto-Peptone water containing 0·5 or 1% of organic acid, was found to be unreliable if the culture was incubated for more than 24 hr. in tests with *d*-tartrate and *l*-tartrate and for more than 4 days in tests with *m*-tartrate. Genotypically fermenting ('positive') strains were rarely misidentified, but genotypically non-fermenting ('negative') strains were commonly read as positive in the prolonged tests due to the emergence of mutants which produced 'late' fermentation. Our observations indicate that most if not all of the strains genotypically non-fermenting with *d*- or *m*-tartrate and some of those non-fermenting with *l*-tartrate are capable of giving rise to fermenting mutants.

Mutants emerged earlier and more frequently in tests of negative strains in which access of air to the surface of the medium was restricted by tight capping of a nearly filled bottle. To identify the negative strains, therefore,

it was necessary to grow the cultures in loosely capped bottles and to read the results after only 24 hr. for *d*- and *l*-tartrates and 4 days for *m*-tartrate. Even so, a few discrepant results were obtained in sets of replicate tests. Thus a few strains genotypically negative for *d*- or *l*-tartrate (e.g. in biotypes 2*d*T − and 5) sometimes gave mutative fermentation within 24 hr., and many *m*-tartrate-positive and *m*-tartrate-negative strains were mistyped in a proportion of tests incubated for 4 days.

The evaluation of other methods established the 24 hr. turbidity test as being very reliable. It gave consistent results in replicate tests of any strain with any of the three tartaric acid isomers. The strain under test was grown for 24 hr. in two loosely stoppered tubes of Oxoid peptone water, one with and the other without the tartrate, and it was judged to be a fermenter if the amount of growth in the tartrate tube was significantly greater than that in the control tube. These tests did not give false-positive results due to the emergence of mutants even when applied to the exceptional strains, such as those of biotypes 2*d*T − and 5, which gave mutative fermentation within 24 hr. in the lead acetate test. The absence of mutative fermentation within 24 hr. in the turbidity test appeared to be due to the use of Oxoid peptone water in this test in the place of Difco Bacto-Peptone water used in the lead acetate test. Bacto-Peptone favoured the emergence of tartrate-fermenting mutants and when turbidity tests were done with Bacto-peptone water, strains of biotypes 2*d*T − and 5 gave some false-positive results due to mutative fermentation within 24 hr. It was not found practicable to avoid early mutative fermentation in the lead acetate test by using Oxoid peptone for that test, because Oxoid peptone differed from the Bacto-peptone in giving a considerable precipitate with lead acetate even when not containing added tartrate.

The turbidity readings indicative of fermentation differed between the *d*- and *l*-tartrates and *m*-tartrate. The degree of stimulation of the growth of positive strains by *d*- and *l*-tartrates over the amount of growth in tartrate-free medium was always more than 100 % and generally about 500 %; the growth of negative strains was not stimulated by these tartrates by more than 50 % and was not inhibited by them. *m*-Tartrate, on the other hand, stimulated positive strains generally only by between 35 and 100 %, and it inhibited *m*-tartrate-negative strains so that they gave 10–60 % less growth than in tartrate-free medium. A strain was therefore judged to ferment *d*- or *l*-tartrate if its growth was stimulated at least 100 % by the tartrate and to ferment *m*-tartrate if its growth was stimulated at least 35 %.

The ability of strains to ferment *l*- or *m*-tartrate in static peptone water was associated with an ability to utilize that tartrate for growth within 7 days on ammonium-salts minimal agar medium. There was no such association in the case of *d*-tartrate and citrate. None of the many strains that fermented *d*-tartrate in the poorly aerobic, static peptone water was able to utilize it for growth under the highly aerobic conditions on plates of minimal agar medium, whilst many of the strains that failed to ferment citrate in static peptone water utilized it for growth on minimal medium. Growth tests on minimal medium were therefore valueless for demonstrating the ability to ferment these two substrates.

Lewis & Stocker's (1971) plate-inhibition test for fermenting ability towards *m*-tartrate depends on the peculiar effect of *m*-tartrate in inhibiting the growth of *m*-tartrate-negative strains of *S. typhimurium* on minimal agar containing citrate or glycerol. We found that this test, read at 48 hr., was as reliable as the 24 hr. turbidity test and, since its results were clearer and more easily read than those of the turbidity test, recommend it as the method of choice for *m*-tartrate.

Application of the turbidity and plate-inhibition tests in biotyping 1435 strains previously typed by the lead acetate method of Brown *et al.* (1924) as used by Kallings & Laurell (1957), showed that many strains had been mistyped by the latter methods. Most of the mistyped strains belonged to types that were genotypically negative for one of the tartrates but gave false-positive, mutative reactions in the lead acetate test. The relative scarcity of strains allotted to *d*-tartrate-negative and *m*-tartrate-negative biotypes in some previously published series may have been due to such mistyping. Use of the turbidity and plate-inhibition tests, moreover, distinguished 16 new biotypes, additional to the 21 in the extended Kristensen scheme (Table 1) and type 19Xd of Morgenroth & Duguid (1968). The new types, which differed from existing ones by giving a negative reaction with one or more of the tartrates, were: 1*l*T−, 1*l*T−*m*T−, 1*m*T−, 2*d*T−, 3*l*T−, 6*l*T−, 8*l*T−, 9*l*T−, 9*m*T−, 10*d*T−, 10*l*T−, 10*d*T−*l*T−, 11*l*T−, 16*d*T−, 16*d*T−*l*T−, and 17*l*T−.

Thus the new tests are not only much more reliable than those previously used, but also subdivide the serotype *S. typhimurium* into a larger number of biotypes.

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EXPLANATION OF PLATE

Fig. 1. Growth of twelve strains of *Salmonella typhimurium* from spot inoculations on citrate minimal agar medium. Six strains (above) from biotype 10 and six (last in second row and five in bottom two rows) from biotype 12 have given good growth in 2 days.

Fig. 2. Growth of same twelve strains as in Fig. 1 from spot inoculations in corresponding positions on minimal agar containing *m*-tartrate in addition to citrate. The six *m*-tartrate-fermenting strains of biotype 10 (above) have given good growth in 2 days, whilst the six *m*-tartrate-non-fermenting strains of biotype 12 (below) have been inhibited and show no growth.

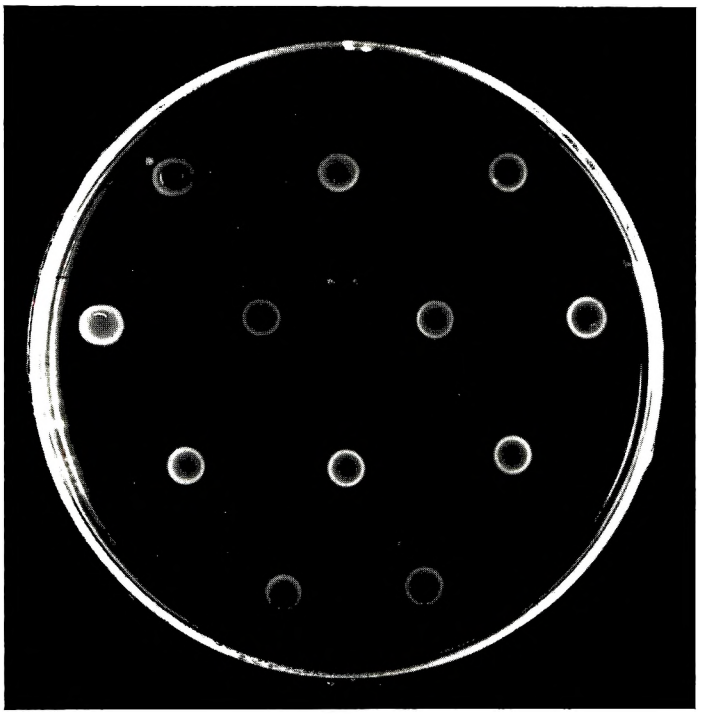

Fig. 1

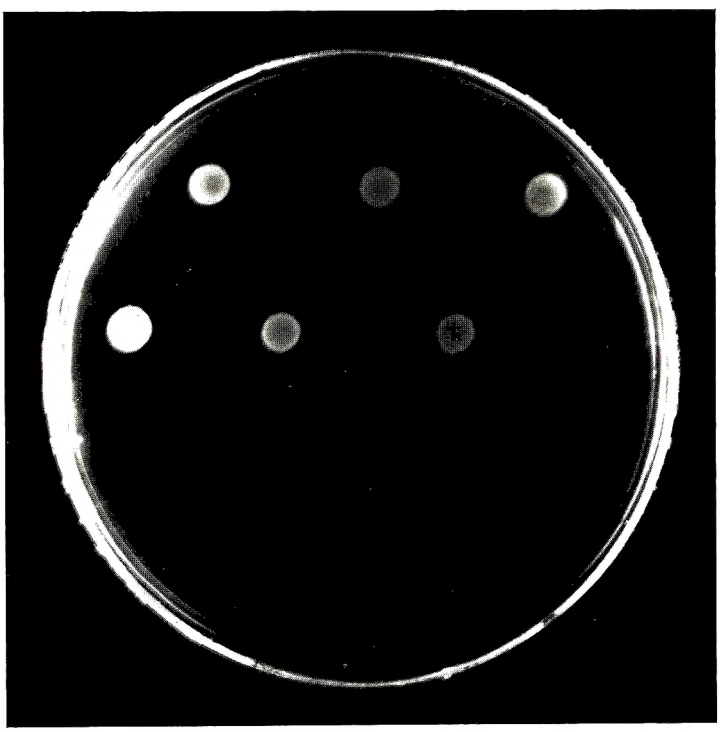

Fig. 2

(*Facing p.* 666)

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Yeasts in a hospital for patients with skin diseases

By DOROTHY A. SOMERVILLE

St John's Hospital for Diseases of the Skin, Institute of Dermatology, Homerton Grove, London, E9 6BX

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SUMMARY

The incidence and acquisition of *Candida albicans* and other yeasts in two wards of a skin hospital is described. Carriage rates on the skin in hospital patients is higher than is generally supposed, and cutaneous sites may act as sources of infection with these organisms.

INTRODUCTION

During a study of cross-infection in two wards for male patients in a skin hospital, the opportunity was taken to study the occurrence and spread of yeasts, with particular reference to *Candida albicans*. Patients with diseases of the skin, especially eczema and mycosis fungoides, have been shown to be major sources of micro-organisms in hospital cross-infection, dispersal taking place on particles of desquamated skin (Noble & Davies, 1965; Wilson, White & Noble, 1971). The greater ability of dermatological patients to disseminate bacteria is due to a greater degree of skin colonization and to the fact that such patients liberate more particles of skin than persons with clinically normal skin (Noble, 1971*a*). *C. albicans* is not found resident on the healthy skin in young adults (Somerville, 1966), but the carriage of this yeast is increased among hospital patients (Vince, 1959; Clayton & Noble, 1966) and it has been suggested that spread of yeasts in the hospital ward occurs in a similar way to the spread of *Staphylococcus aureus* (Clayton & Noble, 1966).

MATERIALS AND METHODS

A total of 286 male patients was included in this study, 117 in Ward A, a ward with 12 beds in single rooms, and 169 in Ward B, an open ward for 16 patients. During the period of the study, patients in Ward A who were not confined to bed used the same dayroom as patients in Ward B.

During the 9 months of the survey, swabs were taken twice weekly from the nose, gums, chest and groins of all patients. An attempt was made to sample areas of skin without obvious lesions, though in extensive cases this proved difficult. The swabs, together with any sent to the laboratories for routine bacteriological examination, were inoculated onto Sabouraud's dextrose agar (pH 4) and then dropped into dextrose yeast extract broth (pH 4) for enrichment culture. Incubation was at 37° C. for 1 week, the broths were then subcultured onto Sabouraud's agar and these cultures incubated for 1 week at 37° C. A semi-quantitative assess-

Table 1. *Details of patients admitted to two male wards during the survey period*

	Ward A	Ward B
No. admitted	117	169
	%	%
Age		
< 15 yr.	3	3
15–39 yr.	27	39
> 39 yr.	70	58
Diagnosis		
Eczema	42	34
Psoriasis	35	31
Others	23	34
Length of stay		
1–7 days	9	17
8–14 days	28	24
15–21 days	23	22
22–29 days	11	14
29–56 days	20	16
> 56 days	9	7
Antibiotic therapy		
Topical	23	17
Systemic	25	17
Total	40	25

ment of the yeasts isolated was made: + +, heavy growth, more than 20 colonies isolated on primary culture; +, 5–20 colonies isolated on primary culture; ±, less than 5 colonies isolated on primary culture or enrichment culture necessary. All yeasts isolated were identified by the criteria of Lodder (1970). These methods of isolation restrict the yeasts obtained to those able to grow at 37° C. and the enrichment technique favours the growth chiefly of *Candida* and *Torulopsis* species. However, these are the species most commonly found associated with man and the ones usually incriminated in opportunist infection. An enrichment technique was used as yeasts generally occur on the skin in small numbers and swabbing, though the most acceptable method of sampling the skin in a hospital, is not entirely efficient. In the event, in most cases yeasts were found in sufficient numbers to be isolated on the primary Sabouraud's medium.

RESULTS

Details of the patients admitted to the wards are shown in Table 1. Two diagnostic categories, eczema and psoriasis, contributed over 60 % of the patients; the remainder were admitted with a variety of diseases, including light sensitivity, varicose ulcers and mycosis fungoides. There was no significant difference in the numbers of psoriatic and eczematous patients admitted to each ward, but the more serious cases of psoriasis were admitted to Ward A and the length of stay of psoriatic patients differed in the two wards, with an average of 31 days in Ward A compared with 23 days in Ward B. Patients admitted to Ward A were on average older than those admitted to Ward B (Table 1), but there was no difference in the

Table 2. *Incidence of yeasts amongst patients on admission*

	No. of patients	No. carrying on				
		Gums	Nose	Chest	Groin	Total
C. albicans						
Patients with						
Eczema	107	39 (36)	2 (2)	2 (2)	3 (3)	39 (36)
Psoriasis	94	24 (26)	1 (1)	2 (2)	6 (6)	27 (29)
Other disease	85	15 (18)	1 (1)	1 (1)	1 (1)	17 (20)
Total	286	78 (27)	4 (1)	5 (2)	10 (4)	83 (29)
Other yeasts						
Patients with						
Eczema	107	15 (14)	4 (4)	4 (4)	9 (8)	24 (22)
Psoriasis	94	5 (5)	3 (3)	6 (6)	6 (6)	14 (15)
Other disease	85	5 (6)	3 (4)	4 (5)	10 (12)	19 (22)
Total	286	25 (9)	10 (3)	14 (5)	25 (9)	55 (19)

The figures in parentheses are percentages.

length of hospital stay between the patients in the two wards. Nursing techniques were the same in both wards and some staff were shared by the two wards.

Carrier status on admission

The carriage rate of yeasts on admission is shown in Table 2: there was no difference between the patients admitted to the different wards. However, the incidence and number of *C. albicans* carried on the gums was significantly greater in patients with eczema (36 %) than in those with psoriasis (26 %) ($1\% < P < 2\%$).

Acquisition

C. albicans

The acquisition rate of *C. albicans* among the disease categories was similar but higher in Ward A than in Ward B. As expected, the chance of acquiring *C. albicans* increased the longer the patient stayed in hospital (Table 3). In the lesions, older patients acquired yeasts more frequently than did younger ones, but this might be explained by the fact that the older patients tended to be in hospital longer (a mean of 25 days as compared with 18 days for the younger group). Although the acquisition rate on the gums did not increase with age, the older patients carried larger numbers of yeasts than did the younger ones ($P < 2\%$).

Over a third of the patients acquired *C. albicans* at some site not colonized on admission and 52 % of all patients carried this yeast at some time during their hospital stay, 45 % on the gums, 12 % on the chest and 20 % in the groins (Table 4). Psoriatic patients carried *C. albicans* on the skin more frequently than did eczema patients, but the difference was not statistically significant. However, patients with eczema who were colonized by the yeast carried them longer than did other types of patients. Carriage of *C. albicans* on the gums and skin was higher in those patients treated with antibiotics (Table 5) and, although the difference was not statistically significant, the carriage rate was highest in those receiving systemic antibiotics.

Table 3. *Acquisition of* C. albicans *in the hospital*

		No. acquiring on						
		Gums*						
	No. of patients	+ +	+	Nose	Chest	Groins	Lesions	Total
Age								
< 40 yr.	106	2 (2)	14 (13)	5 (5)	12 (11)	14 (13)	1 (1)	30 (28)
⩾ 40 yr.	180	17 (9)	19 (11)	15 (8)	18 (10)	32 (18)	12 (7)	67 (37)
Disease								
Eczema	107	12 (11)	10 (9)	6 (6)	10 (9)	18 (17)	6 (6)	43 (40)
Psoriasis	94	1 (1)	14 (15)	9 (10)	11 (12)	18 (19)	5 (5)	29 (31)
Others	85	6 (7)	9 (11)	5 (6)	9 (11)	10 (12)	2 (2)	25 (29)
Length of hospital stay								
< 14 days	114	2 (2)	8 (7)	3 (3)	3 (3)	6 (5)	1 (1)	20 (18)
14–29 days	101	9 (9)	12 (12)	3 (3)	12 (12)	12 (12)	1 (1)	34 (34)
> 29 days	71	8 (11)	13 (18)	14 (20)	15 (21)	28 (39)	11 (15)	43 (61)
Total	286	19 (7)	33 (12)	20 (7)	30 (10)	46 (16)	13 (5)	97 (34)

* + + : ⩾ 20 colonies isolated on primary culture; + : < 20 colonies isolated on primary culture; The figures in parentheses are percentages.

Table 4. *Total carriage of* C. albicans

		No. carrying on						
		Gums						
	No. of patients	+ +	+	Nose	Chest	Groins	Lesions	Total
Age								
< 40 yr.	106	17 (16)	32 (30)	7 (7)	14 (13)	17 (16)	1 (1)	56 (53)
⩾ 40 yr.	180	45 (25)	36 (20)	17 (9)	21 (12)	39 (22)	12 (7)	94 (52)
Diseases								
Eczema	107	34 (32)	27 (25)	8 (7)	12 (11)	20 (19)	6 (6)	69 (64)
Psoriasis	94	12 (13)	27 (29)	10 (11)	13 (14)	25 (27)	5 (5)	45 (48)
Others	85	16 (19)	14 (16)	6 (7)	10 (12)	11 (13)	2 (2)	36 (42)
Total	286	62 (21)	68 (24)	24 (8)	35 (12)	56 (20)	13 (5)	150 (52)

* + + : ⩾ 20 colonies isolated on primary culture; + : < 20 colonies isolated on primary culture. The figures in parentheses are percentages.

Table 5. *Effect of treatment with antibiotics on carriage of yeasts*

		C. albicans			*Other yeasts*		
		No. carrying on			No. carrying on		
	No. of patients	Gums	Skin	Total	Gums	Skin	Total
Topical antibiotics	55	33 (60)	20 (36)	39 (71)	16 (29)	25 (45)	35 (64)
Systemic antibiotics	58	37 (64)	31 (53)	49 (84)	24 (41)	34 (59)	48 (83)
No antibiotics	195	77 (39)	35 (18)	88 (45)	28 (14)	52 (27)	78 (48)

The figures in parentheses are percentages.

Table 6. *Acquisition of yeasts other than* C. albicans

	No. of patients	No. acquiring on					
		Gums	Nose	Chest	Groin	Lesions	Total
Age							
< 40 yr.	106	10 (9)	14 (13)	10 (9)	14 (13)	3 (3)	34 (32)
⩾ 40 yr.	180	37 (21)	37 (21)	43 (24)	44 (24)	14 (8)	89 (49)
Disease							
Eczema	107	19 (18)	20 (19)	20 (19)	26 (24)	6 (6)	50 (47)
Psoriasis	94	13 (14)	18 (19)	21 (22)	20 (21)	6 (6)	43 (46)
Others	85	15 (18)	13 (15)	12 (14)	12 (14)	5 (6)	30 (35)
Length of hospital stay							
< 14 days	114	6 (5)	8 (7)	5 (4)	8 (7)	2 (2)	23 (20)
14–29 days	101	14 (14)	16 (16)	17 (17)	21 (21)	5 (5)	47 (47)
> 29 days	71	27 (38)	27 (38)	31 (44)	29 (41)	10 (14)	53 (75)
Total	286	47 (16)	51 (18)	53 (19)	58 (20)	17 (6)	123 (43)

The figures in parentheses are percentages.

Table 7. *Yeasts other than* C. albicans *isolated from patients (only one isolate from each patient is included)*

	No. isolations				
	Gums	Chest	Groin	Lesions	Total
Candida parapsilosis	19	48	54	10	106
C. tropicalis	4	—	3	—	5
C. pseudotropicalis	3	—	—	1	4
C. krusei	4	—	1	—	5
C. melinii	6	1	1	1	11
C. zeylanoides	1	—	1	—	3
Other *Candida* spp.	2	1	2	3	6
Torulopsis glabrata	30	7	11	2	43
T. famata	7	9	7	3	28
T. inconspicua	13	7	2	—	18
Pityrosporum pachydermatis	13	23	27	5	47

Yeasts other than C. albicans

The acquisition rate of these yeasts was again higher in the older patients ($P < 1\%$) (Table 6), but was not affected by disease category or ward. As with *C. albicans*, the rate increased with length of stay in hospital. Table 7 shows the species of yeasts isolated from the patients and their relative incidences (only one isolate of each species is recorded for each patient). *Pityrosporum pachydermatis*, a non-lipid-requiring *Pityrosporum* species previously reported only from animals was isolated from 47 patients (16%) (Table 8). (I am grateful to the Centraal bureau voor Schimmelcultures, Delft, for confirming the identification of this yeast.) Nine patients were considered to be heavily colonized as large numbers of *P. pachydermatis* could be isolated from the skin over long periods of time. These nine patients had severe, long-standing skin disease, eight had psoriasis and one mycosis fungoides; all were being treated with steroids, six with antibiotics and the condition of six of them warranted treatment with the cytostatic drug, metho-

Table 8. *Isolation of* Pityrosporum pachydermatis

	No. patients	No. patients carrying yeast	No. patients colonized	Diagnosis of those colonized
Ward A	117	23 (20 %)	8	Psoriasis (7), Mycosis fungoides (1)
B	169	24 (14 %)	1	Psoriasis

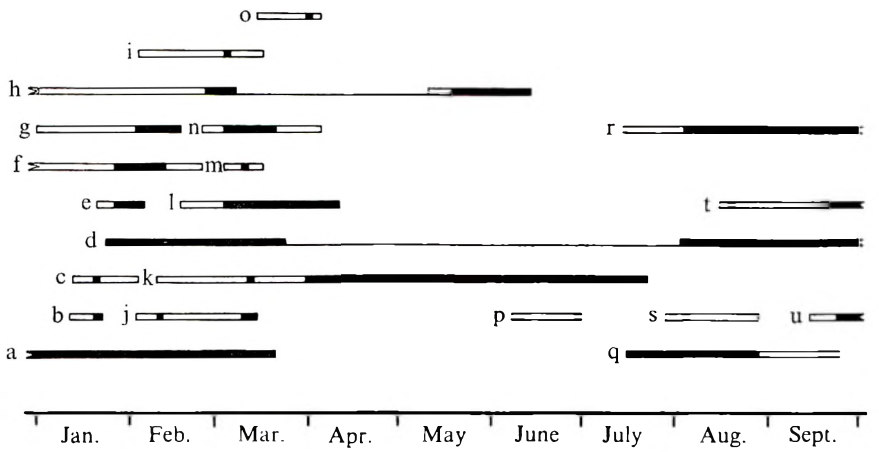

Fig. 1. Spread of *Pityrosporum pachydermatis* in Ward A. The length of a patient's stay is indicated by the horizontal bar, each individual patient is given a letter and blacked in areas indicate periods of carriage of the yeast. Only patients from whom the yeast was recovered are shown in the figure.

trexate. A higher incidence of colonization occurred in Ward A ($\chi^2 = 8{\cdot}90$, $P < 1\%$) than in Ward B, and there was evidence of spread of the yeast in this ward (Fig. 1) though not in Ward B, even though patients mixed freely. Two patients in Ward A with pustular psoriasis were admitted already colonized by this yeast and appeared responsible for its spread through the ward. These two patients had had multiple previous admissions to the hospital and presumably acquired the yeast from some common source.

In contrast to the findings with *C. albicans*, older patients carried other yeasts for longer periods of time than did the younger patients. There was no difference between the three disease groups, but long-stay patients were more likely to carry these yeasts throughout their stay in hospital than those who stayed for less than 29 days ($\chi^2 = 4{\cdot}56$, $P < 5\%$). This is presumably related to the severity of the disease. Antibiotic therapy also increased the numbers of patients with long-term carriage. Significantly more patients receiving systemic rather than topical antibiotics carried yeasts other than *C. albicans*, and the incidence in those patients not treated with antibiotics was much lower than in either of the antibiotic treated groups (Table 5).

Yeasts in pathological material

It is difficult to assess the significance of infection in a skin hospital as there is a high isolation rate of potentially pathogenic organisms from apparently healthy

Table 9. *Incidence of yeasts in infected lesions and other specimens sent to the laboratories*

Specimens	No. of specimens	No. carrying		
		C. albicans	Other *Candida*	Other yeasts
Swabs from				
Ulcers	78	4 (5)	12 (15)	1 (1)
Eyes/ears	78	8 (10)	5 (6)	4 (5)
Axillae/groins	59	11 (19)	5 (3)	1 (2)
Toewebs	29	0	8 (28)	3 (10)
Other areas	809	49 (6)	49 (6)	40 (5)
Sputum	131	62 (47)	7 (5)	20 (15)
Throat swab	306	110 (36)	16 (5)	28 (9)
Nose swab	155	9 (6)	5 (3)	4 (3)

The figures in parentheses are percentages.

skin sites. However, swabs sent to the laboratories under the headings 'infected eczema', 'boil', 'infected ulcer', etc., were considered to come from an infected lesion. Isolation of yeasts from such specimens sent from the two male wards was similar to that from specimens sent from the two female skin wards of the hospital, so the results are considered together in Table 9. There were 1053 lesion swabs submitted from the four wards during the survey period of which 7 % carried *C. albicans*, 8 % other *Candida* species and 5 % other yeasts (Table 9). The differences in isolation rates from the various skin sites for *Candida* species are significant: *C. albicans* was isolated more frequently from lesions in the axillae, groins, ears and eyes; other *Candida* species were more frequent in the toewebs and in ulcers.

DISCUSSION

As in other investigations, the results show that the carriage of yeasts, including yeasts other than *C. albicans*, is more common among hospital patients than in the general population. Skin carriage of *C. albicans*, usually very low in young adults and almost non-existent on the general skin surface (Somerville, 1966) is increased to 15–20 %. Other yeasts are more common on the skin. Carriage on the gums is more common and the results obtained in this survey are similar to those found by workers using similar sampling techniques (Clayton & Noble, 1966; Somerville, 1966; Marples & Somerville, 1968; Barlow & Chattaway, 1969). A much higher incidence of carriage (50 %) is obtained if mouth washings are cultured (Marples & Di Menna, 1952; Somerville, 1964), but this method is not easily carried out in extensive studies. As in the healthy population, carriage of *C. albicans* is more common in the elderly. Skin carriage of the yeasts, especially *C. albicans*, did not merely reflect contamination from the mouth and was highest in the group of patients with psoriasis. The incidence of all yeasts, not only *C. albicans*, is increased in the group of patients treated with antibiotics (Smits, Prior & Arblaster, 1966). As expected, systemic antibiotics produced a larger increase than did topical antibiotics, but increased incidence even in the gums did occur in those patients

treated with topical antibiotics. This is similar to the findings made in a study of acquisition of *Staph. aureus* (Wilson *et al.* 1971), though it may merely reflect the more serious clinical condition. The effect of steroid treatment on yeast carriage could not be assessed as 75% of the patients received steroid therapy, usually topical, during their stay in hospital; those who did not were usually in special patient categories such as light sensitivity.

It is not possible to decide with any certainty the mode of spread of these yeasts in the hospital wards. At present, there is no means of subgrouping yeast species for epidemiological work and the source may be endogenous or exogenous. However, several patients concerned in the spread of *P. pachydermatis* in Ward A were also involved in the spread of other organisms such as *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and antibiotic-resistant *Staph. aureus* (Noble, 1971*b*), and it may be a feature of certain severely affected skin patients that they become easily colonized by any organism in the environment, provide a suitable habitat for its multiplication and act as a good source of 'infection' for other patients in the wards. However, several other long-term severely affected patients admitted to the wards at the same time as these 'dispersers' did not become colonized and Ward B never showed any evidence for significant spread of the yeast from patient to patient, even though there was complete freedom of movement between wards. Spread, not only of *P. pachydermatis*, but also of *C. albicans*, was more widespread in Ward A despite the fact that patients in this ward each had single rooms.

The incidence of yeasts on the skin in patients in hospital is higher than is often supposed. With the increase in the problem of opportunist *Candida* infection in those on treatment with cytotoxic drugs, sites other than the gums and intestine should also be investigated as possible sources of yeasts for infection, given that almost a quarter of the patients in this study carried *C. albicans* on the skin.

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Serological studies in a student population prone to infection with human papilloma virus

By HEATHER A. CUBIE
Department of Bacteriology,
University of Edinburgh

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SUMMARY

Four hundred and sixty-seven serum specimens from the female students at a residential college were examined for the presence of circulating antibody to human wart virus using the technique of counter-current immunoelectroosmophoresis. A significantly higher incidence of antibodies was found in students with a past history of plantar warts than in any other group. Antibody took several months to develop and was detectable in 20–30% of the students up to 9 years after infection. From a few cases of multiple infection, it was shown that reinfection could occur in spite of the presence of circulating antibodies probably of the IgG class. The sensitivity of the test was compared with two recognized techniques for detection of wart virus antibodies, namely gel diffusion and passive haemagglutination.

INTRODUCTION

The high incidence of warts and especially of plantar warts in school children and young adults is well recognized to be associated with the increasing availability of swimming baths (Hall & Burrows, 1968; Allen & Dickenson, 1968; Tranter, 1969). Plantar warts may pass undiagnosed for several months, and they are more often painful and disabling than other types of warts, but it has been considered that one attack may provide protection from subsequent infection. A study into the duration and immunizing properties of the antibody produced after infection has been carried out over a period of 4 years on the students at a girls' residential College of Physical Education, with its own swimming pool and, until recently, a high incidence of plantar warts (Bunney, 1971, 1972).

Several tests for the detection of circulating antibody to human papilloma virus have already been described (Almeida & Goffe, 1965; Ogilvie, 1970*a*). The passive haemagglutination test of Ogilvie is a sensitive method, particularly for the detection of antibodies of the IgM class. It is, however, fairly laborious, technical details are important and there is as yet the difficulty of establishing a base-line for significant titres. This is demonstrated by the fact that 21 out of 34 (61·8%) of girls professing never to have had warts showed titres ranging from 20 to 320; nine of these girls with titres of 80 or above were considered to have significant antibody titres (Ogilvie, 1970*a*). The direct agglutination test described by Almeida and Goffe is relatively extravagant in materials, and the formation of precipitin

lines in agar gel diffusion is a rather insensitive method. It was, however, felt that a method which could increase the sensitivity of the precipitin reaction would be quick and useful for screening fairly large numbers of sera. The technique of counter current immunoelectroosmophoresis has been utilized to detect a variety of antigen-antibody interactions. In 1959, Bussard reported its use with snail haemocyanin and *E. coli* as antigens and mentioned, without giving details, that the technique had been tried successfully elsewhere with virus and homologous antibody. More recently, it has been shown to be an effective method in the detection of serum hepatitis antigen (Pesendorfer, Krasnitsky & Wewalka, 1970) and its increased sensitivity over gel diffusion and practicability of use when samples are small or numbers of specimens large has been demonstrated (Prince & Burke, 1970).

MATERIALS AND METHODS

Population under study

At the beginning of the 1970 and 1971 academic sessions, small samples of blood were obtained from the majority of first-year students. Earlier specimens were collected with the aid of the Blood Transfusion Service which visits the College of Physical Education at 6-monthly intervals. The students donating blood were asked to give a small extra sample at the same time. This meant that of the 1968 and 1969 intakes at the College a smaller number of students were tested, but from many of them two or three samples were obtained at different times. Sera were separated from the clotted bloods and stored at $-20°$ C. Each girl in each year completed a questionnaire requesting details of wart history. The same histories were used by Bunney (1971, 1972) in a trial of protective footwear. In 1971 a more comprehensive history giving more details of each attack and any treatment received was obtained.

Technical methods

Preparation of antigen

Parings from the surface of simple plantar warts were obtained from patients attending the wart clinics at the Skin Department of the Royal Infirmary, Edinburgh, collected in saline containing 200 units penicillin and 200 μg. streptomycin per ml. and stored at $-70°$ C. When a large amount of material had been obtained it was pooled, chopped with scissors and finely ground in a mortar. The extract was clarified by slow centrifugation and then semi-purified by two cycles of ultracentrifugation, the first at 9200 g for 15 min. and the second at 58,000 g for 90 min., in a Spinco Model L ultracentrifuge. The pellet from the second cycle was resuspended in a small volume of saline. The number of particles in the extract was obtained by counting a negatively stained preparation in the electron microscope, with reference to a known latex suspension, using the method of Watson, Russell & Wildy (1963). The antigen preparation was then diluted in saline for use in the electrophoresis tests. Control antigens included an extract of plantar callouses and an extract of a contaminating yeast isolated from one of the wart virus preparations.

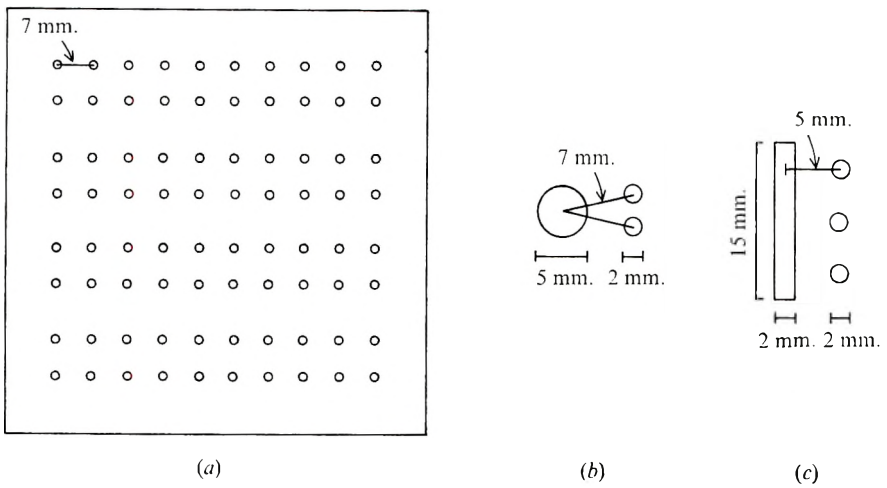

Fig. 1. Templates used in IEOP Tests: (*a*) To test forty serum samples on $3\frac{1}{4} \times 3\frac{1}{4}$ in. slides, (*b*) and (*c*) to enable reactions of identity to be detected.

Antisera from rabbits immunized with extracts of human papilloma virus were kindly provided by Dr M. M. Ogilvie.

Immunoelectroosmophoresis (IEOP)

Cleaned glass plates ($3\frac{1}{4} \times 3\frac{1}{4}$ in.) were coated with 5 ml. of 0·9 % agarose in distilled water. This was allowed to dry down to a thin film, after which 30 ml. of 0·9 % agarose in 0·025 M barbitone buffer, pH 8·6, were applied to the slide held on a levelling tray. A template was used to cut pairs of holes, 2 mm in diameter and with a centre to centre distance of 7 mm. (Fig. 1 (*a*)), thus enabling forty samples to be tested simultaneously, usually as twenty serum specimens with two concentrations of antigen or two different antigenic extracts. Occasionally, microscope slides (3×1 in.) were used, as for example in identity reactions. These were prepared in the same way, using 16 ml. of coating agar and 50 ml. of top agar for batches of eight slides held in a Shandon slide tray.

One of the initial disadvantages of the IEOP test was the inability to produce reactions of identity. Das, Hopkins, Cash & Cumming (1971), however, described a modification of the technique, using a different template consisting of two antigen wells equidistant from a single serum well, which was capable of detecting reactions of identity, partial and non-identity. The template found most satisfactory for identity reactions in this study consisted of a triangle of wells with centre to centre distance of 7 mm, with the serum wells 2 mm in diameter and with the antigen well 5 mm in diameter (Figure 1 (*b*)). A system of wells and trough (Fig. 1 (*c*)) was also used but much greater difficulty was experienced in obtaining complete continuity of the precipitin lines.

The serum wells were filled and the plate placed in a Shandon electrophoresis tank fitted with a cooling platen and containing 800 ml. 0·05 M barbitone buffer, pH 8·6. Wicks of glass fibre paper were used to complete the circuit between plate and buffer.

A constant current of 25 mA. was applied with the polarity such that the

serum was always on the positive side, and the current was run for 10 min. The plate was removed, the serum wells topped up and antigen added to the appropriate well. The plates were re-exposed to a current of 25 mA for 1 hr. after which precipitin lines could be examined by viewing with a bright light against a dark background. The plates were always incubated overnight at 37° C. in case further lines developed.

The plates were normally preserved thereafter by staining as follows: they were washed overnight in several changes of isotonic saline to remove unprecipitated protein. They were then allowed to dry at 37° C, covered with a sheet of filter paper for 4–5 hr., before immersing in 0·2% Ponceau S in 5% trichloracetic acid (TCA) for 3 hr. Excess stain was removed by three washes in 5% TCA followed by a quick rinse in distilled water. The plates were allowed to dry in air. Occasionally, precipitin lines were highlighted by the technique of Alpert, Monroe & Schur (1970) by immersing the plate in 1% tannic acid after the saline washes, leaving for 15 min. and examining directly. Precipitin lines were much more easily observed by this method, but it was only suitable when permanent records were being made by photographing the plates, since staining after tannic acid treatment was not wholly successful.

Determination of immunoglobulin class of antibody

Heavy molecular weight immunoglobulin (IgM antibody) was destroyed using 0·1 M 2-mercaptoethanol (2-ME) in saline with pH adjusted to 7·4. Equal volumes of 2-ME and serum were mixed and held for 24 hr. at 4° C. Before running in the IEOP test, whole sera and 2-ME treated sera were diluted 1/2 and the test carried out as usual, using the mixture and its dilution. The presence of 2-ME did not interfere with the test.

Agar gel diffusion and passive haemagglutination tests

The technique used for both these methods were essentially those described by Ogilvie (1970*a*, *b*). In the gel diffusion test, 0·8% agarose was sometimes used and this was diluted in PBS pH 7·2 or in 0·025 M barbitone buffer, pH 8·6, the latter giving a more direct comparison with the IEOP result.

RESULTS

Antigen concentration

The concentration of antigen in an IEOP test is important. The extract must contain a very large number of particles, but antigen excess can prevent the development of precipitin lines in the area between the wells when tested against a weakly positive serum. On the other hand, a concentration which can detect such sera does not seem to obscure more highly positive sera. Furthermore, antigen excess can cause the production of two precipitin bands or a diffuse drawn-out band. For each antigen extract a small titration was carried out, using a range of sera shown to be positive by precipitation in gel diffusion, and the optimum concentration calculated. Although the dilution factor required to reach this concentration varied from preparation to preparation, the number of particles

Table 1. *Comparison of the techniques of simple gel diffusion and IEOP for the detection of antibody to human wart virus*

Year of collection	No. of specimens tested	IEOP results	Gel diffusion results	
			No. positive	No. negative
1968	55	18 positive	6	12
		37 negative	1	36
1969	40	12 positive	7	5
		28 negative	0	28
1971	36	31 positive	25	6
		5 negative	0	5

Table 2. *Comparison of PHA titres with the results obtained by gel diffusion and IEOP in the detection of antibody to human wart virus*

PHA titre	Gel diffusion, % + ve results	IEOP, % + ve results	No. of specimens tested
10 or less	7·7	23·1	13
20	0·0	16·6	12
40	0·0	46·7	15
80	16·6	50·0	18
160	16·6	100·0	6
320 or greater	100·0	100·0	6
Totals	15·7	47·1	70

present was remarkably consistent, e.g. $1{\cdot}8 \times 10^{11}$ and $1{\cdot}4 \times 10^{11}$ particles/ml in two different preparations. It was found necessary to dilute the antigen in saline rather than in the barbitone buffer since the latter caused the potency of the extract to fall after several freezing and thawing cycles and the particles of papilloma virus to be broken down (Plate 1).

Comparison of IEOP and other serological methods

The increased sensitivity of the IEOP test over simple gel diffusion in agar was shown by direct comparison of 131 samples. Considerably more positive results were obtained by IEOP (61 to 39, see Table 1) and these figures are significant at the 1·0 % level. The 1971 sera which were positive by IEOP were then compared with gel diffusion tests using agarose instead of agar, dissolved in both PBS and barbitone buffer. The results were consistent (25/31 positive in gel diffusion) with one exception – a serum weakly positive by IEOP gave a precipitin line with barbitone-buffered agarose but not with PBS-buffered agarose. No precipitin lines were obtained in IEOP with the antigen from plantar callouses and only one serum gave a weak precipitin line with the yeast antigen.

The IEOP and gel diffusion tests were also compared with the passive haemagglutination (PHA) test. Of the sera used in the present survey, 55 specimens collected in 1968 had previously been tested by Dr M. M. Ogilvie and her PHA and gel diffusion results were made available. A few further PHA and gel diffusion

Table 3. *No. of specimens received from each year*

Year	1st specimens	Repeat specimens	Total
1968	82	49	131
1969	50	21	71
1970	107	49	156
1971	109	—	109
Total	348	119	467

results were obtained from sera collected in 1969. The results of these comparisons are shown in Table 2, where it can be seen that almost 50 % of sera with PHA titres of both 40 and 80 were detected by IEOP, but a titre of greater than 160 was required to ensure detection by gel diffusion.

Specificity of IEOP

It has been possible by means of the IEOP system to obtain reactions of identity between human and human serum, rabbit and rabbit serum and human and rabbit serum against the same antigen using the template shown in Fig. 1(*a*). It was found that a lower concentration of antigen was required to obtain reactions of identity than in straightforward IEOP, presumably because the volume of antigen applied to the large well was greater than normal. Furthermore, reactions of identity with asymmetric arcs (Ouchterlony, 1964) were often obtained when the difference in amount of antibody led to a slight imbalance in the system.

IEOP survey of antibody to human papilloma virus

Specimens of sera were obtained from the girls of the college in the numbers shown in Table 3. No follow-up of the 1971 students has as yet been possible.

The students had been placed in seven groups according to their wart histories for the investigation of the value of protective footwear in preventing the spread of wart infection (Bunney, 1971, 1972). These are shown in Table 4, together with the percentage of students showing antibody in each group, as determined from the first serum samples obtained in each case. Those showing a past history of plantar warts were found to have a significantly higher ($P < 0{\cdot}0005$) level of antibody than those in other groups. The presence of antibodies in some of the girls who said they had never had warts will be discussed.

Development of antibody

As foot inspections were carried out at about 6-monthly intervals at the college and as repeat histories were obtained with second and third samples of serum, it was possible to study the development of precipitating antibody in students with warts present at the time of sampling. The results are shown in Table 5. Although eighty-one specimens were available with all the relevant information, the number in each group was rather small. However, it would appear that most people did not develop antibody to wart virus until the infection had been present for a few months. The overall results for the two sections, namely, plantar warts and other

Table 4. *Group analysis of sera tested by immunoelectroosmophoresis for the presence of circulating antibody to human wart virus*

(All first serum samples from 1968–71 student intakes.)

Group	History	Year	No. of specimens Tested		No. of specimens Positive		% positive	Proportion positive	%
I	Past history, plantar warts	1968	29	88	12	39	44·3	51/112	45·5
		1969	11		3				
		1970	20		7				
		1971	28		17				
II	Past history, plantar and other warts	1968	9	24	4	12	50·0		
		1969	1		0				
		1970	12		6				
		1971	2		2				
III	Past history, hand warts	1968	11	62	3	16	25·8	19/75	25·3
		1969	14		5				
		1970	15		2				
		1971	22		6				
IV	Past history, other warts (legs, face etc.)	1968	4	13	2	3	23·0		
		1969	1		0				
		1970	4		1				
		1971	4		0				
V	No past history of warts	1968	15	94	2	17	18·1	17/94	18·1
		1969	17		5				
		1970	35		4				
		1971	27		6				
VI	Present history of warts other than plantar	1968	10	44	2	14	31·8	19/67	28·4
		1969	5		2				
		1970	8		1				
		1971	21		9				
VII	Present history of plantar warts	1968	4	23	1	5	21·7		
		1969	1		0				
		1970	13		3				
		1971	5		1				

types of wart, were remarkably similar, and it would seem that a second exposure to virus, whatever the site of earlier exposure, was more likely to result in antibody formation.

Duration of antibody

Figure 2 represents in histogram form the duration of antibody as determined from 156 samples from students showing a past history of plantar warts and from 99 samples from those who had had other types, mainly hand warts. No samples were obtained from anyone who had had plantar warts more than 9 years before and only two girls had a history of hand warts less than a year ago. It can be seen from the histograms that the likelihood of developing circulating antibody was slightly greater following plantar wart infection, and once formed, antibody persisted in about 35 % of plantar wart patients for several years, but in less than 20 % of hand wart cases after five years. Antibody was, however, detected in one

Table 5. *Development of antibody during wart virus infection*

Type of wart present	Previous exposures to virus	Duration of lesion in months: < 2 No. tested	< 2 No. +ve	2–6 No. tested	2–6 No. +ve	6–12 No. tested	6–12 No. +ve	12–24 No. tested	12–24 No. +ve	> 24 No. tested	> 24 No. +ve	Total specimens examined No. tested	Total specimens examined No. +ve
Plantar	None	—	—	3	3	3	1	—	—	—	—	6	4
	One or more	9	1	7	2	5	2	2	1	—	—	23	6
	Total	9	1	10	5	8	3	2	1	—	—	29	10
Others mainly hand	None	1	0	3	2	4	1	3	1	4	2	15	6
	One or more	5	2	10	5	6	4	5	3	11	0	37	14
	Total	6	2	13	7	10	5	8	4	15	2	52	20

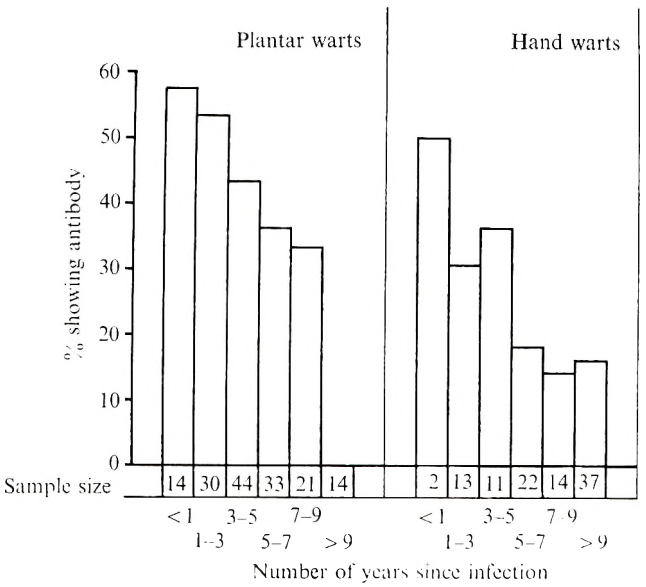

Fig. 2

Table 6. *Change in antibody levels without change in wart history in individual students*

Serum	Group	Detailed history at time of 1st sample	Ab results 1st sample	Ab results × months later 6	12	18	Outcome
E33	I	P4	+	.	.	−	Ab lost in 4–5 years
D210	I	P12, 4, and > 1	++	+	.	.	Ab falling in < 1 year
D205	II	P < 1, H10	++	+	.	.	Ab falling in < 1 year
D67	II	P2, E10,	+	.	−	.	Ab lost in 2–3 years
D84	IV	E9	+	+	.	−	Ab lost in 10–11 years
F110	VI	P3, H present 3/12	−	+	.	.	Ab gained in 3–9 months
F43	VII	P5, H present 3/52	−	.	+	.	Ab gained in < 12 months

Abbreviations: groups as in Table 2; P = plantar wart, H = hand wart, E = wart elsewhere; number following = no. of years since wart, e.g. P2 = 2 years since plantar wart, Ab = antibody; . = not tested.

case as long as 13 years after a history of hand warts (the warts had apparently lasted only about two months and had received no treatment).

It was possible to watch the change in immunological state of a few of the students from whom two or more serum samples were obtained while the wart history remained unchanged. These are detailed in Table 6 and fit in with the general picture shown in Table 5 and Fig. 2. Sera D210 and D205 were tested for the class of immunoglobulin present. Both samples of D210 were shown to contain mercaptoethanol-resistant antibody following treatment with 2-ME; neither sample of D205 showed any precipitin lines after 2-ME treatment. Immunoglobulin class was not tested in the remaining sera shown in Table 6.

Table 7. *Antibody levels following repeated plantar wart infections*

(Abbreviations as in Table 6.)

Serum no.	Group	1st serum specimen: Detailed history	1st serum specimen: Ab result	1st serum specimen: 2ME resistant Ab	2nd serum specimen: Detailed history	2nd serum specimen: Ab result	2nd serum specimen: IgG	3rd serum specimen: Detailed history	3rd serum specimen: Ab result	3rd serum specimen: 2ME resistant Ab
D 17	I	P 6 and 1	+	+	P 6½ and 1½	+	+	P 8, and < 1	+	+
D 47	I	P 7 and 3	+	+	P 9, 5 and 1	−	.	.	.	.
D 53	I	P < 1	+	−	P 3 and < 1	−	.	.	.	.
D 74	I	P 3½	−	.	P 5 and present 1/12	−	.	P 5 and < 1	−	.
D 113	I	P 6½	+	+	P 8 and present 6/12	+	+	.	.	.
D 121	II	P and E 1, H 8	−	.	P and E 3, H 10, P present 3/12	−	.	P and E 3½ H 10½ P < 1	−	.
E 28	II	P and H 7	−	.	P and H 9, H present 12/12	−	.	.	.	.
F 93	II	P 3 and H 7	+	−	P 3½ and H 7½	+	−	P 4, H 8, H present 3/12	+	+

Table 8. *Antibody response in treated and untreated warts*

Group		Plantar warts: Groups I and II Ab + ve	Plantar warts: Groups I and II Ab − ve	Plantar warts: Group VII Ab + ve	Plantar warts: Group VII Ab − ve	Hand and other warts: Groups III and VI Ab + ve	Hand and other warts: Groups III and VI Ab − ve	Hand and other warts: Group VI Ab + ve	Hand and other warts: Group VI Ab − ve	Totals Ab + ve	Totals Ab − ve
Treated	No. specimens tested	14	11	1	2	4	7	3	2	22	22
	Average duration in months	4·1	4·4	—	—	16·2	8·4	—	—	—	—
Untreated	No. specimens tested	4	0	0	0	2	13	6	10	12	23
	Average duration in months	6·0	—	—	—	4·0	6·0	—	—	—	—

Antibody titres after repeated attacks of warts

The variation in the response of girls with repeated histories of plantar warts is shown in Table 7. As might have been expected, the girls subject to repeated attacks would appear to have developed antibody and lost it quickly or not to have developed it at all. The probable presence or absence of IgG in the sera was therefore determined by mercaptoethanol treatment. It can be seen that reinfection could occur in spite of the probable presence of IgG antibodies (girls with serum specimen numbers D17, D47 and D113). With serum D53, and the first two samples of F93, the mercaptoethanol treatment destroyed the precipitin-producing antibody, suggesting that it belonged to the IgM class, although in the latter case, this would be a very long-surviving IgM. In the remaining three cases, no circulating antibodies were detectable at 1, $3\frac{1}{2}$ and 7 years after primary infection and no antibodies were present in any of these students within one year of the most recent lesions.

The effect of treatment

In the 1971 questionnaire, students were asked to give details of the duration of their warts, past as well as present and of any treatment given. None had had their warts removed by curettage, but various chemicals ranging from the commercially available 'Compound W' and formalin to more orthodox treatments such as podophyllin, wart paint and liquid nitrogen had been used. Table 8 shows that more people with plantar warts received treatment than did those with hand and other warts, and that the application of a chemical remedy did not prevent the development of antibody, a finding noted by Bunney, Hunter, Ogilvie and Williams (1971). The presence of antibody in some members of the treated group did not appear to influence the duration of the wart, although the groups tested here were too small for critical analysis.

DISCUSSION

It has been shown by Ogilvie (1970*b*) that plantar warts more often contain sufficient virus to be detected in the electron microscope than other types of wart. This fact, together with the pressure of the body weight on a plantar wart, suggests that the antigenic stimulus would be greater in such warts. It might therefore be expected that more people with plantar warts than with hand warts would develop antibody to the virus. A figure of 18·5% positive in a group of girls professing never to have had warts (Table 4) is disappointing, but plantar warts can occur and resolve spontaneously without recognition. Furthermore, it is surprising how often warts of many years ago are forgotten. Each intake of students was given a lecture about wart virus infections before filling in the form to minimize such errors but, with the 1969 intake, the lecture was given sometime after the first serum samples were collected. About forty girls therefore filled in two forms and 25% of them gave different histories on the two occasions! While the lack of complete consistency with histories obtained directly from the girls may need further clarification experimentally, it seems more likely that questioning the girls

individually would have revealed many forgotten instances of infection (Rasmussen, 1958). Examination of sera taken at intervals following clinically confirmed infections would give better correlation, and this is at present being pursued.

The results showing the development of antibody during wart virus infection (Table 5) suggest that antibody developed to an almost equal extent in plantar and other wart infections, provided there had been an earlier antigenic stimulus. On the other hand, with the first exposure to virus, antibody appeared to develop more readily if the infection occurred on the feet. Although the time taken for antibody to develop appeared to be similar for plantar and non-plantar warts, the former tend to be recognized more quickly because of the pain which often accompanies them and because of the regular foot inspections held in school and college swimming baths. This may mean that the duration of the hand warts reported here has been underestimated.

Eight out of fourteen students continued to show antibody up to a year after plantar warts had disappeared (Fig. 2). A further nine out of ten showed antibody during the period 1–2 years after infection, giving a total figure of 70·8% in the first two years. Although Ogilvie (1970*a*) showed precipitating antibody to be present in all of seven patients tested 3 months to 2 years after cure, Bunney *et al.* (1971), in a controlled treatment trial, found that only 70·5% (24 out of 34) patients whose warts were cured within a 12-week period showed an antibody response at the time of cure. The present survey has shown in addition that the percentage of people continuing to display antibody remained fairly high for 8–9 years, particularly following plantar wart infection.

In both the rabbit papilloma (Kidd, Beard & Rous, 1936) and bovine papilloma (Lee & Olson, 1968) systems, the sera from tumour-bearing animals has been shown to be capable of neutralizing the virus *in vitro* and preventing successful reinoculation. In a series of calves inoculated with bovine papilloma virus, Lee & Olson (1969) found that a precipitating antibody, IgG in nature, was consistently produced. Although IgG antibodies are normally considered to be protective, these workers did not always observe resistance to reinfection. It would appear from the results in Table 7 that a similar situation might exist with human papillomas – new warts developed in three cases in spite of the presence of mercaptoethanol-resistant antibodies.

The nature of the antibody response to human wart virus has been previously examined (Goffe, Almeida & Brown, 1966; Ogilvie, 1970*a*, *b*). Goffe *et al.* showed 72·2% (13 out of 18) patients to have IgM antibodies, two to have IgG only and three to have both types of antibody, thus suggesting that not many of this group were protected. On the other hand, Ogilvie (1970*b*) showed 75% (21 out of 28 patients) with simple plantar warts to have IgG at the time of cure as opposed to 60% (29 out of 49) for all types of warts. In addition, only four out of ten of a group of mosaic wart patients had IgG, again supporting the idea that simple plantar warts offer a greater antigenic stimulus. Possibly if the amount of virus was further increased, as in the above-mentioned results of Lee and Olson with experimentally inoculated calves, IgG would always be produced.

While it is unlikely that antiviral antibody plays any part in the cure of a wart,

it is apparent from the results reported here that some cases of resolution, both spontaneous and following treatment, do develop antibody. The regression of a virus-induced tumour has been shown in many cases to be due to a cell-mediated immune response (Shope papilloma – see Kidd *et al.* 1936; Evans, Weiser & Ito, 1962; Kreider, 1963; bovine papilloma – see Lee & Olson, 1968; rabbit fibroma and other viruses – see Allison, 1967). In particular, Lee & Olson (1968) have shown that existing bovine papillomata continue to grow after humoral antibody has developed and then regress simultaneously with lymphocyte infiltration being a characteristic histological finding. In humans, simultaneous regression of large crops of warts has frequently been observed (Rowson & Mahy, 1967) and a similar method of destruction seems likely.

On a quantitative basis, IEOP was shown by Prince & Burke (1970) to give titres ten times higher than standard Ouchterlony gel diffusion. They also showed that all sera positive by gel diffusion were positive by IEOP, but the latter method detected a significantly higher proportion of positives. While no comparative titrations were done with sera in the present survey, the results were similar – a single specimen was detected by gel diffusion and not by IEOP, while the overall difference in number of positives detected by IEOP was significant at the 1·0 % level (Table 1).

The present results confirm the usefulness of the technique of immuno-electro-osmophoresis, particularly in studies involving large surveys, where speed, ease and sensitivity are important considerations. Much remains to be learnt about wart virus serology, but the techniques described above should make prospective, comprehensive studies feasible.

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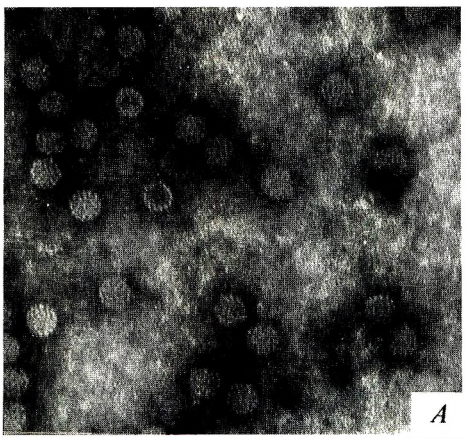
EXPLANATION OF PLATE

Human papilloma virus particles: (A) in saline, (B) partly degraded by several cycles of freezing and thawing in a 0·05 M barbitone buffer. × 80,000.



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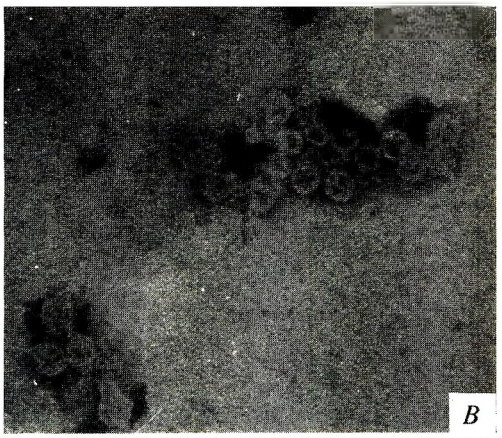

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Comparison of membrane filtration and multiple tube methods for the enumeration of coliform organisms in water

BY THE PUBLIC HEALTH LABORATORY SERVICE STANDING COMMITTEE ON THE BACTERIOLOGICAL EXAMINATION OF WATER SUPPLIES*

(*Received* 26 *April* 1972)

SUMMARY

The membrane methods described in Report 71 on the bacteriological examination of water supplies (Report, 1969) for the enumeration of coliform organisms and *Escherichia coli* in waters, together with a glutamate membrane method, were compared with the glutamate multiple tube method recommended in Report 71 and an incubation procedure similar to that used for membranes with the first 4 hr. at 30° C., and with MacConkey broth in multiple tubes. Although there were some differences between individual laboratories, the combined results from all participating laboratories showed that standard and extended membrane methods gave significantly higher results than the glutamate tube method for coliform organisms in both chlorinated and unchlorinated waters, but significantly lower results for *Esch. coli* with chlorinated waters and equivocal results with unchlorinated waters. Extended membranes gave higher results than glutamate tubes in larger proportions of samples than did standard membranes. Although transport membranes did not do so well as standard membrane methods, the results were usually in agreement with glutamate tubes except for *Esch. coli* in chlorinated waters. The glutamate membranes were unsatisfactory. Preliminary incubation of glutamate at 30° C. made little difference to the results.

INTRODUCTION

In the fourth edition of Report 71 on 'The bacteriological examination of water supplies' (Report, 1969), membrane filtration methods are recommended as alternatives to multiple tube methods for the enumeration of coliform organisms and

* The P.H.L.S. Standing Committee on the Bacteriological Examination of Water Supplies is composed of the following members of the P.H.L.S. Staff: Dr W. H. H. Jebb (Oxford), Chairman (until 30 September 1971); Dr B. Moore (Exeter), Chairman (from 1 October 1971); Dr L. A. Little (Wakefield), Secretary; Dr G. I. Barrow (Truro); Dr R. D. Gray (Newport); Dr J. E. Jameson (Brighton); Dr J. H. McCoy (Hull); Dr R. Pilsworth (Chelmsford); Dr J. A. Rycroft (Southend); Dr A. J. Kingsley Smith (Conway); Mrs H. E. Tillett (Colindale), Statistician (from 17 July 1971); Miss J. M. Watkinson (Manchester); together with Dr R. G. Allen, Water Research Association, for whom Mr R. W. Collingwood acted; Dr F. W. Bunting, Society of Medical Officers of Health; Dr N. P. Burman, Metropolitan Water Board; Dr G. U. Houghton, South Essex Waterworks company (until 26 November 1971); Dr A. E. Martin, Ministry of Health; Dr E. Windle Taylor, Metropolitan Water Board.

Requests for reprints should be addressed to Dr L. A. Little, Public Health Laboratory, Wood Street, Wakefield.

Escherichia coli in water. The recommendations were based on extensive work from 1953 to 1969 carried out at the Metropolitan Water Board (MWB) together with the experience of other laboratories where these methods have been adopted (Windle Taylor, 1953–70; Burman, 1960; Windle Taylor & Burman, 1964; Burman, 1967). The Public Health Laboratory Service Standing Committee on the Bacteriological Examination of Water Supplies therefore conducted multi-laboratory trials to compare these membrane methods with the recommended multiple tube techniques. As a result of previous multi-laboratory trials (PHLS, 1968, 1969) a glutamate medium for the multiple tube technique was recommended in the fourth edition of Report 71 in preference to MacConkey broth. The assessment of membrane culture techniques by the MWB was however carried out mainly in comparison with MacConkey broth and only later were membrane results compared with some of the earlier modifications of glutamate medium. For the purpose of the present trials therefore, the membrane techniques were compared with both MacConkey broth and glutamate medium using multiple tube methods.

The membrane filtration technique involves a preliminary incubation of two membranes for 4 hr. at 30° C. followed by 14 hr. at 35° C. for the total coliform count and 44° C. for the *Esch. coli* count. This had been shown to give higher counts, particularly of coliform organisms or attenuated organisms (Burman, 1967). Preliminary trials at the MWB (Windle Taylor, 1965–6) applying a similar incubation procedure to glutamate medium in the multiple tube method also yielded increased coliform counts without any reduction in those of *Esch. coli*. This modified multiple tube incubation technique was therefore also included in the present series of trials. Repeated attempts were made to incorporate the advantages of glutamate into the membrane filtration technique, although considerable difficulty was experienced. The best modification of glutamate media suitable for membranes devised at the MWB was included in these trials. The standard membrane technique and the two modifications of it described in Report 71 were therefore used, together with the glutamate membrane method, and compared with the three multiple tube methods for enumerating coliform organisms and *Esch. coli* in water samples. Seven laboratories in different parts of the country participated in these trials but not all of them used every method or both chlorinated and unchlorinated water.

MATERIAL AND METHODS

Membrane methods

The three basic membrane filtration techniques as described in Report 71 were used for this investigation.

These were:

(1) The standard technique with incubation of both membranes at 30° C. on 0·4 % enriched Teepol medium (0·4 ET) for 4 hr. followed by 14 hr. at 35 and 44° C. respectively.

(2) The extended technique with incubation of both membranes at 25° C. on 0·4 ET for 6 hr. followed by 18 hr. at 35 and 44° C. respectively.

(3) The transport technique with incubation of both membranes on transport medium (TM) for 24 hr. at 25° C. followed by their transfer to 0·4 ET and incubation at 35 and 44° C. respectively for a further 18 hr. In addition, a membrane glutamate medium (MG) developed at the MWB was used (Windle Taylor, 1967–8). This contained L(+)-glutamic acid sodium salt, 6·5 g.; lactose, 30 g.; L(+)-arginine monohydrochloride, 0·02 g.; L(−)-aspartic acid, 0·024 g.; L(−)-cystine, 0·02 g.; K_2HPO_4, 1 g.; NH_4Cl, 2·5 g.; $MgSO_4.7H_2O$, 0·1 g.; $CaCl_2.2H_2O$, 0·02 g.; ferric ammonium citrate, 0·05 g.; thiamin (aneurin hydrochloride), 0·001 g.; bromocresol purple (1% ethanolic solution), 12 ml.; distilled water to 1000 ml.; pH after sterilization, 6·7; penicillin 100 μg./ml. was added immediately before use. The general method of preparation and sterilization of this medium was as described for glutamate medium in Report 71. Membranes were incubated on this medium for 4 hr. at 30° C. followed by 20 hr. at 35 and 44° C. respectively, and then a further 18 hr. at 35 and 44° C. Counts of yellow colonies were made after 24 and 42 hr. incubation.

As some of the laboratories participating in the trial did not have the equipment required for automatic temperature changes after the resuscitation periods, the temperature changes were made by hand and the 14 hr. incubation periods were extended to 18 hr. to avoid inconvenient incubation times. This was unlikely to affect the counts unless large colonies coalesced and obscured smaller colonies.

Multiple tube method

The multiple tube technique with MacConkey broth and improved formate lactose glutamate medium (IFLG) was used exactly as described in Report 71. All presumptive positive results at 18 hr. were recorded as coliform organisms but those at 24 and 48 hr. were confirmed as coliform organisms by subculture to lactose ricinoleate broth (LRB) incubated at 37° C. The presence of *Esch. coli* was confirmed by subculture of all presumptive positive tubes to LRB for gas formation and to peptone water for indole production, both incubated at 44 ± 0·25° C. Some laboratories examined an additional series in IFLG incubated at 30° C. for the first 4 hr. (IFLG (4 hr., 30°)).

Sources of media

The 0·4 ET, the IFLG and the MacConkey broth used were from single batches of Oxoid dehydrated media purchased in bulk and distributed to each laboratory where they were made up and sterilized. The membrane glutamate medium was made in one batch at the MWB for distribution. The membrane transport medium and the LRB were prepared by the methods normally in use at each laboratory.

Water samples

Unchlorinated water samples were used as available and, if necessary, were diluted so as to give some tubes with negative results among one 50 ml., five 10 ml., and five 1 ml. portions. Samples of chlorinated water were prepared from raw waters by the marginal chlorination method (PHLS, 1968). This was based on chlorination in the presence of excess ammonia at very low temperatures

Table 1. *Relation between degrees of bacterial content and bacterial counts*

Degree of bacterial content	Most probable numbers by multiple tube methods	Counts by membrane methods
1	0	0
2	1	1
3	2	2
4	3	3
5	4	4
6	5	5
7	6	6
8	7	7
9	8	8
10	9	9
11	10	10
12	11	11
13	12	12
14	13	13
15	14	14, 15
16	17	16, 17
17	18	18, 19
18	20	20–22
19	25	23–27
20	30	28–32
21	35	33–37
22	40	38–45
23	50	46–70
24	90	71–125
25	160	126–180
26	> 180	> 180

and for times sufficient to allow the survival of at least some coliform organisms detectable by one or more of the methods under investigation.

Randomization of order of examination

In order to avoid the possibility of the results being influenced by the order of setting up the samples by the various methods, each laboratory was supplied with a series of randomized letters representing the sequence in which the methods were to be used for each sample. This order was then recorded on each result sheet.

RESULTS

The most probable number of organisms (MPN) was obtained by the use of McCrady's tables as printed in appendix C, table II, of Report 71. For the 11 tubes inoculated the MPN gives one or other of 26 possible results which, for the purpose of this analysis, have been expressed as degrees of bacterial content (DBC). The counts on membranes were also transformed into the corresponding 26 degrees of bacterial content (Table 1). Most of the comparisons between the methods have been based on and expressed in these 26 degrees of bacterial content. Rough interpolation was used for the two combinations of positive tubes which are not included in the set of tables used. Degrees of bacterial content are convenient for

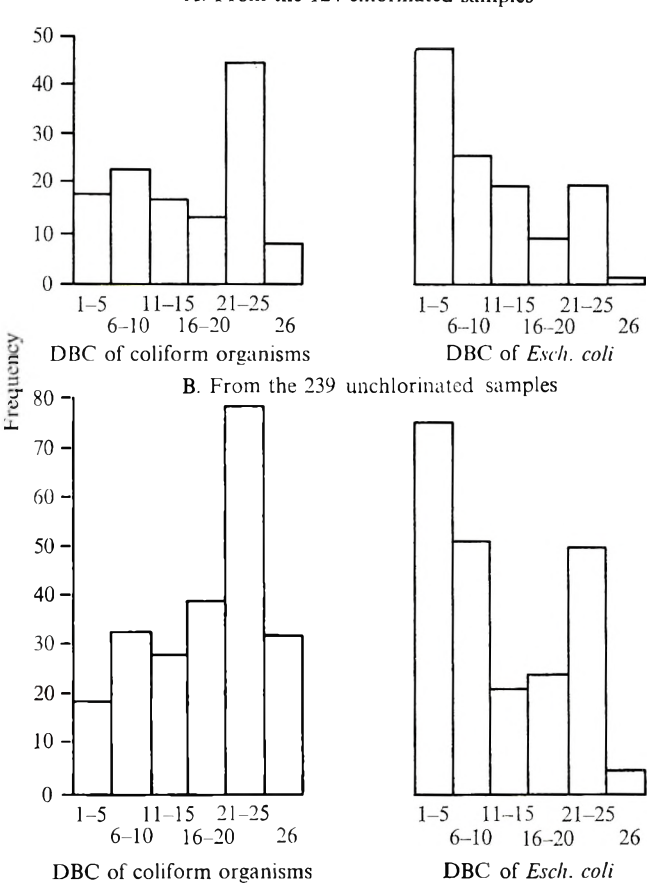

Fig. 1. Distribution of DBC of coliform organisms and *Esch. coli* as found by IFLG method.

statistical analysis and, like the actual numbers of tubes yielding acid and gas which were used in a previous trial (Report, 1969), avoid giving undue weight to high counts.

It became clear after preliminary analysis that the difference between DBC given by tube and membrane techniques depended on whether samples were of chlorinated or unchlorinated water and whether coliform organisms or *Esch. coli* were sought. The type of sample and bacterium sought were therefore analysed separately.

As was to be expected the DBC of samples from widely varying sources did not follow any regular distribution (Fig. 1), nor were DBC results from any single laboratory regular in their distribution. Comparisons were therefore based on the more normally distributed differences between DBC of paired results from two methods applied to the same sample. For each water sample the difference between the DBC given by IFLG and the other method under comparison was calculated and these differences were averaged for all samples. If this average difference is significantly greater or less than zero, the two methods can be said to give different results.

Table 2. *Chlorinated water samples; differences of DBC for paired multiple tube methods*

Methods of testing	Laboratory	Coliform organisms			*Esch. coli*		
		No. of samples	Mean difference of DBC	Standard error of mean	No. of samples	Mean difference of DBC	Standard error of mean
IFLG, MacConkey	Manchester	12	4·0*	1·6	12	4·1*	1·1
	MWB	14	−0·1	1·2	14	4·4*	1·5
	Newport	—	—	—	—	—	—
	Oxford	9	−2·6	2·1	9	1·4	1·2
	Wakefield	19	0·1	0·8	18	2·4*	1·0
	All laboratories	54	0·4	0·69	53	3·1*	0·62
IFLG, IFLG (4 hr., 30°)	Manchester	13	−0·4	0·9	13	1·5	1·1
	MWB	14	−2·3*	1·0	14	−0·6	0·7
	Newport	—	—	—	—	—	—
	Oxford	3	4·0	—	3	0·0	—
	Wakefield	15	−0·9	0·8	15	1·7	0·9
	All laboratories	45	−0·9	0·56	45	0·8	0·50

* Mean difference significantly greater than zero ($P < 0·05$).

Chlorinated waters

Between November 1969 and June 1971 five laboratories reported results from a total of 124 chlorinated water samples, in each of which coliform organisms and *Esch. coli* had been enumerated by at least two methods. The possible methods were MacConkey, IFLG and IFLG (4 hr., 30°) multiple tube methods and standard, extended, transport and glutamate membrane filtration methods. The last was little used.

Table 2 shows no overall significant difference between the DBC of coliform organisms obtained with IFLG and MacConkey media or IFLG and IFLG (4 hr., 30°), although on average IFLG gave slightly higher DBC than MacConkey and slightly lower than IFLG (4 hr., 30°). The DBC of *Esch. coli* with IFLG were on average very significantly higher than with MacConkey and slightly, but not significantly, higher than with IFLG (4 hr., 30°).

A comparison was made between the IFLG method and the four membrane methods in turn. Table 3 shows that for all laboratories combined, the average DBC of coliform organisms was significantly lower for IFLG than standard and extended membranes, but the difference between IFLG and transport membranes was not significant. Glutamate membrane gave significantly lower average DBC than IFLG. In contrast, the average DBC of *Esch. coli* with IFLG was significantly higher than with any of the four membrane filtration methods.

It should be noted that, although there were definite differences in average DBC between methods, the actual variation between individual samples was considerable. Fig. 2 demonstrates this variability for three pairs of methods for coliform organisms and *Esch. coli*. The proportions are shown of paired results from samples where both methods gave the same DBC; where the first method

Table 3. *Chlorinated water samples; differences of DBC for paired IFLG and membrane methods*

Methods of testing	Laboratory	Coliform organisms			*Esch. coli*		
		No. of samples	Mean difference of DBC	Standard error of mean	No. of samples	Mean difference of DBC	Standard error of mean
IFLG, standard membrane	Manchester	29	−3·8*	0·9	29	0·6	1·2
	MWB	33	−1·9	1·2	33	5·9*	1·1
	Newport	10	−0·3	1·8	10	3·5	1·6
	Oxford	20	−2·0	1·3	20	1·1	0·9
	Wakefield	32	−2·8*	0·9	31	4·5*	0·9
	All laboratories	124	−2·5*	0·52	123	3·3*	0·49
IFLG, extended membrane	Manchester	27	−3·8*	1·0	27	−0·1	1·2
	MWB	33	−4·1*	1·4	33	5·8*	1·1
	Newport	—	—	—	—	—	—
	Oxford	12	−1·5	1·8	12	1·6	1·4
	Wakefield	32	−3·3*	1·0	31	4·7*	0·9
	All laboratories	104	−3·5*	0·61	103	3·4*	0·57
IFLG, transport membrane	Manchester	29	−3·7*	1·6	27	2·0	1·6
	MWB	33	1·2	1·5	33	7·2*	1·2
	Newport	—	—	—	—	—	—
	Oxford	3	5·0	—	3	7·3	—
	Wakefield	24	−0·2	1·2	24	6·8*	1·4
	All laboratories	89	−0·7	0·85	87	5·5*	0·80
IFLG, glutamate membrane	All laboratories	27	5·4*	1·57	26	6·0*	1·31

* Mean difference significantly greater than zero ($P < 0{\cdot}05$).

gave greater or smaller DBC of up to five degrees; and where the difference was six or more. These diagrams show that even where one method gave a significantly higher average DBC than a second method, the difference for some samples was in favour of the second method. For example, figure 2A (i) shows that, although IFLG gave significantly lower average DBC of coliform organisms than standard membrane, IFLG gave higher DBC in 26% of the samples. The proportion of samples where the membrane method gave a higher coliform or *Esch. coli* DBC than IFLG was slightly greater for extended membrane than for standard membrane. Transport membrane gave the lowest proportion.

As the five laboratories sampled different water supplies, their results were dissimilar in many ways. However, in the comparison of methods none of the combined findings for chlorinated water samples were significantly contradicted by the results from any individual laboratory.

One method sometimes showed the presence of coliform organisms which were not found in the same sample by a different method. This could mean that the actual method used for detecting the presence of coliform organisms and *Esch. coli* could influence any action to be taken. Taking all the chlorinated water samples examined, IFLG and most of the other methods detected coliform organisms in a similar number of samples, although there was not complete agreement about

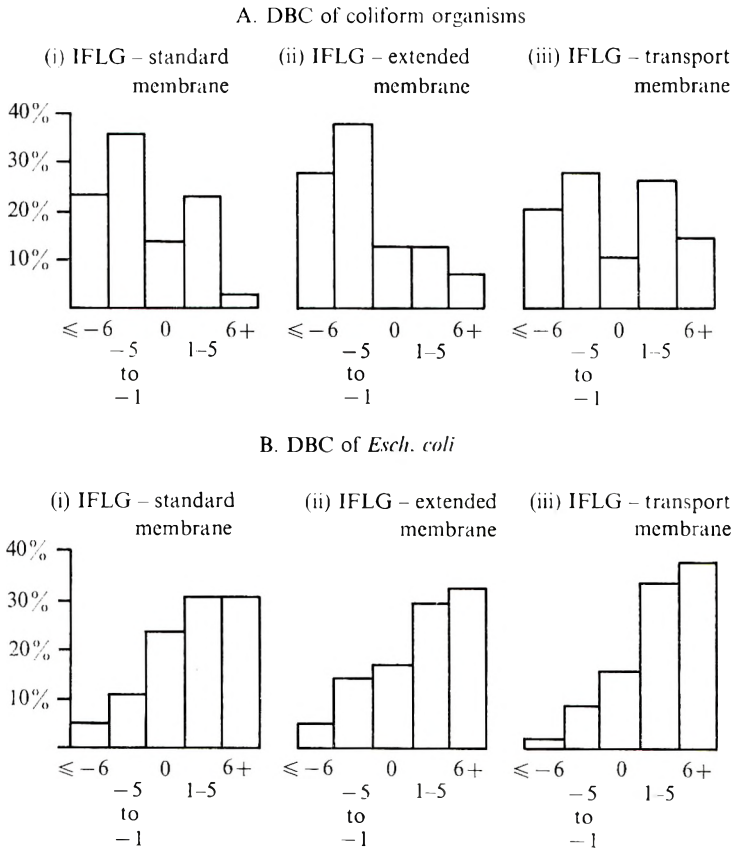

Fig. 2. Distribution of difference in paired results from chlorinated water samples.

Table 4. *Chlorinated water samples; number of samples in which organisms were detected or not detected by IFLG and other methods*

	Coliform organisms				*Esch. coli*			
	Found by IFLG		Not found by IFLG		Found by IFLG		Not found by IFLG	
Other method	Found by other method	Not found by other method	Found by other method	Not found by other method	Found by other method	Not found by other method	Found by other method	Not found by other method
MacConkey	51	1	0	2	40	5	1	7
IFLG (4 hr., 30°)	42	0	2	1	37	2	1	5
Standard membrane	114	5	2	3	80	24	3	16
Extended membrane	97	2	2	3	66	21	4	12
Transport membrane	77	7	2	3	46	32	0	9
Glutamate membrane	15	9	1	2	18	8	0	0

which samples contained the organisms (Table 4). It was uncommon for the other two multiple tube methods to yield organisms where IFLG did not and vice versa. Coliform organisms, detected in samples by IFLG, were not found in five (4 %) of 119 samples by standard, in two (2 %) of 99 samples by extended, in seven (8 %) of 84 samples by transport and in nine (38 %) of 24 samples by glutamate membrane. However, IFLG detected *Esch. coli* in larger numbers of samples than did the other methods. Where *Esch. coli* was found by IFLG, they were not detected in five (11 %) of 45 samples by MacConkey, in two (5 %) of 39 samples by IFLG (4 hr., 30°), in 24 (23 %) of 104 samples by standard, in 21 (24 %) of 87 samples by extended, in 32 (41 %) of 78 samples by transport and in eight (31 %) of 26 samples by glutamate membrane. There were few chlorinated water samples in which no coliform organisms were detected by IFLG, but in which they were detected by other methods. The number of such samples was too small to evaluate relative frequencies.

Analysis of repeatability

Sixty of the chlorinated waters were tested in duplicate in an attempt to assess the repeatability of four methods. Each of the 60 samples was examined twice by IFLG and twice by standard membrane; most of them were also examined twice by extended and transport membrane. Only the first result of each pair was used for the main analysis already described. The results of the analysis of repeatability showed that none of the methods invariably gave the same DBC twice. Order of examination and magnitude of DBC were not associated with the size of the difference. The differences of DBC of coliform organisms and of *Esch. coli* were similar and were therefore combined. The variability was significantly less in membrane filtration methods than in multiple tubes with IFLG. In 120 duplicate multiple tube tests for coliform organisms or *Esch. coli* using IFLG, there was no difference between the two results in 31 (26 %) samples; in 52 (43 %) there was no difference or a difference of one DBC, and in 100 (83 %) the difference was not more than five DBC. The corresponding proportions for the 292 duplicate tests with membrane filtration methods were 40, 70 and 93 %. It is claimed as an advantage of membrane filtration that the results are likely to lie within narrower limits of variation than with a multiple tube method which is dependent on a MPN. These variations are discussed in Report 71. The present experimental results detected less variation of DBC with membranes in duplicate tests on the same samples.

Unchlorinated waters

Between November 1969 and November 1970 seven laboratories reported results from a total of 239 unchlorinated water samples. Each sample had been tested by at least two of the methods already mentioned for coliform organisms and *Esch. coli*.

Table 5 shows that the overall DBC of coliform organisms was significantly higher for IFLG than for MacConkey. There was no overall significant difference between IFLG and IFLG (4 hr., 30°). IFLG gave a significantly higher average DBC of *Esch. coli* than MacConkey and a slightly but not significantly lower average than IFLG (4 hr., 30°).

Table 5. *Unchlorinated water samples; difference of DBC for paired multiple tube methods*

Methods of testing	Laboratory	Coliform organisms: No. of samples	Coliform organisms: Mean difference of DBC	Coliform organisms: Standard error of mean	*Esch. coli*: No. of samples	*Esch. coli*: Mean difference of DBC	*Esch. coli*: Standard error of mean
IFLG, MacConkey	Conway	9	7·0*	2·3	9	6·2*	2·3
	Manchester	84	1·2*	0·5	85	1·1	0·6
	MWB	24	1·3*	0·5	24	2·5*	1·0
	Newport	—	—	—	—	—	—
	Oxford	11	0·2	1·6	11	0·3	0·6
	Southend	39	3·6*	1·1	39	3·8*	1·0
	Wakefield	29	1·2	0·9	29	0·4	0·9
	All laboratories	196	1·9*	0·37	197	1·9*	0·38
IFLG, IFLG (4 hr., 30°)	Conway	—	—	—	—	—	—
	Manchester	85	−0·7	0·6	85	−0·6	0·5
	MWB	24	−0·1	0·6	24	−1·0	1·1
	Newport	—	—	—	—	—	—
	Oxford	5	−1·2	—	5	0·8	—
	Southend	13	2·6*	1·0	13	1·7	1·4
	Wakefield	23	−0·8	1·1	23	−1·5	1·1
	All laboratories	150	−0·3	0·39	150	−0·6	0·35

* Mean difference significantly greater than zero ($P < 0·05$).

Table 6 compares the results from IFLG and membrane filtration methods for unchlorinated water samples; with these, laboratories did not always agree on which method gave the highest average DBC. With chlorinated waters, however, each laboratory gave results which were either significantly in favour of the same method or else inconclusive.

IFLG gave significantly lower average DBC of coliform organisms than either standard or extended membranes. The overall average differences in DBC of coliform organisms between IFLG and transport membrane were not significant, although two laboratories gave significantly lower and one laboratory significantly higher results with IFLG. The differences in average DBC of *Esch. coli* between IFLG and the membrane methods were less conclusive. Overall, IFLG gave higher results than standard membrane but in one laboratory it gave significantly lower results. There was no significant overall difference between IFLG and extended membrane, although one laboratory favoured IFLG and one extended membrane. The comparison with transport membrane was similar to that with standard membrane – overall results in favour of IFLG but one laboratory in favour of the membrane method. It has been suggested by one participating laboratory that the laboratory differences with transport membranes could be due to the lack of buffering and consequent difficulty of stabilizing the pH. IFLG gave a very significantly higher average DBC than glutamate membrane for both coliform organisms and *Esch. coli*.

The distribution of differences between results from IFLG and three of the

Table 6. *Unchlorinated water samples; differences of DBC for paired IFLG and membrane methods*

Methods of testing	Laboratory	Coliform organisms: No. of samples	Coliform organisms: Mean difference of DBC	Coliform organisms: Standard error of mean	*Esch. coli*: No. of samples	*Esch. coli*: Mean difference of DBC	*Esch. coli*: Standard error of mean
IFLG, standard membrane	Conway	14	1·2	1·9	14	2·1	2·2
	Manchester	83	−3·8*	0·7	81	−2·0*	0·8
	MWB	24	−0·5	0·4	24	2·5*	1·0
	Newport	27	1·5	0·8	27	5·7*	0·9
	Oxford	11	−6·3*	1·3	11	−1·6	0·9
	Southend	39	−2·4*	0·9	39	1·3	0·9
	Wakefield	39	−5·2*	1·0	36	3·9*	1·5
	All laboratories	237	−2·7*	0·35	232	1·1*	0·44
IFLG, extended membrane	Conway	—	—	—	—	—	—
	Manchester	83	−4·1*	0·7	83	−1·9*	0·9
	MWB	24	−0·7	0·6	24	3·2*	1·1
	Newport	—	—	—	—	—	—
	Oxford	9	−6·8*	1·3	9	−1·1	1·1
	Southend	—	—	—	—	—	—
	Wakefield	7	−5·6*	1·5	6	6·0	2·7
	All laboratories	123	−3·7*	0·47	122	−0·4	0·63
IFLG, transport membrane	Conway	—	—	—	—	—	—
	Manchester	80	−3·8*	0·6	81	−2·4*	0·8
	MWB	24	2·8*	1·1	24	8·4*	1·4
	Newport	—	—	—	—	—	—
	Oxford	4	1·0	—	4	0·0	—
	Southend	35	0·9	1·3	35	4·2*	0·9
	Wakefield	24	−5·3*	1·5	26	5·3*	1·7
	All laboratories	167	−0·3	0·39	170	1·7*	0·54
IFLG, glutamate membrane	All laboratories	91	6·9*	0·85	84	5·2*	0·86

* Mean difference significantly greater than zero ($P < 0·05$).

membrane methods are shown in figure 3. Standard and extended membranes gave significantly higher average DBC of coliform organisms than IFLG but in 23 and 13 % of samples respectively their results were lower. Standard and transport membranes gave significantly lower average DBC of *Esch. coli* than IFLG, but gave higher results in 35 and 30 % of samples respectively. As with the chlorinated water samples (see figure 2) extended membrane gave higher results than IFLG in a slightly greater proportion of samples than did standard membrane, whereas transport membrane gave the smallest proportion. In the comparison of methods illustrated in figures 2 and 3 there are larger proportions to the left of the diagrams – where membrane methods gave higher results than IFLG – for unchlorinated water than for chlorinated water samples.

Table 7 shows the presence or absence of these bacteria in the unchlorinated water samples as found by IFLG and the other methods. With the exception of glutamate membrane, each method detected coliform organisms in nearly all of

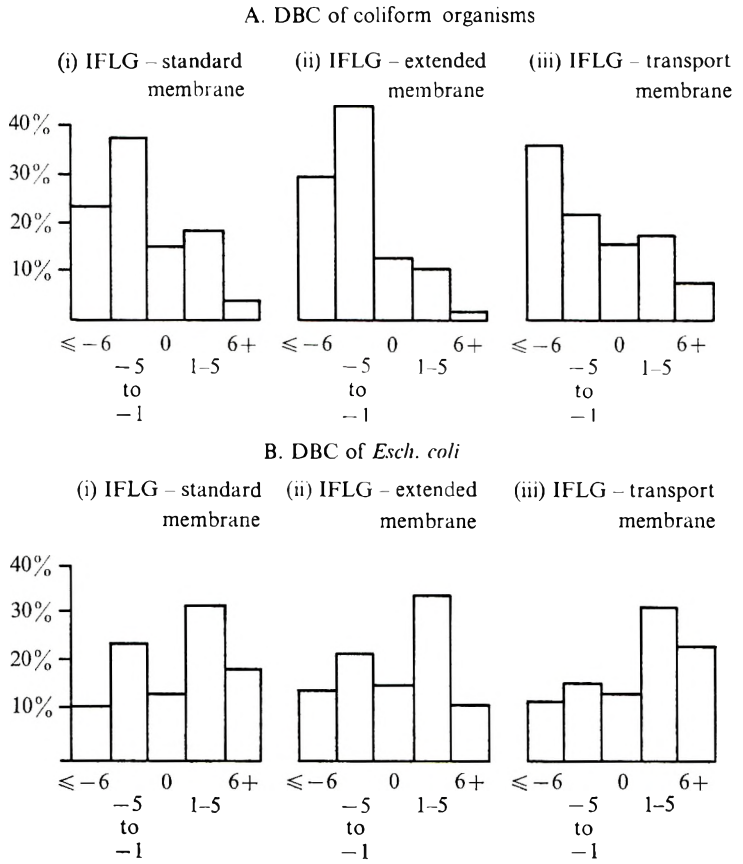

Fig. 3. Distribution of differences in paired results from unchlorinated water samples.

Table 7. *Unchlorinated water samples; number of samples in which organisms were detected or not detected by IFLG and other methods*

	Coliform organisms				*Esch. coli*			
	Found by IFLG		Not found by IFLG		Found by IFLG		Not found by IFLG	
Other method	Found by other method	Not found by other method	Found by other method	Not found by other method	Found by other method	Not found by other method	Found by other method	Not found by other method
MacConkey	190	2	1	3	144	21	11	21
IFLG (4 hr., 30 °)	148	0	2	0	116	9	12	13
Standard membrane	233	0	4	0	175	23	22	12
Extended membrane	121	0	2	0	91	7	16	8
Transport membrane	162	2	1	2	118	29	11	12
Glutamate membrane	57	22	2	1	32	38	3	11

these water samples. Agreement between methods on the presence or absence of *Esch. coli*, however, was not so close. Altogether, *Esch. coli* was detected in more of the samples by IFLG than by other methods, except for standard membrane, where the numbers were similar, and IFLG (4 hr., 30°) and extended membrane which both detected *Esch. coli* in more samples than IFLG. *Esch. coli*, found in samples by IFLG, were not detected in 21 (13 %) of 165 samples by MacConkey, in 9 (7 %) of 125 samples by IFLG (4 hr., 30°), in 23 (12 %) of 198 samples by standard, in 7 (7 %) of 98 samples by extended, in 29 (20 %) of 147 samples by transport or in 38 (54 %) of 70 samples by glutamate membrane. Where *Esch. coli* was not found by IFLG they were more often than not detected by standard or extended membrane.

DISCUSSION

A comparison has been made between the results from four membrane methods and those from IFLG with the same water samples. At the same time some comparisons have been made between IFLG, MacConkey and IFLG (4 hr., 30°) multiple tube methods. Seven laboratories took part, although they did not all carry out every test. Laboratories sometimes differed significantly in the comparisons obtained between membranes and multiple tube methods. The reasons for such differences between laboratories cannot be explained by any one factor, but they confirm the recommendation in Report 71 that 'It is essential that before adopting membrane filtration as a routine procedure in any laboratory or with any particular water supply, an adequate parallel series of tests should be run comparing membranes with multiple tubes, in order to establish their equivalence or the superiority of one or the other.' Despite the differences between individual laboratories, the combined results from all laboratories showed some significant differences in the results obtained with different media.

The three multiple tube methods gave average results of comparable magnitude for coliform organisms except that IFLG detected in general more organisms more frequently than MacConkey in unchlorinated waters. IFLG was better in that it gave significantly higher average DBC of *Esch. coli* than MacConkey with both unchlorinated and chlorinated waters, whereas it was only slightly better than IFLG (4 hr., 30°) with chlorinated waters and slightly worse with unchlorinated waters. IFLG (4 hr., 30°) was slightly better than MacConkey at detecting organisms not found by IFLG and in not failing to detect them when found by IFLG.

The findings of the four membrane methods compared with IFLG show that two of them – standard and extended membrane – achieved better results in some respects than IFLG. Transport membrane was never significantly better than IFLG on the combined results for all laboratories and the results from glutamate membrane were consistently poor. Standard and extended membranes were significantly better than IFLG for coliform organisms for both unchlorinated and chlorinated waters, but were significantly poorer for *Esch. coli* in chlorinated waters; they gave equivocal results with unchlorinated waters. Throughout the trial, extended membrane gave better results than IFLG in larger proportions of

samples than did standard membrane. These two membrane methods seldom failed to detect the presence of coliform organisms found by IFLG, but failures did occur with *Esch. coli*, especially in chlorinated waters where the failure rates for both methods were nearly 25 %; however, they did sometimes detect *Esch. coli* where IFLG had failed. In unchlorinated waters both these membrane methods yielded *Esch. coli* in two thirds of the samples.

As the recommendations on membrane filtration in the fourth edition of Report 71 were based mainly on work done at the Metropolitan Water Board, the results of the present trials were examined to see if they supported those recommendations. As stated in the introduction, the original MWB work used MacConkey broth in multiple tubes as the standard reference method. The current work has used IFLG as the standard method and all the other methods, including MacConkey, have been compared with it.

Considering overall results with unchlorinated waters for coliform organisms, standard and extended membranes gave significantly higher results than MacConkey; for *Esch. coli*, standard membranes gave lower results than IFLG, and there was no significant difference between extended membranes and IFLG. The standard membrane results, however, were not significantly lower than those with MacConkey in any laboratory. These results were, therefore, in accord with the earlier work at the MWB that with unchlorinated waters standard and extended membranes gave results as high as or higher than MacConkey for coliform organisms and *Esch. coli*.

With chlorinated waters for coliform organisms, standard and extended membranes gave higher results than IFLG, and there was no significant difference between IFLG and MacConkey; these two membrane methods thus gave higher results than MacConkey. This result is more favourable for membranes with coliform organisms in chlorinated waters than previously found at the MWB. With chlorinated waters for *Esch. coli*, on the other hand, IFLG gave significantly higher results than any membrane method or MacConkey. In this trial, there was no overall significant difference in results for *Esch. coli* in comparison between extended membranes and MacConkey with chlorinated waters.

It may be inferred from these results that IFLG would be better than the present membrane methods for detecting *Esch. coli* in chlorinated waters. But these results have also shown that standard and extended membranes gave higher results than IFLG tubes for coliform organisms in chlorinated waters. Coliform organisms are not, however, a separate group but include *Esch. coli*. This would suggest therefore that higher numbers of *Esch. coli* could be obtained by confirming the identity of the colonies on the coliform membranes. This would take no longer and involve no more work than the equivalent IFLG tube method. The failure of the membrane methods for *Esch. coli* in chlorinated waters, appears therefore to be related to incubation at 44° C. as it does not occur at 37° C. Chlorinated waters should not contain coliform organisms or *Esch. coli* and as any such organisms found would normally be subcultured for confirmation, reliance on the coliform membranes for detecting *Esch. coli* would not involve any additional work. Furthermore, such results occur so infrequently in normal quality testing at waterworks that local

comparisons of membrane and multiple tube methods on routine samples would be unlikely to show any significant differences in actual practice. Indeed, the differences found in this paper were only demonstrated by deliberately producing inadequately chlorinated samples of water in which there were some surviving organisms.

Certain assumptions have however been made in reaching these conclusions. Although *Esch. coli* is included in coliform counts, it does not necessarily follow that the higher coliform counts obtained in the present work with membranes did in fact include *Esch. coli* because confirmation was not carried out. Other possibilities are that the membrane coliform counts consisted largely of false positive results due to yellow colonies other than coliform organisms or of coliform organisms other than *Esch. coli*, which would imply that IFLG is detecting *Esch. coli* but not some other coliform organisms. This is less likely but can only be resolved by indentification of the coliform organisms on the membranes from chlorinated samples. Meanwhile, it is recommended that membranes may continue to be used for chlorinated samples provided that any coliform organisms isolated at 35 or 37° C. are subcultured for confirmation as *Esch. coli*. In practice, whatever laboratory methods are used, any chlorinated waters which yield coliform organisms but not *Esch. coli* should be resampled as a routine.

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Passive protection of mice against intracerebral infections with *Bordetella pertussis*

By JEAN M. DOLBY
The Lister Institute of Preventive Medicine, Elstree, Hertfordshire

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SUMMARY

The passive protection of mice against an intracerebral infection with *Bordetella pertussis*, by antiserum introduced directly into the brain with the infecting organisms, was compared with the protection afforded by intraperitoneal antiserum. The antibody effective by the intracerebral route is that which is adsorbed onto the infecting organisms, although it does not affect the viability of the organisms *in vitro* in the absence of complement.

Passive protection against organisms introduced intracerebrally takes place in one of two ways, depending on the size of the challenge: (1) after 3–4 days' growth, the growth rate declines so that the number of organisms does not reach the figure lethal for the mouse; (2) the organisms do not appear to multiply, as their numbers decline from the moment of injection, so that the brain is sterile after 2 days.

Many of the mice protected against a challenge of 50,000 organisms (*ca.* 100 LD50) by intraperitoneal or intracerebral antiserum recover by the first mechanism. The second mechanism operates after a smaller challenge of 5000 organisms (*ca.* 10 LD50), irrespective of whether the antiserum is given intracerebrally with the challenge, or intraperitoneally within several hours of challenge. Too much antiserum given intracerebrally with a 50,000 challenge, but not with a 5000 challenge, inhibits protection.

In some mice, virulent antibody-treated organisms which have not been killed, grow very slowly over a long period, but are eventually eliminated from the brain.

INTRODUCTION

Spasojević (1962) measured the protective potency of *Bordetella pertussis* antisera for mice by means of an intracerebral passive protection test in which bacteria were incubated at 37° C. for 1 hr. with graded dilutions of antiserum, and injected intracerebrally into mice. (This will be referred to as the 'IC mix' test.) She obtained consistent and reproducible results with both rabbit sera and convalescent human sera from children; the dose–response curves were good and some sera were protective at very high dilutions. She also found (private communication) that the number of sterile brains 2 days after challenge equalled the number of survivors. Adams (1968) found that over half the mice that were to survive had sterile brains 2 days after challenge.

We were unable to reproduce the consistency and dose–response curves of

Spasojević's experiments; often the same number of mice died in each of a wide range of dilutions, giving a plateau-like or even inhibitory zone effect over lower antiserum dilutions. The preliminary investigations which were made on the course of infection in mice given serum and organisms together also indicated that the brains of recovering mice were seldom sterilized before 6 days; the curve of infection and recovery was similar to that described for intraperitoneal serum and intracerebral challenge (Dolby & Standfast, 1961).

It was felt that, apart from explaining differences between workers, a reinvestigation of the 'IC mix' test was worth while for both practical and academic reasons. Only very small amounts of serum are needed for the test and if it were measuring the same antibody as that causing protection when much larger doses are administered intraperitoneally, 3 hr. before challenge (IP/IC test), then it would be useful. If, on the other hand, there are two mechanisms for passive protection, then it is of interest to learn more about the differences.

METHODS

Strains used for challenge

Bordetella pertussis strain 18–323 was used for infections by the intracerebral route and *B. pertussis* strain Gl. 353 for infections by the intranasal route.

The required suspension was made by homogenizing the growth after 20 hr. at 36°C. from Bordet-Gengou plates in 1% (w/v) Difco Casamino Acids (vitamin free) and diluting the suspension to 10 International Opacity Units (equal to $10{,}000 \times 10^6$ *B. pertussis* per ml.). Further dilutions were made as required in 1% Difco Casamino Acids. Routine viable counts by the method of Miles & Misra (1938) showed that about 10% of the 18–323 suspensions and 20% of the Gl. 353 suspensions were viable. Doses are all cited as total, not viable, numbers of organisms. The LD 50 of strain 18–323 injected intracerebrally was 500–1000 organisms; lethal doses of 5000 and 50,000 organisms were used. Strain Gl. 353 was instilled intranasally in sublethal doses of 50,000 organisms (about 0·01 LD 50).

Mice

White mice, 17–19 g., of Schneider-Webster, ICI, Theiler's Original, and TF 1 strains were used. Mice of one strain and one sex were used in any one experiment. The first three strains were easily protected by intraperitoneal vaccine against an intracerebral challenge 14 days later. Halfway through this work, the Schneider-Webster and TF 1 mice became more difficult to immunize; the implications of this are discussed below.

Antisera

Two rabbit antisera were used. Batch number 6660, a lyophilized serum used in most experiments, was a pool of sera from a dozen or so rabbits given phase I *B. pertussis*; 0·2 ml. of undiluted serum given intraperitoneally at about the same time as the intracerebral challenge usually protected all mice, whereas 0·2 ml. of a 1/10 dilution protected rather less than half. Batch number E014, a similar liquid serum, was used in a few experiments. Sera were heated at 56° C. for 30 min.

before use. The amount needed to protect half the mice (PD 50) was calculated by the method of Reed & Muench (1938).

Passive protection tests with intraperitoneal serum and intracerebral challenge (IP/IC)

Mice received 0·2 ml. of the serum dilution (in saline) intraperitoneally 3 hr. before the challenge. The intracerebral challenge of 50,000 or 5000 organisms was given in 0·03 ml. of 1 % Casamino Acids. This method is referred to in brief as 'IP/IC 50,000' or 'IP/IC 5000'.

Passive protection tests with intracerebral serum and challenge ('IC mix')

Suitable dilutions of serum and challenge suspensions, both in 1 % Casamino Acids, were mixed in equal volumes so that 0·03 ml. contained either 50,000 or 5000 organisms as required. The mixtures were injected into the mice usually after incubation for 30 min. at 37° C., although incubation seemed to make very little difference to the results. There was no diminution in viable count due to *in vitro* serum treatment. This method is abbreviated to 'IC mix 50,000' or 'IC mix 5000'.

Sensitized organisms were prepared from the serum +organisms mixtures by centrifuging at 3000 rev./min. (on an MSE angle head laboratory bench model centrifuge) and replacing the supernatant with an equal volume of 1 % Casamino Acids. In one experiment, sensitized organisms were prepared by incubating serum with concentrated suspension at 10^{10} organisms per ml. and then diluting to 50,000 organisms in 0·03 ml.

Sublethal intranasal challenge

Normal mice were given 50,000 organisms of strain Gl. 353 in 0·04 ml., instilled intranasally under light anaesthesia, producing a sublethal lung infection in which the bacterial count increased up to about the 8th day and then decreased (Dolby, Thow & Standfast, 1961). Active or passive immunity suppressed the sublethal infection and counts of viable organisms in the lungs of such mice provided a sensitive method for measuring the degree of protection.

Estimation of the degree of protection and of viable organisms in brains and lungs

Dead and living animals were recorded daily in cages of treated mice. Groups of mice were taken at intervals from duplicate cages and killed with coal gas for counts. Brains or lungs were removed aseptically, each into a 2 oz. universal bottle containing 9 ml. 1 % Casamino Acids and 2 ml. 5 mm. (diam.) glass beads. The bottles were shaken on a vertical shaker ($2\frac{3}{4}$ in. throw, 325 rev./min.), 3 min. for brains and 10–15 min. for lungs. Tenfold dilutions were made of the homogenates in 1 % Casamino Acids and the dilutions counted (Miles & Misra, 1938) on solid medium (see below) which was examined after 5 days at 36° C. Volumes of 0·5 ml. of the undiluted homogenate were spread on plates for counts when low counts, between 10 and 100 organisms per brain, were expected. Brain counts of less than 10 viable organisms per 0·5 ml. were recorded as sterile.

The solid medium was made from Cohen & Wheeler (1946) liquid medium, modified by the use of 0·1 % casein hydrolysate acid (Oxoid), 0·5 % glutamic acid, 5 % blood and 1·3 % New Zeland agar.

The PD 50 dose of antiserum was calculated by the method of Reed & Muench (1938).

In vitro *measurement of circulating antibodies in mouse serum*

Bactericidal antibody was measured by incubating together for 40 min. at 37° C. 0·2 ml. antiserum dilution, 0·2 ml. *Bordetell apertussis* strain 18–323, containing 10^6 organisms, and 0·2 ml. guinea-pig serum at 1/15 dilution; all dilutions were made in Casamino Acids (Difco 1 %). After incubation, mixtures were diluted in 1 % Casamino Acids containing 7·7 % (w/v) NaCl to stop the action of complement and plated on Cohen & Wheeler blood plates. A fivefold reduction of viable count was considered significant.

Agglutinins were measured by incubating together equal volumes of antiserum dilutions and suspensions of organisms, strain 18–323 at 5×10^9 organisms per ml., in Dreyer tubes at 37° C. for 4 hr. The tubes were held overnight at room temperature and titres of over 1/50, read the following day with a hand lens, were considered significant.

RESULTS

The effect of intraperitoneal and intracerebral antiserum on a challenge of 50,000 *intracerebral organisms* (100 *LD* 50)

Fig. 1 shows the effect of graded amounts of antiserum E 014 by different routes. It will be seen that 0·015 ml. of serum per mouse intracerebrally had the same effect as ten times that volume intraperitoneally, and 0·02 ml. serum given intraperitoneally was inactive, and the increase in viable organisms in mice given this dose was indistinguishable from that in the controls.

The course of intracerebral infection by 50,000 *organisms* (100 *LD* 50) *mixed with antiserum*

Table 1 shows the results for dilutions of 1/2 to 1/250 of the rabbit antiserum 6660. Fig. 2 shows the individual mouse counts for 1/2 serum with the intracerebral challenge of 50,000 organisms. From Fig. 2 it can be seen that at day 1 the serum-treated organisms were fewer than the control organisms, with viable counts of 100–1000 compared with 1000–10,000 in the controls. This reduction in count in the serum-treated mice was not maintained, however, and only 2/10 had sterile brains at 3 days, whereas 92 % of the mice survived. Many of the mice which would have survived were therefore fairly heavily infected at 3 and 10 days with brain counts of up to $10^{6\cdot0}$. Recovery took place following a pattern similar to, but more extended than, what happens in mice vaccinated intraperitoneally 14 days before intracerebral challenge or given antiserum intraperitoneally near the time of challenge (Dolby & Standfast, 1961).

To determine whether adsorbed or unadsorbed antibody was effective, a com-

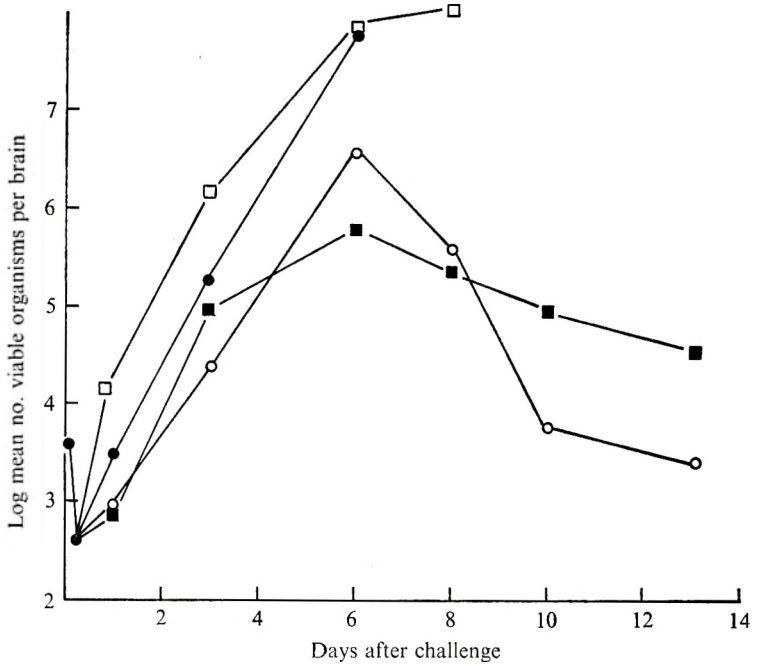

Fig. 1. Growth curves of 50,000 organisms of *Bordetella pertussis*, strain 18–323, in the brains of mice treated with antiserum E014 in various ways and in untreated controls. Each point represents the average count in five mice including healthy, ill and dead individuals, selected in proportion. ●—●, Control mice untreated; 80% dead by the 14th day. □—□, 0·02 ml. antiserum intraperitoneally 3 hr. before challenge; 74% dead by the 14th day. ■—■, 0·2 ml. antiserum intraperitoneally 3 hr. before challenge; 10% dead by the 14th day. ○—○, 0·015 ml. antiserum intracerebrally with challenge; 42% dead by the 14th day.

Table 1. *Data for mice injected intracerebrally with* 0·015 ml. *antiserum* 6660 *mixed with* B. pertussis 18–323, 50,000 *organisms per mouse*

(The mice were kept for 35 days. Fig. 2 shows the viable count of organisms in mice receiving 1 in 2 dilution of antiserum.)

Initial serum dilution	Survivors/ total at 35 days	Percentage survivors	Average time to death (days)
1/2	23/25	92	15·7
1/10	4/14	29	16·4
1/50	5/15	33	12·9
1/250	1/15	7	9·1
No serum	0/10	0	6·6

31/32 survivors from first three cages had sterile brains 35 days after infection. One mouse given 1/2 serum was infected with 80 organisms at the 35th day.

parison was made of the protective effect of antiserum and bacteria, and bacteria alone, after sensitization *in vitro* (Table 2); there was little difference in protection, indicating that the free antiserum in the mixture, which was presumably rapidly eliminated from the brain, took no part in protection. Such small amounts given intraperitoneally were not effective (Fig. 1). The degree of infection by sensitized

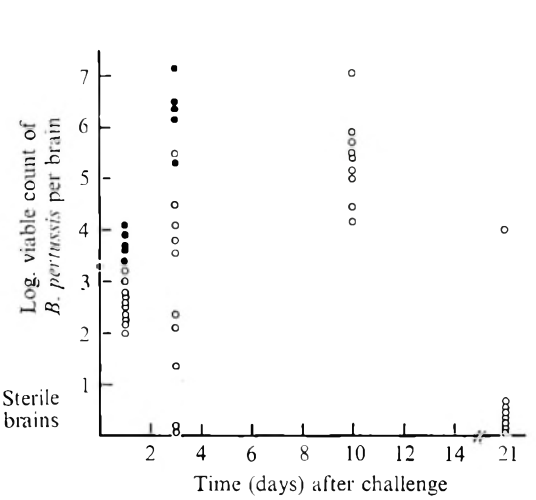

Fig. 2

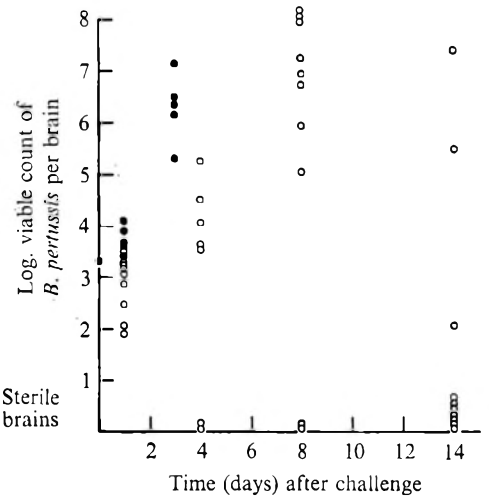

Fig. 3

Fig. 2. *Bordetella pertussis*, strain 18–323, in the brains of individual mice given antiserum 6660 dilution 1/2 (final) and 50,000 organisms intracerebrally. ○, Infection with a mixture of antiserum and organisms; 92 % survived the 21st day. ●, Control mice given infecting organisms only; none survived beyond the 6th day.

Fig. 3. *Bordetella pertussis*, strain 18–323, in the brains of individual mice given, intracerebrally, 50,000 organisms which had been sensitized with antiserum 6660 at a dilution of 1/2 (final), centrifuged and resuspended in Casamino Acids. ○, Infection with sensitized organisms; 50 % survived the 14th day. ●, Control mice given unsensitized organisms only; none survived beyond the 6th day.

Table 2. *Comparison of 'IC mix' passive protection tests using antiserum–organisms mixture or sensitized organisms resuspended in Casamino acids*

(Each challenge volume of 0·03 ml. contained 50,000 organisms of strain 18–323.)

Initial serum dilution	State of organisms on injection	No. of viable organisms in inoculum	Survivors/ total at 21 days	Degree of infection in brains of survivors at 21 days		
				No. investigated	No. sterile	No. of organisms in infected brain
1/2	Mixed with antiserum	1270	5/12	5	4	3200
1/2	Mixed with antiserum, washed and resuspended	1110	6/12	6	6	—
1/100	Mixed with antiserum	1170	9/12	5	5	—
1/100	Mixed with antiserum, washed and resuspended	1160	7/12	5	4	40

organisms is shown in Fig. 3. In this group, mortality was 50 %, yet all mice were infected at days 1 and 8.

Undiluted serum, mixed with organisms at 10,000 times the challenge strength, was unable to sensitize them enough to protect the mice.

It was surprising how long strain 18–323 could persist in the brain after 'IC mix 50,000' experiments. The organisms isolated after 35 days from two brains of

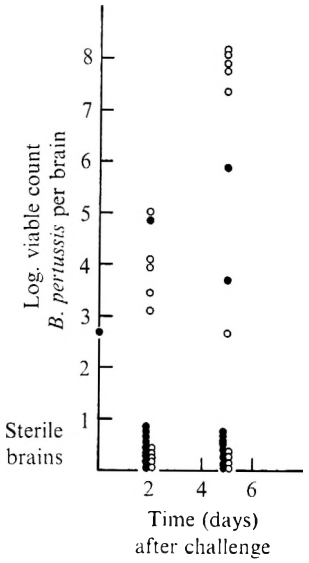

Fig. 4. *Bordetella pertussis*, strain 18–323, in the brains of individual mice given 5000 organisms intracerebrally with: ○, 6660 at a dilution of 1/250 (final), 50% survived; ●, 6660 at a dilution of 1/5 (final), 90% survived.

mice given 1/10 antiserum were typical smooth *Bordetella pertussis*, but another isolate from a mouse given 1/2 antiserum was less easily agglutinated by the antiserum (to almost the same titre, but very weakly) and was 3–4 times less virulent.

Since some mice with these prolonged infections survived, one might expect them to possess some demonstrable active immunity. This was tested on the survivors of 'IC mix 50,000' experiments, after treatment with various amounts of antiserum, by reinfecting with 50,000 organisms intracerebrally; none survived and the course of infection was identical with that in untreated control mice. Neither serum bactericidal antibody nor agglutinins nor active response of the mice to a sublethal lung infection was positive in mice surviving 'IC mix 50,000' experiments with 1/20 or 1/200 antiserum, but serum antibodies and active immunity to small lung infection were detected in mice surviving 'IC mix 50,000' experiments with 1/2 antiserum.

These positive results were due to an active response of the mice and not to persistent antibody from the top dose of antiserum injected. When mice were given 1/2 antiserum intracerebrally and tested 4 weeks later, they had no immunity to a sublethal intranasal infection, nor were antibodies demonstrable *in vitro*.

The course of intracerebral infection by 5000 *organisms* (10 *LD* 50) *mixed with antiserum*

With the smaller, but still lethal, challenge the result was different (Fig. 4). In 'IC mix 5000' experiments with 1/5 and 1/250 antiserum, the viable count dropped from day 0 and the proportion of mice free of organisms on the second day corresponded to the proportion of ultimate survivors. These results are similar to those of Spasojević (1962) and quite different from those in Figs. 2 and 3.

Table 3. *Passive protection experiments with* 6660 *antiserum in Theiler's Original mice against an intracerebral challenge of* 18–323.

(Paired results from two similar experiments: 20 mice on each dose; brain counts at two days; LD 50 of 18–323 was 1300; PD 50 IP/IC against 50,000 challenge was 0·06 ml., against 5000 challenge was 0·006 ml.; 0·2 international units of vaccine protected 50% of these mice.)

		Brains at 2 days				Brains at 2 days			
		Infected				Infected			
	Dilutions of antiserum	No. infected	Log. geom. mean of organisms	No. sterile brains	S/T*	No. infected	Log. geom. mean of organisms	No. sterile brains	S/T*
IP/IC serum in 0·2ml.	1/1	4	2·0	16	10/10	14	4·0	6	16/20
	1/5	6	2·9	14	18/20	16	4·6	4	7/19
	1/25	14	3·5	6	10/19	16	4·6	4	2/10
	1/125	16	3·5	4	8/19				
	0/	18	3·6	2	3/19	18	5·0	2	1/20
'IC mix' in 0·03ml. total	1/2	0	—	20	16/20	14	3·6	6	10/20
	1/10	4	4·6	16	18/20	18	3·6	2	6/19
	1/50	3	3·0	16	17/21	11	3·6	9	11/20
	1/250	3	3·7	17	19/22	13	3·8	6	14/22
	1/250	8	2·3	12	9/20	18	4·4	2	3/20
	0	17	3·9	3	4/21	18	4·8	2	3/20
		5000 challenge				50,000 challenge			

* S/T Survivers over total in groups of mice kept for 14 days.

Passive protection experiments, comparing the effect of the route of administration of antiserum and size of challenge on type and degree of protection

Serum was given intraperitoneally followed by an intracerebral challenge ('IP/IC 50,000' or 'IP/IC 5000') or mixed with the challenge ('IC mix 50,000' or 'IC mix 5000'). Two days after infection, half the mice were killed for brain counts; the other half were observed for 14 days (Table 3).

In the left-hand side of Table 3, giving the 5000 challenge results, the proportion of sterile brains at 2 days for all these mice over a wide range of serum doses injected either intraperitoneally or intracerebrally mixed with the challenge, was fairly close to the proportion of survivors at 14 days, though less close in mice receiving intraperitoneal serum. Survival after a 5000 challenge was therefore associated with the ability of the animal to overcome the infection very quickly.

The top right-hand side of Table 3, showing the results of serum given intraperitoneally against a 50,000 challenge, indicates (as did Fig. 1) that for 2 days the organisms increased in numbers only slightly more slowly than in control mice, but that many of the infected mice did not die; a killing mechanism was effective at later than 2 days.

The 'IC mix 50,000' results (lower right-hand part of Table 3), though typical

Table 4. *Comparison of passive protection (pooled results) by serum* 6660 *against an intracerebral challenge of* 18–323 *in Theiler's Original (TO) and TF* 1 *mice.*

Route and challenge	Serum dilution	Percentage survival in TO	Percentage survival in TF1
'IC mix' 5000	1/2	70	60–70
	1/20	80	75
	1/200	70	60–70
'IC mix' 50,000	1/2	45	10–20
	1/20	40	10–20
	1/200	70	10–20
IP/IC 50,000	1/1	96	25
	1/5	33	17
	1/25	20	4

of most of these experiments, are more difficult to interpret. Although the proportion of survivors at 14 days was greater than the proportion of sterile brains at 2 days with 1/2, 1/10 and 1/250 antiserum, as with intraperitoneal serum and a 50,000 challenge, there was no consistent association of serum dose and its effect. At the 1/50 and 1/250 serum dilutions, the proportion of sterile brains was greater than at other dilutions, and at these dilutions the corresponding proportion of survivors was greater.

The PD50 for the antiserum given intraperitoneally was 10 times less against the smaller challenge than the larger; such a comparison was impossible in the 'IC mix' test because of the lack of dose response, but 1/250 at both challenge levels produced the maximum protection with a decrease in protection at the next dilution used.

The influence of mouse strain on the degree of protection conferred

During the course of this work, it became increasingly more difficult to protect the Schneider–Webster strain of mice against an intracerebral challenge with intraperitoneal antiserum. At the same time, the ImD50 of pertussis vaccine increased by more than 10 times. The strain TF1 was also difficult both to immunize and to protect passively IP/IC. The amount of vaccine needed to protect the TF1 mice to the same extent as Theiler's Original (TO) mice was 6–10 times; but the virulence of 18–323 for the two strains of mice was the same. The Schneider–Webster mice, on the other hand, were very susceptible to 18–323 and for this reason less suitable for comparison with the TO mice.

Table 4 shows the passive protection in TO and TF1 mice. There is little difference in the ability of antiserum to protect both strains of mice when the antiserum was mixed with a 5000 challenge, where the mechanism of protection appears to be an immediate sterilization. There was only a little protection of TF1 mice against a 50,000 challenge, with antiserum either intraperitoneally or intracerebrally.

Blake & Wardlaw (1969) showed that the immunosuppressive reagent, cyclophosphamide, inhibited both active and passive protection of mice by vaccine or

intraperitoneal serum against a 50,000 challenge. In one experiment by my colleague, Dr J. P. Ackers, cyclophosphamide-treated TO mice given intraperitoneal antiserum were protected only to the same degree as TF 1 mice.

Serum from TF 1 mice 14 days after vaccination had, however, as high an agglutinin titre and content of complement-mediated bactericidal antibody *in vitro* as TO mice similarly vaccinated, whereas antibodies were not found in cyclophosphamide-treated TO mice. The TF 1 mice were not, therefore, behaving as immunosuppressed animals, even though the superficial results in passive protection experiments were similar.

DISCUSSION

In 'IC mix' experiments, we are dealing with the fate of sensitized organisms as antiserum given with infecting organisms intracerebrally is effective only when adsorbed on to the organisms. In spite of this, the protective effects of antiserum against 50,000 and 5000 organisms are completely different, and the difference is independent of the route of administration of antiserum. All effective doses of antiserum given either IP or IC with 50,000 organisms intracerebrally sterilize the brains after an initial rise in the bacterial content of the brain; all effective doses of antiserum given IP or IC with 5000 organisms sterilize the brain immediately. The difference does not seem to be an expression of the ratio of organisms to serum. A 1/250 dilution by the 'IC mix' route protects nearly all the mice by immediate sterilization of a 5000 challenge; over 100 times more antiserum by the same route protects half the mice against a ten-times bigger challenge, but not by immediate sterilization.

There is an apparent inhibition of the protective effect with strong antiserum, mixed with a 50,000 challenge, for more dilute serum gives the better protection. The sharing out during bacterial division of not-immediately-lethal antibody that has been adsorbed to bacteria was beautifully demonstrated for *Salmonella typhi* by Cole (1964). The inhibition zone of poor protection (i.e. non-killing of the bacteria *in vivo*) is so like the non-killing zone in the complement-mediated bactericidal action of antibacterial sera *in vitro*, the Neisser–Wechsberg phenomenon (Dolby, 1965), that it was tempting to consider that both *in vivo* and *in vitro* bactericidal inhibition were caused by the same antibody. Attempts were made to correlate the *in vivo* and *in vitro* bactericidal inhibition zones, but were not very successful, a result perhaps only to be expected in dealing with a system of more than one effective antibody. Moreover, if such a correlation existed, then an inhibition zone would be expected against a 5000 as well as a 50,000 challenge (Table 3, bottom left-hand side); it is not evident here. All of this suggests that the bactericidal antibody which acts *in vitro* may have little to do with the *in vivo* killing of bacteria. This question is investigated more fully elsewhere (Ackers & Dolby, 1972; J. M. Dolby & S. Stephens, to be published).

Although we were able to isolate 18–323 consistently from the brains of surviving mice in passive protection tests after 14 days, in active immunization experiments 18–323 was isolated only once from 50 survivors after the 14th day (C. J.

Shanbury, personal communication). Dolby & Standfast (1961) showed that a single organism of 18–323 which lodged in the brain of a 'normal' mouse would cause death in 12–14 days and that it was rare for deaths to occur later. In these passive protection 'IC mix' experiments, however, deaths occurred up to 28 days and 18–323 was isolated as late as 35 days after challenge. There must have been organisms which escaped being killed at 6–8 days but whose growth was slowed down so that either death resulted eventually or a host–parasite balance was achieved. Small amounts of circulating antibody were detected in long-surviving, infected mice. Organisms isolated 3 weeks after challenge from passively protected mice were fully virulent (C. J. Shanbury, personal communication), but two out of three strains isolated 5 weeks after had decreased virulence. Avirulent infections which are too low to stimulate a general response in the host can cause local immunity which eventually overcomes the infection (Standfast & Dolby, 1972), but no local immunity to a re-infection could be detected in mice with long drawn-out infections with 18–323.

The experiments reported here only seem to add to the complexity of relating active and passive immunity. They also emphasize the importance of the size of the challenge dose in the 'IC mix' passive protection test. It is the immediately acting antibody which is effective only against the smaller challenge and organisms not killed in this way grow up to be victims of the second antibody at 4 days. Antisera such as those we used contained both antibodies. If the two antibodies were separable, then it might be possible to use a serum having only immediate or long term activities, but not both.

As judged by the course of infection in passively protected mice, the 'IP/IC 50,000' and 'IC mix 50,000' tests resemble the results after active immunization IP. The role of antibody in the active protection test is, however, controversial and the possibilities have been set out by Blake & Wardlaw (1969). The inability to protect passively mice that do not immunize supports their experiments, suggesting that protection of mice by pertussis antiserum may not be such a passive phenomenon as is generally thought.

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Routine administration of oral polio vaccine in a subtropical area. Factors possibly influencing sero-conversion rates*

By T. A. SWARTZ

Department of Clinical Epidemiology, Asaf Harofe Hospital, Tel-Aviv University Medical School, Zerifin, Beer Yaakov, Israel

PAULINA SKALSKA and C. G. GERICHTER

Central Virological Laboratory, Ministry of Health, Jaffa, Israel

and W. C. COCKBURN

Virus Diseases Unit, World Health Organization, Geneva, Switzerland

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SUMMARY

Poliomyelitis is an important problem of public health in warm-climate countries. Studies of serological responses to vaccination in these countries have given conflicting results but in many investigations the rates have been considerably less than in countries with temperate climates. In this study three possible factors influencing sero-conversion were investigated – the season of the year when vaccine was given, the social status of the mother (as indicated by the number of years of schooling) and the presence of non-poliomyelitis viruses (NPV) in the gut when vaccine was given.

Over 200 children about 2 months of age were included in the study. Each was given three doses of trivalent vaccine at 6-week intervals.

The sero-conversion rates of the groups fed in winter were excellent but were slightly less good in summer. The differences were greatest in children in the lower socio-economic groups and in children excreting other enteroviruses.

The conclusions are that, provided a potent vaccine is used, the factors which diminish the effectiveness of immunization in warm-climate countries can be overcome: (1) by giving three doses of trivalent vaccine; (2) by beginning vaccination at the earliest possible age (when enteroviruses are fewest); (3) by concentrating special attention on the lower socio-economic groups and if necessary by giving a reinforcing dose several months after the third dose has been given – preferably in the colder months.

INTRODUCTION

In the past twenty years paralytic poliomyelitis has been recognized as a problem of increasing importance in the public health of numerous countries with warm climates. Clinical and epidemiological observations have been complemented by serological investigations and by isolations of polioviruses. In several

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tropical and subtropical countries the incidence in recent years has followed trends similar to those observed in countries with temperate climates before the introduction of vaccination. The age distribution has so far shown little change from the classical pattern, most cases occurring in the 0–4 age group, and though outbreaks and sporadic cases due to types 2 and 3 are not uncommon, most cases are due to type 1 (Drozdov & Cockburn, 1967, 1971).

Vaccination with live oral poliomyelitis vaccine has resulted in control of the disease in those tropical countries in which a sufficient proportion of the susceptible age groups has been given adequate dosage (Sabin *et al.* 1960), but studies based on sero-conversion rates have often indicated that the titres are much less satisfactory than in temperate countries (Cockburn & Drozdov, 1970). Among several possible reasons for the poor serological conversion rates in warm climates interference between natural enteroviral infections and the live vaccine viruses has been considered important (Montefiore, 1971) and it is well known that the incidence of gastrointestinal infections in tropical countries is high and is related both to social conditions and to the hot season. Routine poliomyelitis vaccination with oral vaccine has since 1961 reduced the incidence of the disease to a very low level in Israel (Ministry of Health, 1968). However, the considerable range in temperature between summer and winter and differences in the socio-economic conditions of certain groups in Israel provided an opportunity to study the effects of these factors on the excretion of enteroviruses and sero-conversion rates after poliovirus vaccination.

METHODS AND MATERIALS

Field procedures

The studies were made from December 1969 to April 1970 (winter trial) and May to September 1970 (summer trial). In the Tel-Aviv area the lowest average day temperatures for each month from December to March were between 13·2° and 15·6° C. and the highest from May to October were between 20·7° and 26·4 °C.

Infants living under normal home conditions and registered at six well-baby clinics in or near Tel-Aviv and Ashdod were included. The clinics were selected without prior knowledge of the incidence of acute gastrointestinal infections in the surrounding areas. The winter trial comprised 117 infants born in September and October 1969 and the summer trial 109 infants born in February and March 1970. They were allocated to one or other of two socio-economic groups according to the number of years the mothers had attended school. In Group A were children whose mothers had 12 or more years at school (12 years is the minimum before higher education can begin in Israel) and in Group B children whose mothers had less than 12 years at school. It is believed that a mother's knowledge of health and hygiene is related to her educational level, and presumably has a direct influence on the health of the child especially in the first year of life.

Administration of the vaccine

Two drops of commercially prepared trivalent live oral poliovirus vaccine* (OPV) were given to each child in a spoon containing sugared water. Beginning at

* Donated by the Wellcome Research Laboratories.

2 months of age three doses were given with an interval of 6 weeks between doses. The vaccine was held at 4° C until administered and the infectivity titres, expressed as TCID 50 per dose, were: type 1, 10^6; type 2, 10^5; type 3, $10^{5\cdot5}$.

Collection of samples

Stool samples were collected from each infant immediately before and 1 week after each dose of vaccine. They were sent to the laboratory on the same day and stored at −20° C. until investigated.

Capillary blood was collected on filter-paper disks (Reed & Brody, 1965) from about half the children in each group before each of the three vaccinations and 2 months after the third dose, when venous blood was obtained at the same time from a sample of 36 children.

Laboratory methods

The stool specimens were examined for the presence of polioviruses and other enteroviruses which in this paper are described as non-polioviruses (NPV).

For the tests attenuated strains of polioviruses* types 1, 2 and 3 were used. Virus stocks were prepared in primary cell cultures of African green monkey kidney and stored at −20° C. until required. Monkey kidney tissue cultures were employed throughout for the isolation and identification of strains.

Ten per cent. stool suspensions were centrifuged at 2500 rev./min. and 0·2 ml. of supernatant was inoculated into each of six culture tubes (3 undiluted and 3 diluted 10^{-2}). Inoculated cultures were incubated and examined for cytopathic effects (CPE) for a period of 10 days. The cultures were harvested for typing if CPE occurred. Tissue cultures harvested after 6 days of incubation were passaged once before titration. Typing of all viruses isolated in monkey kidney cell cultures was done by neutralization with antisera against the three types of poliovirus. A calculated dose of 100 TCID 50 was used in all neutralization tests.

For the serological tests three disks were placed in 1 ml. phosphate-buffered saline containing penicillin and streptomycin and held at 4° C. overnight. The soaked disks were then dropped into the chamber of a disposable syringe and the fluid squeezed back into the eluate. About 0·75 ml. of eluate, considered to be a 1/5 dilution, was obtained and was centrifuged for 10 min. at 2500 rev./min. to sediment particles of paper. The supernatant fluid was then poured off and used in the test.

The neutralization test was done in primary vervet monkey kidney tissue culture in tubes. Serial fourfold dilutions of the eluate ranging from 1/10 to 1/160 were tested against approximately 100 TCID 50 per 0·1 ml. of each of the poliovirus types.

The virus-serum mixtures were allowed to react during 4 hr. at 37° C. and overnight at 4° C., and were then inoculated in 0·2 ml. amounts into each of two monkey kidney tissue culture tubes.

Control virus was titrated with 0·1 ml. per tube (six tubes per ten-fold dilution).

Tests were read first when all virus control tubes containing 100 TCID 50

* Donated by the Wellcome Research Laboratories.

Table 1. *Subjects in study according to the social group and season of trial*

	Winter		Summer	
Social group	Virological investigations	Serological investigations	Virological investigations	Serological investigations
A	51	28	51	27
B	66	38	58	28

Table 2. *Preimmunization neutralizing antibody in the study groups*

	Neutralizing antibody					
	Type 1		Type 2		Type 3	
Subjects in study	% with antibody	G.M.*	% with antibody	G.M.	% with antibody	G.M.
Winter						
Social group A	60·0	12·3	70·0	16·2	45·0	7·3
Social group B	36·4	8·3	63·6	11·3	27·3	6·6
Summer						
Social group A	50·0	9·3	75·0	14·6	35·0	6·8
Social group B	62·5	12·2	66·6	14·1	30·9	9·0

* Geometric mean antibody titre.

exhibited a definite cytopathic effect (CPE). The titration and neutralization tests were usually completed by 7 days. The antibody titre was expressed as the reciprocal of the highest dilution of serum giving complete neutralization of the virus.

Titration of neutralizing antibody in the capillary and venous blood specimens obtained at the final bleeding was carried out simultaneously.

A neutralizing antibody titre of $\geqslant 1/10$ was considered as indicating immunity. Titres of $< 1/10$ were considered as evidence of lack of immunity. For the calculation of geometric means (GM), titres of $< 1/10$ were considered as 5, and those $\geqslant 1/160$ were considered as 160.

Distribution of the infants

The distribution of the 226 infants by social grouping and study season is given in Table 1.

RESULTS

Measurement of neutralizing antibody in simultaneously drawn capillary and venous blood specimens from 36 children gave similar antibody titres for each of the three types of poliovirus.

The state of immunity of the infants at 2 months of age, immediately before their first dose of vaccine, is shown in Table 2. The percentage of infants with antibodies (presumably maternal) varies, but in each subgroup the geometric mean titre (GM) is low for each of the three types of poliovirus.

Table 3. *Percentage of seroconversion after administration of live poliovirus vaccine*

	After one dose. Type 1		After two doses. Type 1		After three doses					
					Type 1		Type 2		Type 3	
Subjects in study	%	G.M.*	%	G.M.	%	G.M.	%	G.M.	%	G.M.
Winter										
Social group A	81·2	24·0	85·7	26·2	100·0	152·0	100·0	144·9	100·0	129·2
Social group B	67·1	15·2	92·5	46·0	100·0	115·0	100·0	142·9	100·0	132·8
Summer										
Social group A	77·7	25·9	85·7	91·3	96·4	109·4	100·0	144·9	92·8	82·7
Social group B	64·2	13·1	80·6	40·6	86·3	70·6	95·4	105·1	81·8	52·6

* Geometric mean antibody titre.

Table 4. *NPV excretion*

Study group	NPV excretion (%) in samples taken at time of administration of	
Winter		
Social group A	First dose	1·9
	Second dose	0·0
	Third dose	2·4
Social group B	First dose	9·3
	Second dose	5·1
	Third dose	8·9
Summer		
Social group A	First dose	23·0
	Second dose	15·3
	Third dose	7·6
Social group B	First dose	33·3
	Second dose	20·0
	Third dose	13·3

Excellent sero-conversion was observed after three vaccinations in the winter season in both groups (Table 3). High GM titres to the three poliovirus types were also recorded, though the GM titres to poliovirus type 1 were somewhat lower in group B.

In the summer trial the sero-conversion rates and GM titres in both groups A and B are lower, except for the GM titres to poliovirus type 2 in group A which were the same in both trials.

Also in Table 3 differences are shown between the groups in the sero-conversion rates to each of the three types of poliovirus in the summer trial. The differences are emphasized by comparing the corresponding GM titres which are much lower in group B than in group A.

In both groups many more children excreted NPV in summer than in winter and the incidence of NPV excretion was higher in group B than in group A in both seasons (Table 4).

In children who did not excrete NPV (Table 5) only slight differences in the

Table 5. *Relationship of polio neutralizing antibody to N.P.V. excretion*

Study group	Distribution of polio neutralizing antibody: Without N.P.V. excretion — Type 1 %	Type 1 G.M.*	Type 2 %	Type 2 G.M.	Type 3 %	Type 3 G.M.	With N.P.V. excretion — Type 1 %	Type 1 G.M.	Type 2 %	Type 2 G.M.	Type 3 %	Type 3 G.M.
Winter												
Social group A	100·0	127	100·0	151	100·0	151	100·0	108	100·0	160	100·0	108
Social group B	100·0	116	100·0	147	97·0	118	100·0	108	100·0	108	100·0	108
Summer												
Social group A	95·0	109	100·0	149	100·0	149	83·3	45	100·0	127	83·3	45
Social group B	94·1	102	100·0	160	94·1	129	50·0	20	83·3	61	65·0	30

* Geometric mean antibody titre.

Table 6. *Excretion of polioviruses*

Study group	Stools collected 7 days after	Percentage of stools yielding virus belonging to Type 1	Type 2	Type 3
Winter				
Social group A	First dose	40·0	77·7	42·2
	Second dose	48·8	9·3	62·7
	Third dose	14·6	4·8	26·8
Social group B	First dose	51·6	85·4	33·8
	Second dose	45·1	9·8	49·0
	Third dose	5·3	3·5	14·3
Summer				
Social group A	First dose	53·8	61·5	53·8
	Second dose	38·0	15·3	30·7
	Third dose	23·0	15·3	7·7
Social group B	First dose	40·0	80·2	40·0
	Second dose	33·3	13·3	6·7
	Third dose	6·7	6·7	6·7

sero-conversion rates and GM titres were observed between groups A and B in either season. In contrast, in NPV excreters the sero-conversion rates and GM titres in the two groups were definitely lower in summer than in winter, with the lowest values in group B in summer.

Information on the isolation of polioviruses from stools collected 7 days after each dose of vaccine was administered is given in Table 6.

In contrast to the correlation between NPV excretion and serological response, there was no consistent difference between groups or between seasons as measured by the excretion of the vaccine viruses.

DISCUSSION

The sero-conversion rates obtained in this study were excellent in winter and good in summer. Such high rates have been reported in only a few earlier studies in warm climates (e.g. Sabin *et al.* 1960). They are in distinct contrast to those in many other investigations in some of which the conversion rate for type 1 has been below 30% in children previously without antibody (Report, 1966).

Despite the overall good results, conversion rates and GM titres were better in children vaccinated in winter than in summer and in the higher social group (A) than in the lower social group (B). Excretion of NPV was more frequent in summer than in winter and in group B than in group A.

Also the GM titres in children not excreting NPV were generally much higher than in children excreting NPV both in the winter and summer trials, and this is perhaps the most interesting observation arising from this study. It supports the conclusions of workers who have laid great stress on interference between NPV and the vaccine strains of polioviruses. At the same time it appears quite clear that three doses of well-spaced potent oral vaccine will give satisfactory sero-conversion rates even in hot weather. In practice, however, it would be advisable

to vaccinate in the cool season as far as possible, and to take particular care that infants in the lower social groups are given at least three doses. Where vaccination is done in the warm season a fourth dose might be given, preferably in the cool season.

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The serum and conjunctival antibody response to trachoma in Gambian children

By L. H. COLLIER, J. SOWA and SHIONA SOWA
Medical Research Council Trachoma Unit, Lister Institute of Preventive Medicine, London, S.W.1, and M.R.C. Laboratories, Fajara, The Gambia

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SUMMARY

Ninety-nine young Gambian children were studied for 61 weeks. About half of them had trachoma at the outset, and 80% of the remainder acquired the disease while under observation. IgG trachoma antibody in the serum and IgG and IgA antibodies in the conjunctival secretions (CS) were titrated by an indirect immunofluorescence method. In serum samples obtained in capillary tubes the mean titre was slightly higher than in samples collected on filter paper. Serum antibody at titres $\geqslant 1/10$ was invariably associated with a clinical diagnosis of trachoma; it increased both in frequency and titre as the disease progressed, and was present in about half of those with Tr II. In CS, IgG antibody was present less often and at lower titres than in serum, and IgA antibody was detected even less frequently. There was some evidence of correlation between the titres of IgG and IgA antibodies in CS, but none for a relationship between the titres of the antibodies in serum and those in CS. Antibodies were almost never present in the absence of conjunctival follicles, but their titres were unrelated to the degree of follicular hyperplasia; there was no obvious relationship between the serological findings and corneal lesions. In children diagnosed clinically as trachoma, serum antibody was present in almost all those with conjunctival inclusions, and in a proportion of inclusion-negative subjects; the mean titre was much higher in the inclusion-positive group.

These findings do not settle whether CS antibodies are made locally, or are derived partly or wholly from the blood. They suggest that the indirect immunofluorescence test may be a useful diagnostic aid in trachoma, particularly in view of the rarity of false positive reactions; but there is at present little to choose between it and complement-fixation tests in terms of sensitivity.

INTRODUCTION

Many workers have studied the serological response to naturally acquired trachoma; most of their reports deal with serum antibody that fixes complement with the heat-stable *Chlamydia* group antigen, but the consensus of opinion seems to be that the appearance of such antibody is insufficiently constant to be of much use in diagnosis (see, for example, Tarizzo, Nabli & Labonne, 1968). Little is

Table 1. *Classification of children by age and clinical diagnosis at start of the investigation (Survey 1)*

Age group (months)	No. of children	Clinical diagnosis		
		N	Ab	Early active trachoma
0–6	33	25	2	6
7–12	20	3	5	12
13–18	14	3	3	8
19–24	9	2	0	7
25–36	23	4	1	18
Totals	99	37	11	51

N = normal eyes. Ab = minor abnormalities (e.g. papillae, scanty follicles at angles) not suggestive of TRIC infection.

known about the time-course of the antibody response to natural infection, and the role of antibody in immunity, pathogenesis and recovery is even more obscure.

In 1966, Bernkopf, Orfila & Maythar first reported the presence of antibody in the conjunctival secretions of people with active trachoma. Anti-TRIC antibody that stained with anti-human gamma globulin conjugated with fluorescein isothiocyanate was found in the conjunctival fluids of 10 of 21 patients; the titres ranged from 1/10 to 1/120, and in some instances were higher than those in the blood sera. Stimulated by these findings, we began in 1968 an investigation to elucidate the following points:

(1) Whether IgA as well as IgG trachoma antibody is present in conjunctival secretions.

(2) The relationship between conjunctival and serum antibody.

(3) The relationship of the presence of antibody to (*a*) clinical diagnosis and (*b*) presence of conjunctival inclusion bodies.

(4) The diagnostic value of trachoma antibody detectable by immunofluorescence.

(5) The relationship between presence of antibody and physical signs such as follicles and corneal lesions.

In addition, the opportunity was taken to compare the merits of the immunofluorescence and iodine techniques for detecting conjunctival inclusions; this study has already been reported (Sowa, Collier & Sowa, 1971).

MATERIALS AND METHODS

General plan

After preliminary work on immunofluorescence methods, a group of 99 infants was selected in Salekini, a large Gambian village with 3500 inhabitants (Sowa, Sowa, Collier & Blyth, 1969; Sowa *et al.* 1971). Thirty-seven of these children had normal (N) eyes (Table 1); the remainder had early active trachoma with or without corneal lesions, or minor conjunctival abnormalities not suggestive of TRIC infection (Ab). The intention was to make observations not only on well-established trachoma, but also on the appearance of physical signs and antibody in

Table 2. *Comparison of antibody titres in blood samples obtained in parallel by the filter-paper and capillary methods from* 46 *subjects* (*Survey* 4)

Anti-TRIC IgG antibody titre*	No. of samples	
	Filter paper	Capillary
< 5	33	31
5	1	2
10	1	0
20	3	4
40	6	4
80	2	5
No. with titre ⩾ 1/5	13	15

* Reciprocal of end-point serum dilution by indirect FA method: dilutions of samples eluted from filter paper are notional (see text).

infants acquiring the disease during the period of study. The initial survey (S1) was made in January 1969, and the children were examined on five subsequent occasions (S2–6) during the next 61 weeks at the intervals shown in Fig. 1. At each survey, the eyes of every infant were examined by slit-lamp, and conjunctival scrapings were examined for inclusions. At S1, 2, 4 and 5 the blood sera and conjunctival secretions were tested for antibodies to TRIC agent.

Clinical examination

The eyes were examined with a Haag–Streit slit-lamp and physical signs were scored by the method of Sowa *et al.* (1969). All observations were made by one ophthalmologist (S. S.); the clinical diagnoses and scores were recorded without reference to the results of previous examinations or to the laboratory findings.

Tests for inclusion bodies

Conjunctival scrapings were taken in duplicate and stained at random either with iodine or by an indirect fluorescent antibody (FA) method (Sowa *et al.* 1971). For the purpose of the present investigation, the result was taken as positive if inclusions were found by either method.

Blood samples

Blood was taken by finger prick, or, from very young babies, by heel prick. At S1 and S2 it was collected on 6 × 24 mm. strips of Whatman no. 1 filter paper, each of which required 0·075 ml. fluid for saturation. At S4, the method was compared with collection in 2·6 × 75 mm. glass capillary tubes. Table 2 compares the antibody titres in blood samples collected by the two methods; the geometric means for the filter paper and capillary methods are 1/29 and 1/32 respectively; they do not differ significantly (Student's t = 0·271 with 26 D.F.), but since the capillary method appeared to hold a slight advantage it was used exclusively for S5. Strips and capillaries were transported from the village to the laboratory on wet ice. Thereafter, strips were stored in screw-capped bottles at −60° C. pending

tests for antibody. Capillaries were left for 24 hr. at 4° C., and then centrifuged at 800 *g* for 10 min.; the serum was removed, and kept at −60° C. until needed.

Conjunctival secretions

At S1 and S2, conjunctival secretions (CS) were collected by exploiting the technique originally described by Schirmer (1903) for measuring tear flow. The end of a filter-paper strip similar to those used for blood samples was inserted into the lower fornix of each eye; after saturation was complete, each pair of strips was placed in a screw-capped bottle. At S5, a capillary method was used: one drop (0·075 ml.) of sterile 0·85% (w/v) NaCl solution was instilled into the eye; 5–10 sec. later it was collected at the outer canthus into a capillary tube. The fluids from both eyes were pooled. The conditions of transport and storage were like those for blood.

Tests for antibody by indirect immunofluoresence method

At each survey, blood samples were tested for specific anti-TRIC IgG antibody, and CS for both IgG and IgA antibodies.

Phosphate-buffered saline (PBS) (Fothergill, 1964) was used as diluent throughout, and for all washing procedures.

TRIC agent slide antigen. BHK-21 cells were grown as monolayers on 9 × 35 mm. cover-slips in Leighton tubes. When just confluent they were seeded with the 'fast-killing' variant (Reeve & Taverne, 1963) of TRIC/2/GB/MRC-4/ON (formerly LB4) at a concentration that infected most of the cells. After a further 36–38 hr. incubation at 37° C., cover-slips bearing cells containing mature inclusions were fixed in acetone for 10 min. at room temperature; preliminary washing was omitted because it impaired fluorescence staining, probably by leaching out soluble antigen. Cover-slips were stored at −60° C. until required.

The inclusions in these monolayers were stained by the indirect FA method with both cross-absorbed (type-specific) and unabsorbed antisera prepared in donkeys against TRIC agents grown in yolk sac, and with an anti-donkey FITC conjugate; these reagents were obtained through the courtesy of Dr Roger Nichols, Harvard School of Public Health. The fast-killing variant MRC-4*f* used in these experiments stained well with unabsorbed sera directed against serotypes 1, 1b and 2 and with anti-type 2 serum absorbed with type 1 antigen; but not at all with low dilutions ($\leqslant 1/3$) of anti-type 1 or 1b sera cross-absorbed respectively with type 1b and 1 antigens. This variant thus types like its parent strain as type 2 (Sowa *et al.* 1971) but reacts with unabsorbed antisera to types 1 and 1b.

Anti-human IgA conjugate was kindly made by Dr W. D. Brighton (National Institute for Medical Research). The serum was from a sheep which had been immunized with human serum IgA according to the schedule recommended by a Medical Research Council subcommittee (1966). The serum was fractionated first by precipitation with neutralized 1·7 M-$(NH_4)_2SO_4$, pH 7·2, then by elution from DEAE Sephadex to produce a globulin fraction. Half of this globulin fraction was conjugated with fluorescein isothiocyanate (FITC) by the method of Brighton (1966).

The original serum was tested for freedom from cross-reactivity with IgG and IgM by double diffusion in agarose gel. The globulin fraction was tested for freedom from other serum proteins by immunoelectrophoresis and the conjugate for freedom from unconjugated fluorochrome by thin layer chromatography on Sephadex G 200 gel. The contaminants were below the maximal levels recommended by Brighton & Lampard (1970).

Anti-human IgG conjugated with FITC prepared in goats was obtained from Microbiological Associates, Bethesda, Md, U.S.A. and used at an optimum dilution of 1/20 determined by chess-board titration with a known positive serum.

Antibody titrations. At each survey, samples were stored until observations were completed, and then tested together within a period of a few days. CS and blood sera collected in capillaries were serially diluted in PBS. Samples obtained on filter paper were eluted by adding four drops of PBS to each vial, so that the strips were immersed, and holding at 4° C. for 24 hr. The eluate was regarded as a starting 1/5 dilution for further twofold steps. Cover-slips were cut into four equal sections, each of which was used to test one dilution; for staining, the sections were lightly attached with adhesive, cells uppermost, to microscope slides. Cover-slips were treated with dilutions of CS or blood sera, washed in PBS, and then stained with the appropriate dilution of anti-IgG or anti-IgA conjugate. Incubation and washing procedures were as described for yolk sac slide antigens (Sowa *et al.* 1971), except that counterstaining with Evans's blue was omitted. For microscopy, the cover-slips were mounted cell-side down on fresh slides in glycerol-PBS, 9:1. They were examined under dark field illumination with a Zeiss Photomicroscope equipped with an HB 200 ultraviolet lamp; exciter filter BG 12/4 was used in combination with barrier filters 47 and −65. The intensity of fluorescence was read with a X 40 apochromat objective, and scored with the aid of a set of graded density filters (Collier, 1968). The titration end-point was taken as the dilution giving a score of 2.

RESULTS

Diagnostic criteria for trachoma

In our earlier descriptions of the group of children participating in this study, we stated that '37 had normal (N) eyes, 34 had active trachoma (Tr I or II), 10 had physical signs suggestive of early trachoma (Tr D), and 18 had minor conjunctival abnormalities not suggestive of trachoma (Ab)' (Sowa *et al.* 1971). In describing the criteria on which this classification was based (Sowa *et al.* 1969) we mentioned that the diagnosis of trachoma was made in accordance with the recommendations of the WHO Expert Committee on Trachoma (1962); we also noted that children with a diagnosis of Ab were more likely to acquire classical trachoma than N children. This tendency was pronounced in the study described here; furthermore, all children diagnosed clinically as 'trachoma dubium' (Tr D) (i.e. who had physical signs suggestive of TRIC infection, but who failed to meet the WHO criterion of at least two of the cardinal signs) sooner or later developed the classical disease. We therefore considered that a more realistic classification might be arrived at by considering the clinical history of each child individually

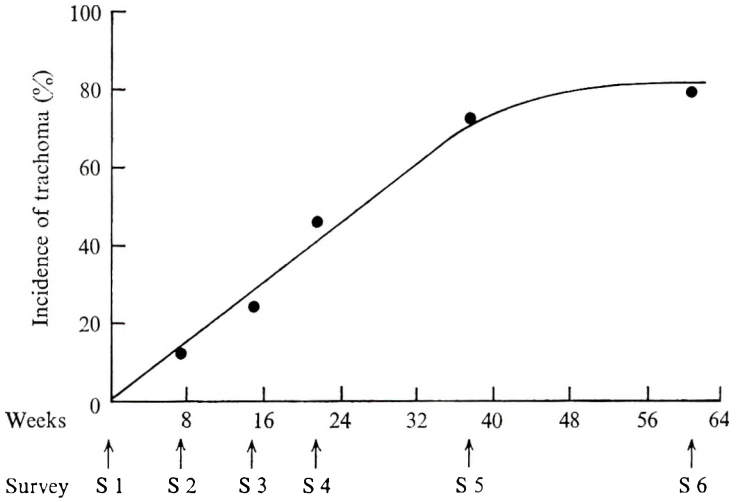

Fig. 1. Cumulative incidence of trachoma in 37 children diagnosed as normal at Survey 1.

at the end of the investigation, taking into account the results of tests for inclusions but without any reference to the results of the antibody studies. At each survey, children were diagnosed as trachoma if they fell into one or more of the following categories:

(*a*) Those with an unequivocal clinical diagnosis of trachoma with at least two of the cardinal signs (follicular hyperplasia, keratitis, pannus, cicatrization).

(*b*) Those with conjunctival inclusions.

(*c*) All those, originally diagnosed as Ab and Tr D, who developed classical trachoma at later surveys; but not children in whom the diagnosis of classical trachoma was immediately preceded by a diagnosis of N (normal eyes).

The reasoning underlying (*c*) is that Ab and Tr D children converting without interruption to classical trachoma were probably infected with TRIC agent from the outset; but this is less likely in children who reverted to N in the interim. An example may help: at six successive examinations, child A was diagnosed as N, N, Ab, Ab, Tr I, Tr II; child B was diagnosed as N, Ab, N, N, Tr I, Tr II. For the purposes of this paper, A is regarded as having acquired trachoma at the 3rd examination, and child B at the 5th.

Table 1 shows the composition of the group at S 1; the 51 children with definite or putative TRIC infections are now classified together as 'early active trachoma'; 34 of them had pannus. Fig. 1 shows the trachoma attack rate in children diagnosed as normal at the outset. Use of the criteria described above for the clinical diagnosis of trachoma gave a plot of the cumulative incidence that conformed better with the expected curve than one based on rigid adherence to the WHO recommendations. The incidence increased regularly until about the 40th week, after which flattening of the curve suggested that almost all the susceptible children had become infected.

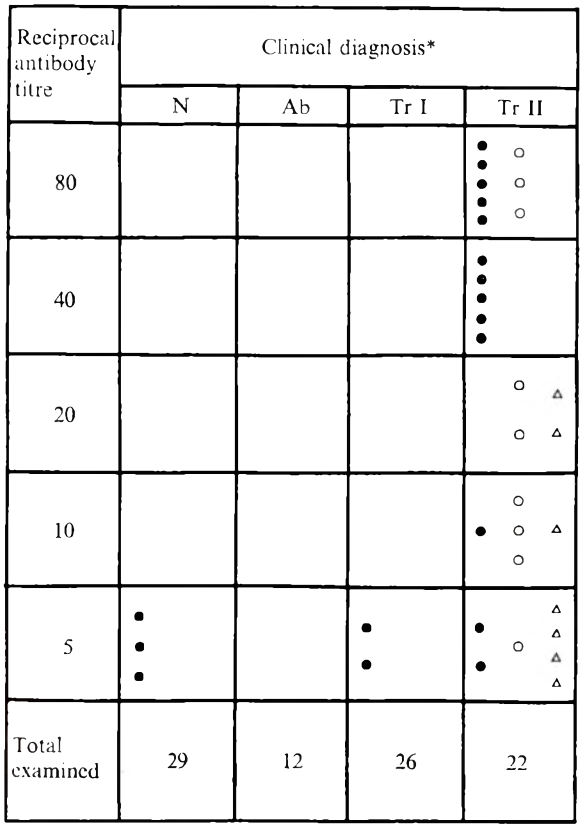

Fig. 2. Relationship of antibodies in sera and conjunctival secretions to clinical diagnosis (Survey 2), ●, IgG antibody in serum; ○, IgG antibody in CS; △, IgA antibody in CS. * See text for abbreviations.

Relationship of antibodies to clinical diagnosis

Figs. 2 and 3 are examples of the results obtained as the investigation proceeded; they show the titres of antibodies recorded at S 2 and S 5, 8 and 38 weeks respectively after the start of the investigation.

IgG antibody in serum. A titre of 1/5 was occasionally recorded in non-trachomatous children. Titres of 1/10 and over were invariably associated with a clinical diagnosis of trachoma, and this figure will henceforth be regarded as the limiting value in statements about the presence or absence of antibody in the serum. As the disease progressed from Stage I to Stage II, antibody increased both in frequency and in average titre. In S 5, for example, it was present in 29 % of 24 patients with Tr I, with a geometric mean titre of 1/16; in 35 children with Tr II, the corresponding figures were 46 % and 1/28. Antibody appeared to be diminishing in children who had progressed to Tr III, but there were not enough of them to permit a definite conclusion on this point.

IgG antibody in CS was present less frequently and at lower titre than the corresponding serum antibody. At S 5, it was present at a titre $\geqslant$ 1/10 in only 6 (17 %) of the 35 children with Tr II. Almost invariably, children with IgG antibody in their CS also had a significant titre of serum antibody.

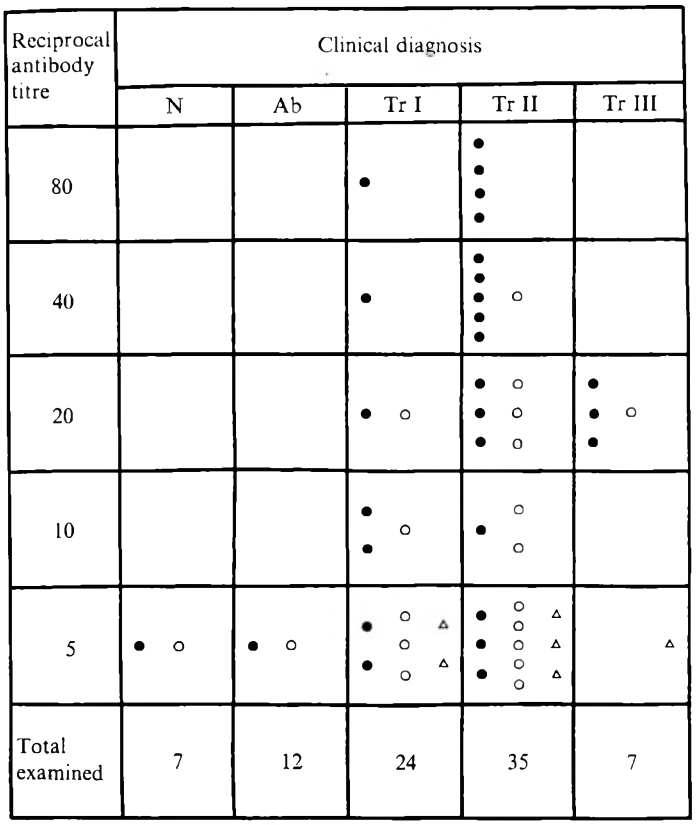

Fig. 3. Relationship of antibodies in sera and conjunctival secretions to clinical diagnosis (Survey 5). Abbreviations and symbols as in Fig. 2.

IgA antibody in CS was detected even less frequently than IgG. It was never detected in non-trachomatous children, even at the lowest dilution tested (1/5). In children with trachoma, the titre never exceeded 1/20.

Relationship between antibody titres in serum and CS

Fig. 4 is an example, taken from Survey 4, of the interrelationships of the various titres in 15 children with antibodies both in blood and CS. The titre of IgG antibody in the blood was not related to those of the IgG or IgA antibodies in CS. In this survey there was a significant positive correlation between the titres of the two sorts of antibody in the conjunctival secretions; but in other surveys the correlation coefficient for these two variables did not always attain a statistically significant value.

Relationship of antibodies to clinical and microbiological signs of trachoma

Follicular hyperplasia. There was no evidence that in terms of prevalence or titre the antibodies in the sera or CS were related to the degree of conjunctival follicular hyperplasia; it may, however, be significant that although follicles were often present without detectable antibody, in only one instance was antibody found – in the serum – in the absence of follicles.

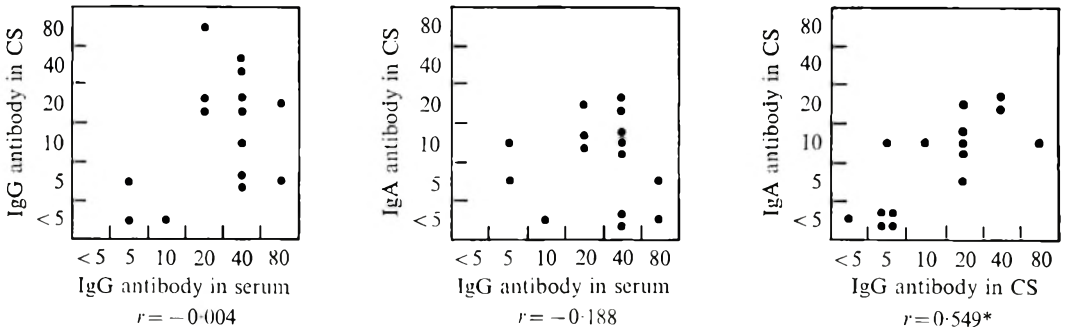

Fig. 4. Relationships of anti-TRIC antibody titres in sera and conjunctival secretions (CS) (Survey 4). Titres are given in terms of reciprocals of end-point dilutions by indirect FA method. *Correlation coefficient significant at 5% level.

Table 3. *Relationship of titre of IgG antibody in serum to presence of conjunctival inclusions in children with clinical trachoma*

	Inclusion positive		Inclusion negative	
Survey	No. of children	Mean titre*	No. of children	Mean titre*
S 1	10	54	41	2
S 2	2	41	46	2
S 4	8	22	56	2
S 5	16	22	50	2

* Geometric means of reciprocal titres.

Table 4. *Relationship of presence of IgG antibody in serum to that of conjunctival inclusion bodies in children with clinical trachoma*

		No. of children with antibody titre	
Survey	Inclusions	⩾ 1/10	< 1/10
S 1	+	10	0
	−	4	37
S 2	+	2	0
	−	9	37
S 4	+	7	1
	−	6	50
S 5	+	13	3
	−	8	42

Corneal lesions. Both keratitis and pannus were often present before antibody appeared, but there was no constant relationship between these physical signs and the serological findings.

Inclusions. Table 3 shows that in children with trachoma the geometric mean titre of IgG antibody in the serum was much higher in inclusion-positive than in inclusion-negative subjects; but although serum antibody was present in almost all those with inclusions, it was also detectable in a significant proportion of inclusion-negative subjects diagnosed clinically as trachomatous (Table 4). Since

serum antibody was not present in children without clinical trachoma (Figs. 2, 3) its presence at a titre $\geqslant 1/10$ may be a more sensitive laboratory test than the finding of inclusions.

DISCUSSION

Since this investigation was started, other reports have appeared on the use of immunofluorescence for detecting trachoma antibodies in man and in animals. McComb & Nichols (1969) examined Saudi Arab children. With a system employing yolk-sac slide antigens and rabbit anti-human globulin, they found, like us, that the prevalence and mean titre of antibody in CS collected on filter-paper strips were similar to those in a series of samples collected by eye dropper. No antibody was found in 30 non-Arab control subjects; of 81 trachomatous Arab children, 50 had antibody in CS at titres ranging from 1/2 to 1/320, with a geometric mean of 1/48. These figures are of the same order as ours; a further point of agreement was the finding by McComb and Nichols of a highly significant correlation between the presence of antibody and the finding of TRIC agent in the conjunctiva. These workers detected gamma globulin antibody in the CS of 89% of children with Tr II, compared with our figures of 45–60% for IgG globulin (Figs. 2, 3); However, the lowest dilution tested by them was 1/2, compared with 1/5 in our experiments.

Hathaway & Peters (1971) reported, without quoting titres, that in 25 Saudi Arab children with trachoma antibody in their CS, it was of the IgG type in all and of the IgA type in 20; IgE antibody was also present in 20 (not necessarily the same subjects). By contrast, none of 20 samples from Navajo Indians (presumably trachomatous, although this is not specifically stated) contained IgG or IgE antibody; this group was not tested for IgA antibody.

The findings of Jawetz *et al.* (1971) also varied according to the population studied. In 143 trachomatous Tunisian children IgG antibody was present in the CS of 16 at titres ranging from 1/4 to 1/64. Nine of these children also had IgA antibody in CS with titres of 1/4 to 1/16, but IgM antibody to trachoma was not detectable; complement-fixing antibodies were present in the sera of seven children. Of the 16 children with antibody in CS, five were inclusion-positive; but another 31 children with inclusions had no detectable antibody in their CS. By contrast, the prevalence of antibody in sera and CS was higher in patients diagnosed in San Francisco as having acute TRIC ocular infection; but antibody was found in only 1 of 47 American Indians with very mild trachoma. In the San Francisco series IgG antibody in CS was again present more frequently and at higher titres than was IgA antibody. Jawetz and his colleagues suggested that, since in some patients the antibody titre in the blood is considerably higher than in CS, there is a possibility of transudation from serum to CS through the inflamed conjunctiva; they also put forward the speculation that the presence of antibody in tears may be related to the amount of viable TRIC agent in the conjunctiva.

Some of the findings in man have their counterparts in the results of animal experiments. Wang & Grayston (1971) demonstrated strain-specific antibody in the eyes of Taiwan monkeys infected with TRIC agents; the titre often reflected the

severity of infection. These authors' observations led them to opposing, but not necessarily mutually exclusive explanations of the source of CS antibody. Its titre appeared to be related to that in the serum, and a preliminary experiment indicated that the IgG antibodies in sera and CS were of the same serotype; these results suggested to Wang & Grayston that 'the most important source of eye secretion trachoma antibody is the serum'. On the other hand, the finding of IgA but not IgM antibody in CS also led them to suppose that 'the eye antibody is not directly from the serum, and may to some extent be locally produced'.

Murray *et al.* (1971) used a 4-layer indirect FA technique to study antibodies in owl monkeys; the classes of specific immunoglobulin induced were not defined. Antibodies apparently specific for *Chlamydia* were detected in the sera of some monkeys on receipt in the laboratory. Ophthalmic infection with either of two serotypes of TRIC agent induced antibody both in CS and serum, and protection against challenge with the homologous or heterologous strains; it was suggested that resistance to re-infection might be related to the presence of CS antibody. These observations were confirmed and extended by McComb *et al.* (1971), using similar methods. They demonstrated that owl monkeys with antibody in their sera before exposure to trachoma antigens sometimes had it in their CS as well, but CS antibody was not detectable when it was absent from the serum. There was a positive correlation between the titre of serum antibody and resistance to ophthalmic infection. Killed trachoma antigens instilled into the conjunctival sac induced small rises in CS antibody titres and much greater rises in serum titres; the degree of antibody response was related to resistance to challenge, measured in terms of the numbers of inclusions in conjunctival scrapings. McComb and co-workers concluded that 'the relationship in primates between circulating antibody and response to challenge in the eye with trachoma organisms must be considered established'.

Murray & Charbonnet (1971) adduced further evidence that CS antibody may be related to immunity to *Chlamydia*. In guinea-pigs both conjunctival inoculation of live guinea-pig inclusion conjunctivitis agent (Gp-ic) and intraperitoneal injection of formalin-inactivated organisms induced serum antibody that fixed complement; the conjunctival inoculation also induced IgA antibody in the CS and resistance to challenge, whereas the injection of killed organisms evoked neither.

Turning now to our own findings, we must first refer to the necessity for defining clearly the criteria on which the diagnosis of ophthalmic TRIC infection is based; unless this is done the validity of statements about the relation between antibody and clinical syndromes is open to question. We recognize that the criteria described in this paper depend in part on sequential studies and are thus unsuitable for general use; but we believe that they give a truer picture of the incidence of trachoma in the population investigated than do those of the WHO Expert Committee (1962).

We have previously mentioned (Sowa *et al.* 1971) that the only serotypes of TRIC agent isolated in Salekini, and in another Gambian village, were types 1 and 2 (Bell, Snyder & Murray 1959; Bell & McComb, 1967). Neither type 1b of Bell & McComb nor types D, E and F (Alexander, Wang & Grayston, 1967) have ever

been isolated by us in this area. Since the MRC-4*f* slide antigen used in our tests reacted adequately with unabsorbed antisera prepared against types 1 and 2, it is unlikely that it failed to detect any serum antibodies prevalent in this population.

The preceding summary of other workers' findings indicates that differing views may be held about the origin of antibody in the serum and conjunctival secretions; but these interpretations are as yet based on circumstantial evidence only. Our observations agree well with those of others, but do not settle the question of where these antibodies are formed. Anti-TRIC IgG antibody in the serum seems always to be present at higher titre than the corresponding antibody in CS, and thus may be formed elsewhere than in the eye; although the possibility that it is formed locally but passes more readily into the blood than into the CS cannot be discounted. By analogy with other infections of mucous membranes it seems reasonable to suppose that the IgA antibody in CS, which was shown by Hathaway & Peters (1971) to be of the secretory type, is produced locally. Our finding of a rather tenuous relationship between the titres of IgA and IgG antibody in the CS may be evidence that some at least of the IgG antibody is produced locally; on the other hand, the observation that IgG antibody in CS is detectable only when it is also present in the serum argues for the possibility, mentioned by various authors, of transudation through the inflamed conjunctiva. If antibody is produced locally the obvious site is the conjunctival follicle, which was shown by Jones (1971) to produce antibodies to adenovirus and to the causal agents of molluscum contagiosum and cat scratch disease; in our children conjunctival antibody was never found in the absence of follicular hyperplasia, although the reverse was by no means always true.

We found, like others, that antibody is more likely to be present both in serum and CS if TRIC agent can be demonstrated in the conjunctiva. This relationship may however be indirect since the detection of both antibody and TRIC agent appears to depend on the severity of infection. A consideration of this sort might also apply to inferences about the relation of antibody to immunity; although there is a strong *prima facie* case for supposing that it does play a part, there is at this stage an obvious danger in making *post hoc* inferences about the concomitant appearance of antibody and resistance to infection; cellular factors may be of equal or even greater importance.

One of us (Collier, 1967) suggested that trachomatous pannus might be due to an antigen/antibody reaction at the limbus, but the finding that there was no constant relationship between the appearance of corneal lesions and that of conjunctival antibody does not sustain this supposition.

One important result of these researches is that the presence of IgG antibody in the serum at a titre of 1/10 or greater appeared to be a more sensitive diagnostic indication of trachoma than the finding of conjunctival inclusions, either by the iodine or FA methods. It should, however, be noted that in this investigation the proportion of inclusion-positive subjects was lower than in some of our previous studies in The Gambia (see, for example, Sowa, Sowa, Collier & Blyth, 1965). Tests for trachoma antibody by the indirect immunofluorescence technique may be most useful when the physical signs are atypical or not fully developed, and

when it is difficult to demonstrate TRIC agent in the conjunctiva; the rarity of false positive reactions is a particular advantage. Since the antibody titres reported by those who used complement fixation (CF) methods are of the same order as those measured by FA techniques, it might be inferred that these tests do not differ greatly in sensitivity. However, Hanna *et al.* (1972) recently published the results of parallel CF and FA tests on '150 random sera from Tunisia'. Nearly 20% were positive for antibody to TRIC agent (at titres $\geqslant 1/8$) by both methods; 48·7% were positive by FA and negative by CF, whereas only 3·3% were positive by CF and negative by FA. Nevertheless, all workers seem to agree that every method so far used in field studies fails to detect antibody in a significant proportion of people diagnosed clinically as trachoma, especially when the disease is mild. It may well be that these relatively crude techniques will be complemented, or even superseded, by more sensitive methods such as the radioisotope precipitation test first used for psittacosis antibody by Gerloff & Watson (1967), or the binding assay with ^{125}I-labelled Fab' molecules recently described for TRIC agents by Macdonald & Barenfanger (1971); if so, inferences from the results of immunofluorescence tests may have to be modified.

We are grateful to Professor R. Nichols (Harvard School of Public Health, Boston, U.S.A.) and Dr W. D. Brighton (National Institute for Medical Research, London, England) for providing serological reagents; to Mr Malcolm Race for his competent technical help; and to Dr I. A. McGregor and his staff at the M.R.C. Laboratories, Fajara, for assistance with various facilities.

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Experimental aspergillosis in mice: aspects of resistance

By G. R. SMITH

Nuffield Institute of Comparative Medicine,
*The Zoological Society of London, Regent's Park, London N.W.*1

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SUMMARY

Intravenous inoculation of *Aspergillus fumigatus* spores was used to study experimentally induced and natural resistance. Slight resistance resulted in increased survival time and higher resistance produced in addition a decreased infection rate.

Sublethal doses of living spores gave significant protection against challenge 3 weeks later, but large doses of heat-killed spores had no demonstrable effect.

Mice from one source showed a single, dramatic decrease in dose response to a deep-frozen strain of the organism over a period of 34 months. The dose response initially resembled that described by Scholer (1959) in which one million spores killed the majority of mice. The change was almost certainly due to an increase in resistance of the mice due to environmental factors, and the resistance was probably also effective against other strains of the organism. Although not proved, it seemed likely that the resistance was due to increased natural contact with *A. fumigatus* or related fungi. Possibly for a similar reason, mice of the same stock bred on different premises differed in their susceptibility to infection. The results indicated that environmental resistance-producing factors may have been operating simultaneously on a number of premises housing laboratory animals in south-east England. These findings may have significance in relation to the occurrence of natural aspergillosis of mammals and birds.

Of five *A. fumigatus* strains, four were of closely similar virulence; the fifth strain grew more slowly *in vitro* and was somewhat less virulent. Isolates from mice which died sporadically after small doses of spores were of no greater virulence than the inoculated strain. Although the susceptibility of mice aged 3 weeks was not uniform under all conditions, such animals were less resistant than young adult mice. Mice from six different sources showed only slight differences in susceptibility between each other, or from mice known to have developed a natural resistance.

INTRODUCTION

Vaccination against mycoses is almost untried (Smith, 1969) and experiments on immunization (Kong & Levine, 1967) have usually been concerned with fungi whose growth *in vivo* is characterized by multiplication of individual organisms.

Aspergillus fumigatus, although basically saprophytic, is an important pathogen of birds and mammals. When growth occurs in the depths of animal tissues, it

does so without increase in the number of organisms. Human allergic disease and the demonstration of antibodies *in vitro* (Austwick, 1965; Pepys, 1969) have attracted considerable study, but precise information on increased resistance to infection as a result of previous contact with the organism or its products is scanty and often inconclusive.

Henrici (1939) considered that hyperimmunization with endotoxin immunized four out of six rabbits against inoculation of spores, but Tilden, Hatton, Freeman & Williamson (1957) and Tilden *et al.* (1961) reported that rabbits which had proved immune to large doses of toxin failed to resist the intravenous inoculation of 10^7 spores. They also found that neither a previous non-fatal infection nor inoculation of heated spores protected rabbits against fatal doses. Asakura, Nakagawa, Masui & Yasuda (1962) stated that resistance seemed to be conferred on a group of five ducks by subcutaneous inoculation of living spores: although all showed symptoms after intravenous challenge only one died, whereas three out of five control birds succumbed. Austwick (1962) reported that 66 % of slaughtered dairy cows showed minute pulmonary lesions termed 'asteroid bodies' and that *A. fumigatus* was isolated; similar bodies were found by King, Munday & Hartley (1965) who considered that the pathology indicated an immune response. O'Meara & Chute (1959) and Klimeš & Rosa (1964) found that chicks developed considerable resistance within the first few days of life to infection by inhalation.

Scholer (1959) studied aspergillosis induced in mice by intravenous inoculation of spores and found that, although slight variations in virulence occurred among six strains of *A. fumigatus*, most animals died within 20 days when given one million or more spores. Ford & Friedman (1967) reported that three strains of *A. fumigatus* were of similar virulence for mice by intravenous inoculation, but the dose response may have been somewhat lower than that described by Scholer (1959).

Although such infections are highly artificial, the ready availability of mice constitutes an important advantage in studies which demand large numbers of animals. This paper describes the use of mice inoculated intravenously with spores for the investigation of certain aspects of resistance to *A. fumigatus*.

MATERIALS AND METHODS

Purification and preservation of A. fumigatus *strains*

Cultures were received at the laboratory after storage at room temperature for 9–90 days. On receipt, the purity of each strain was ensured as follows. Serial dilutions of a nutrient broth suspension of well-separated spores were sampled on Sabouraud's dextrose agar (Oxoid, CM 41). After incubation at 37° C. for about 21 hr., a piece of agar bearing a single unsporulated colony was transferred to a fresh sterile container and incubated for a further 24 hr. The spores thus produced were suspended in saline and used to inoculate a large batch of Sabouraud's dextrose agar cultures which, after incubation for 3 days, was preserved at either −20° C. or 4° C.

Strains of A. fumigatus

Strain AF_1 was isolated from a black-footed penguin which died in the Zoological Society's Collection at Regent's Park from pulmonary aspergillosis. This strain was used exclusively for all experiments except one. The work was carried out during a period of 34 months and, for each experiment, the organism was obtained from a single batch of purified cultures stored at $-20°$ C.

Strains AF_2 and AF_3 were isolated from pulmonary aspergillosis in a citron crested cockatoo at Whipsnade and a Chilean flamingo in Gloucestershire; after purification, these strains were kept at 4° C. and used 1 month later. Strain AF_4 was isolated from pulmonary aspergillosis in a yellow-fronted Amazon parrot in Yorkshire and, after purification, was stored for 3 weeks at $-20°$ C. before use. Strain AF_5 was cultured from the pneumonic lung of a day-old Demidoff's bush-baby which died at Regent's Park, but its pathogenic significance was uncertain; it was purified and stored in the same way as strain AF_4.

Preparation of spore suspension for intravenous inoculation

Sabouraud's dextrose agar was poured into horizontal medical flat bottles. The bottles were inoculated with spore suspension and incubated at 37° C. for 3 days. Nutrient broth (Oxoid, CM 67) was then introduced through the rubber liners of perforated bottle caps by means of a hypodermic syringe and needle. After thorough agitation, the bottles were inverted and spore suspension was withdrawn by syringe from the centre of the fluid column, thus avoiding clumps of spores, the majority of which either rose to the surface or sedimented. After further thorough agitation, the spores were washed 3 times, resuspended in nutrient broth to the opacity of Brown's tube 19 and checked microscopically to ensure that the great majority were singly dispersed. Such a suspension could be expected to contain approximately 2×10^8 viable spores per ml., and dilutions in nutrient broth were prepared for the immediate intravenous inoculation of mice in 0·25 ml. volumes. Because spores sedimented rapidly, suspensions were quickly shaken on each occasion before filling a syringe. A retrospective check on viable count was always carried out, by sampling 0·1 ml. volumes of decimal dilutions on Sabouraud's dextrose agar plates and counting colonies after 21 hr. incubation; the mean of three replicate counts was taken as the true value.

Heat-killed spore suspension

A 100 ml. medical flat bottle containing 30 ml. of a nutrient broth spore suspension ($24·8 \times 10^6$ viable spores per ml.) was immersed for 15 min. in a water bath at 65° C. and then immediately cooled. Sterility was checked by culturing 0·3 ml. in Sabouraud's liquid medium (Oxoid, CM 147).

Mice

Unless stated otherwise, the mice consisted of females weighing approximately 20 g., purchased from supplier A ('SA' mice). Mice designated SB, SC, SD, SE_1, SE_2 and SF were purchased from five further suppliers. All animals were Swiss

white mice from outbred, closed colonies and they consisted of different strains with the exception of SD and SE_1 mice, which represented two different lines of the same strain. The SE_2 and SF mice were specific pathogen free, but they were not barrier-maintained at the Institute. Mice were used within a week of arrival, except where stated otherwise. At the Institute, mice were fed a pelleted form of diet 86 (Howie, 1952), obtained throughout from a single manufacturer.

Cultural examination of mice

In initial studies on the nature of the infection, a loopful of brain tissue and the cut surfaces of lung, liver, spleen and kidney were smeared on Sabouraud's dextrose agar and incubated for 2 days at 37° C. In later studies, renal tissue was examined by culturing any obvious lesion and a large loopful of homogenized suspension of both kidneys. Carcasses were frequently stored at −20° C. before examination.

RESULTS

Experimental aspergillosis in mice

The pathology of the disease which follows intravenous inoculation of *A. fumigatus* spores has already been described by Scholer (1959) and additional observations only are given here. Detailed information on the effects of various doses on mortality is presented later.

Lung, liver, spleen, kidney and brain from mice which died in 1 or 2 days as a result of large doses of spores were almost invariably positive on culture. Multiple, minute, whitish foci were visible in both kidneys of animals dying after the second or third day, and these became progressively larger as the time to death increased; the lesions were very obvious in mice dying after about 7 days. As judged by culture, infection was progressively lost, first from the lungs, then spleen and liver, and somewhat later from brain, so that in animals dying 10 or more days after inoculation, infection was almost invariably confined to the kidneys. Mice dying after several weeks usually showed large lesions in both kidneys, and survivors slaughtered after prolonged periods were usually either normal at post-mortem or severely affected in one kidney only. A week or more after inoculation, there was very close correlation between the presence or absence of macroscopic kidney lesions and positive or negative cultures.

Experimental production of resistance

Two groups of 104 SA mice were inoculated intravenously with either 62,000 or 6200 living spores of strain AF_1. Two further large groups were similarly treated in dose volumes of 0·25 ml. with either $6{\cdot}2 \times 10^6$ heat-killed spores or sterile nutrient broth (controls). After 3 weeks, during which the larger dose of living spores produced nine deaths (8·6%) and the smaller dose none, the four main groups were used to supply subgroups of 11–15 mice for intravenous challenge with doses of 60, 30, 6, 3, 0·6, 0·06 and 0·006 million spores. Deaths were recorded at least once a day, and all survivors were killed 64 days after challenge. To monitor the effects of the original doses of living spores, 13 and 20 mice treated respectively with

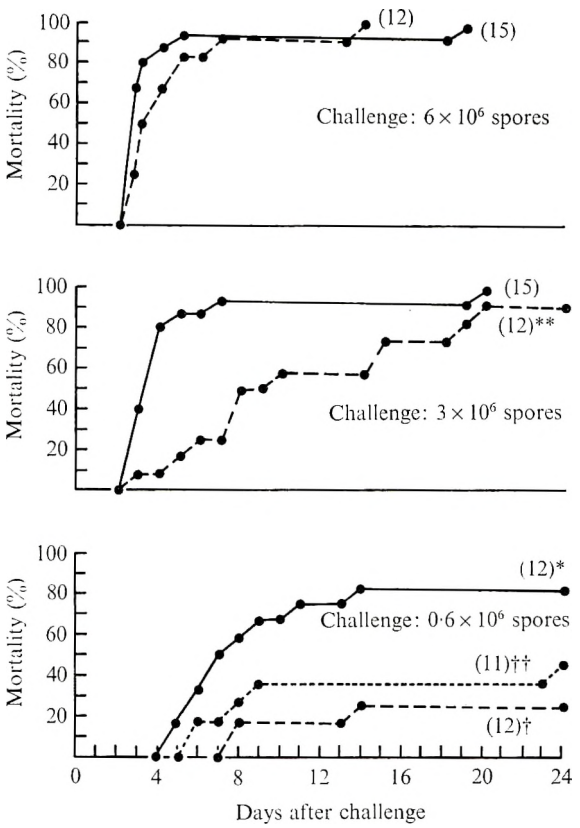

Fig. 1. Production of resistance to *A. fumigatus* by intravenous inoculation of sublethal doses of living spores. Mice treated with 62,000 spores (– – –), 6200 spores (- - - - -) and sterile nutrient broth (——) 3 weeks before intravenous challenge. Figures in parentheses indicate number of mice in group. ** = 100% mortality 64 days after challenge; * = 83% mortality 64 days after challenge; †† = 64% mortality 64 days after challenge; † = 50% mortality 64 days after challenge.

62,000 and 6200 spores were left unchallenged; the larger dose produced two deaths from renal aspergillosis and a further four infected animals were found at slaughter, but the smaller dose was without apparent effect.

Challenge doses of 60×10^6 and 30×10^6 spores were fatal to all mice within 5 days, while $0{\cdot}06 \times 10^6$ had killed only three of 12 controls, and $0{\cdot}006 \times 10^6$ none of 12 controls, by the end of the experiment. However, doses of an appropriate size showed clearly (Fig. 1) that an earlier sublethal infection produced resistance, manifested in this experiment as an increase in survival time. A dose of 6200 spores produced resistance to challenge with $0{\cdot}6 \times 10^6$ spores ($P < 0{\cdot}025$ on days 14–23), but the larger dose of 62,000 was more effective ($P < 0{\cdot}005$ on days 11 to 24). This larger dose also resulted in increased survival time of mice challenged with 3×10^6 spores ($P < 0{\cdot}0005$ on day 4 to $P < 0{\cdot}0125$ on day 9) and 6×10^6 spores ($P < 0{\cdot}04$ at 65 hr.), but the observable effect diminished as challenge increased. The dose of 6200 spores failed to protect against challenge of 3×10^6 and 6×10^6 spores. The 8·6% mortality which occurred before challenge in mice pretreated

Table 1. *Increased resistance of mice following inoculation of a sublethal dose of living spores*

	Total deaths at intervals (days) after challenge				
	5	10	14	28	69
Control mice	17/30	24/30	26/30	29/30	29/30
Pre-infected mice	3/12*	5/12	5/12	6/12	7/12
$P <$	NS	0·02	0·003	0·0005	0·002

Intravenous challenge of $0{\cdot}9 \times 10^6$ spores given 3 weeks after a pre-infecting dose of $0{\cdot}03 \times 10^6$ spores.

* Two of the deaths shown occurred before challenge; they were due to renal aspergillosis.

NS = not significant.

with 62,000 spores may have removed a small number of the more susceptible animals, but this could not have accounted for the considerable resistance produced. Moreover, any mortality occurring after challenge as a result of pretreatment with 62,000 spores would have biased the experiment against the demonstration of protection. When killed, the survivors of the groups depicted in Fig. 1 almost always showed a lesion in one kidney, from which *A. fumigatus* could be isolated. The narrowness of the range of challenge doses over which resistance could be demonstrated was probably a reflexion of the organism's inability to multiply *in vivo*. The experiment gave no indication that mice pretreated with heat-killed spores differed from the controls.

A further test was made with mice kept, in this instance, at the Nuffield Institute for 4 weeks between purchase and the commencement of the experiment. Twelve mice were pretreated with $0{\cdot}03 \times 10^6$ spores intravenously and 30 control mice were left untreated. The animals were challenged intravenously with $0{\cdot}9 \times 10^6$ spores after three weeks, during which period two test mice died from renal aspergillosis. Despite the mortality produced by the preinfecting dose, the test mice showed significantly lower mortality than the controls from the tenth day after challenge until the experiment was terminated on day 69 (Table 1). Thus, significant protection was of greater duration than in the first experiment. Of the five surviving test mice, four were found at slaughter to have *A. fumigatus* infection of one kidney.

Natural occurrence of resistance

The work described above formed part of a series of experiments on aspergillosis in mice carried out between April 1969 and January 1972. Six experiments performed during the period April 1969 to August 1970, each demonstrated a dose response similar to that reported by Scholer (1959) in that a dose of about 10^6 spores was rapidly fatal to most mice. The experiment shown in Table 1 was carried out at the end of August 1970, and is worthy of special mention as the control mice showed the usual susceptibility, even though they had in this instance been kept at the Institute for 7 weeks between purchase at 20 g. weight and inoculation. Again, the control mice shown in Fig. 1 were kept at the Institute for 4 weeks before challenge, yet $0{\cdot}6 \times 10^6$ spores produced 83 % mortality in 14 days.

Table 2. *Spontaneous change in response of mice from a closed colony to* Aspergillus fumigatus *spores grown from a single batch of frozen cultures*

Reciprocal dilution of spores	Days after inoculation: 2		5		8		64	
	A*	B*	A	B	A	B	A	B
1	12	7	12	12	12	12	12	12
2	12	0	12	8	12	8	12	12
4	9	0	12	6	12	10	12	12
8	5	0	11	0	11	6	11 (1)	11 (1)
16	0	0	12	0	12	4	12	7 (4)
32	1	0	8	0	11	0	12	1 (5)
64	0	0	9	0	10	0	11 (0)	3 (1)
128	0	0	3	0	5	0	9 (2)	0 (7)
256	0	0	1	0	3	0	8 (3)	1 (2)
512	0	0	0	0	0	0	3 (6)	0 (0)
1,024	0	0	1	0	1	0	2 (7)	0 (1)
2,048	0	0	0	0	0	0	0 (3)	0 (1)
4,096	0	0	0	0	0	0	0 (2)	0 (1)
8,192	0	0	0	0	0	0	0 (2)	0 (0)
16,384	0	0	0	0	0	0	0 (0)	0 (0)

Figures indicate deaths in groups of 12 mice after inoculation with dilutions of spore suspension.

Figures in parentheses indicate number of survivors showing active *A. fumigatus* lesions at slaughter, 64 days after inoculation.

* Expt. A was begun in May 1970: the 1/32 dose contained $1{\cdot}43 \times 10^6$ viable spores. Expt. B was begun in May 1971: the 1/32 dose contained $1{\cdot}71 \times 10^6$ viable spores.

At some time between August and November 1970, the response of the SA mice to spores grown from the deep-frozen AF_1 cultures underwent a change, and from December 1970 to January 1972, each of 15 experiments showed a dose response which was greatly reduced and, though it fluctuated slightly on occasion, was on the whole remarkably constant. One effect was that 10^6 spores no longer rapidly killed the majority of mice.

The magnitude of the change is indicated in Table 2, which shows virulence titrations carried out in May 1970 (Expt. A) and May 1971 (Expt. B), under conditions as nearly identical as possible. The dramatic change in dose response was related to increased survival time and also to reduced infection rate, as was shown by examination of survivors killed 64 days after inoculation. The final LD50 values (Reed & Muench, 1938) in Expts. A and B were $0{\cdot}146 \times 10^6$ and $2{\cdot}75 \times 10^6$ spores respectively, differing by a factor of approximately 18. The corresponding ID50 values were $0{\cdot}034 \times 10^6$ and $0{\cdot}9 \times 10^6$, differing by a factor of approximately 26. These differences are impressive in view of the organism's inability to multiply *in vivo*.

Every effort was made to adhere throughout to standard technique in the preparation and inoculation of spore suspensions. Nevertheless, an examination of technique was made and the results, which were entirely negative, are summarized as follows. Spores grown on two different manufacturer's batches of

Table 3. *Virulence of five strains of* Aspergillus fumigatus *for SA mice*

Dilutions of Brown's no. 19 spore suspension	Deaths in groups of 12 mice after inoculation with dilutions of spore suspensions prepared from five strains					
	Expt. 1: results recorded 21 days after inoculation			Expt. 2: results recorded 28 days after inoculation		
	AF_1	AF_2	AF_3	AF_1	AF_4	AF_5
1/5	12	5 (5)	12	11 (1)	11 (1)	12
1/10	11 (1)	2 (9)	4 (7)	8 (3)	6 (6)	9 (3)
1/20	4 (8)	2 (2)	3 (7)	8 (3)	6 (3)	5 (4)
1/40	1 (5)	0 (4)	3 (4)	2 (7)	3 (4)	3 (5)
1/80	1 (7)	2 (4)	2 (8)	2 (6)	2 (3)	4 (4)
1/160	0 (4)	0 (2)	1 (2)	1 (5)	1 (2)	0 (4)

Figures in parentheses indicate survivors showing active *A. fumigatus* lesions at slaughter. The 1/40 doses contained the following numbers of viable spores (millions): AF_1 (Expt. 1), 1·46; AF_2, 1·15; AF_3, 1·05; AF_1 (Expt. 2), 1·32; AF_4, 1·39; AF_5, 1·47.

Sabouraud's dextrose agar, and on medium subjected to excessive autoclaving, did not differ significantly in their effects on mice. Slight differences in the age of cultures from which spores were obtained did not affect mortality. Spore suspensions made up independently by two individuals produced similar mortality.

Supplier A, a well-known and reputable breeder, gave a categorical assurance that he had not consciously changed the management, feeding or breeding of the SA closed colony.

The explanation of the events observed clearly lay either in a decrease in virulence of strain AF_1 or in an increase in the resistance of the mice due to environmental factors; increased resistance due to genetical factors appeared to be ruled out by the circumstances. The following experiments were designed to investigate the cause of the change in dose response and to give a background of information against which it could be viewed.

Comparison of the virulence for SA mice of five strains of A. fumigatus

Loss of virulence of strain AF_1 always seemed unlikely because the purified seed cultures were stored at $-20°$ C.; in addition, the change in dose response took place suddenly rather than gradually. Nevertheless, it was conceivable that slow progressive loss of viability at $-20°$ C. in a possibly heterogeneous population of spores might have resulted in selection leading to reduced virulence. This was examined in the light of Scholer's (1959) observation, based on examination of six strains, that 10^6 spores killed the majority of mice – a dose response which resembled that of the SA mice to strain AF_1 before the autumn of 1970. Accordingly, comparisons between AF_1 and four more recently isolated strains were made in the course of two experiments separated by an interval of 16 weeks (Table 3). It seemed reasonable to argue that if strain AF_1 had lost virulence during storage, it should be noticeably less virulent than at least some, if not all, of the four additional strains. Table 3 shows that this was not so.

Doses slightly greater than 10^6 spores produced consistently low mortality and

Table 4. *Virulence of strain AF_1 after one mouse passage*

Days after inoculation	Deaths in mice inoculated with doses of spores from: Original strain AF_1 $1{\cdot}1 \times 10^6$	six isolates of strain AF_1 after passage through mice: $1{\cdot}7 \times 10^6$	$1{\cdot}3 \times 10^6$	$1{\cdot}7 \times 10^6$	1×10^6	1×10^6	$0{\cdot}9 \times 10^6$
12	3/12	2/8	1/8	1/8	2/8	2/8	1/8
28	4/12	2/8	4/8	2/8	2/8	2/8	2/8

no significant differences in virulence emerged except that, in Expt. 1, strain AF_2 produced a somewhat lower mortality 21 days after inoculation than did strains AF_1 and AF_3. Thus, while $9{\cdot}2 \times 10^6$ AF_2 spores proved fatal to only five of a group of 12 mice, $5{\cdot}8 \times 10^6$ AF_1 spores and $8{\cdot}4 \times 10^6$ AF_3 spores produced, respectively, 11 deaths ($P = 0{\cdot}01$) and 12 deaths ($P < 0{\cdot}0025$). It should be noted that strain AF_2 differed in growth characteristics from the other strains used: colonies which grew on Sabouraud's dextrose agar from well-separated spores did not exceed 1 mm. in diameter after 21 hr. incubation, whereas most colonies of the other four strains were at least twice as large; furthermore, after 2 and 3 days incubation the spores of strain AF_2 produced a greenish blue colour which was much lighter in shade than that produced by the other strains.

Thus it seemed unlikely that strain AF_1 had lost virulence during storage, and further information on this point was supplied by the next experiment.

Virulence of strain AF_1 after isolation from mice

Although the dose response after the autumn of 1970 was greatly reduced, small numbers of sporadic deaths occurred, sometimes surprisingly soon after inoculation, in mice treated with low doses. To exclude the possibility that these deaths might have been due to a small proportion of virulent spores in a conceivably heterogeneous inoculum, AF_1 isolates from six such mice were examined. The animals chosen consisted of four mice which died after doses ranging from $0{\cdot}7 \times 10^6$ to $0{\cdot}1 \times 10^6$ spores, and two of five mice which died from a group of 50 mice inoculated with $1{\cdot}3 \times 10^6$ spores.

Each isolate was purified and, 10 days after isolation, spore suspensions prepared in the usual way were inoculated intravenously into groups of eight SA mice. A further group of 12 mice received spores grown from the original, deep-frozen AF_1 cultures. The doses ranged from $0{\cdot}9 \times 10^6$ to $1{\cdot}7 \times 10^6$, and reference to Table 2 and the related text shows that, before the end of August 1970, such doses would have killed almost all mice within 8 days.

The results given in Table 4 show that after a single passage through mice strain AF_1 did not differ significantly in virulence from the original strain. Since growth without multiplication of the organism occurs in the depth of tissues, no attempt was made to raise virulence by repeated passage.

Table 5. *Effect of environment and age on resistance of mice to* Aspergillus fumigatus

Expt.	Days after intravenous inoculation	Dose of spores ($\times 10^6$)	Deaths of SA mice in four separate experiments: Mice bred by supplier A	Mice bred at Nuffield Institute	Significance (P) of difference in mortality between mice bred on different premises
			♂ mice weighing 20 g.	♂ mice weighing 10 g. aged 3 weeks	
A	6	5·6	2/15	12/12	< 0·00005
		1·4	0/15	8/12	< 0·0005
	28	5·6	12/15 (3)	12/12	NS
		1·4	2/15 (7)	*11/12 (1)	< 0·0001
			♀ mice weighing 22 g.	♀ mice weighing 26 g. aged 6 weeks	
B	4	5·3	0/15	4/12	< 0·017
		1·3	0/15	0/12	NS
	6	5·3	6/15	6/12	NS
		1·3	2/15	1/12	NS
	28	5·3	15/15	12/12	NS
		1·3	8/15 (3)	9/12 (3)	NS
			♀ mice weighing 21 g.	♀ mice weighing 30 g. aged 9 weeks	
C	6	5·3	5/15	11/12	< 0·003
		1·3	1/15	4/12	NS
	28	5·3	11/15 (3)	12/12	NS
		1·3	3/15 (9)	11/12 (1)	< 0·0005
			♂ mice weighing 20 g.	♂ mice weighing 37 g. aged 13 weeks	
D	6	1·6	1/15	6/22	NS
	10	1·6	2/15	12/22	< 0·0125
	28	1·6	3/15 (8)	15/22 (7)	< 0·005

NS = not significant.

Figures in parentheses indicate survivors showing active *A. fumigatus* lesions at slaughter, 28 days after infection.

* Result complete by 10th day.

Effect of environment and age on resistance

In view of the evidence that AF_1 had not lost virulence, it seemed certain that the resistance of the SA mice must have increased. As already explained, the circumstances indicated that increased resistance could not have resulted from genetical factors. The importance of environment is apparent from the next experiment.

Adult SA breeding mice were purchased from supplier A. Their offspring, bred at the Institute, were challenged with strain AF_1 immediately after weaning at the age of 3 weeks (mean weight 10 g.) and also at the ages of 6 weeks (weight 26 g.), 9 weeks (weight 30 g.) and 13 weeks (weight 37 g.). On each of these four occasions, mice weighing 20–22 g. were obtained from supplier A 24 hr. earlier and challenged simultaneously (Table 5).

As judged by mortality, mice bred at the Institute were more susceptible than

20–22 g. mice obtained from supplier A; this was so not only when they were smaller and younger than the purchased mice, but also when they were larger and older. The difference was very clear in each test except the second (Expt. B, Table 5), in which there was merely a slight difference in survival time; in this instance, the mortality of the purchased mice may have indicated a temporary upward fluctuation in the normally low susceptibility of the 20 g. SA mice, to a degree which was unique in the 15 experiments carried out after the autumn of 1970. It is clear from the cultures made on slaughtered survivors that Institute-bred mice, unlike those bred by supplier A, invariably became infected after inoculation of 10^6 to 2×10^6 spores.

The early onset of symptoms and the mortality pattern of Institute-bred mice aged 3 weeks (Expt. A, Table 5) indicated high susceptibility similar to that consistently found in adult SA mice before the end of August 1970 (see Table 2 and related text). Institute-bred mice of older age groups were probably less susceptible, and a further experiment on age and resistance was carried out using mice bred by supplier A.

Fifteen SA males immediately after weaning at 3 weeks (mean weight 11·7 g.) and 15 older males (mean weight 20 g.) were inoculated with $1{\cdot}6 \times 10^6$ spores. Total deaths in the 11·7 g. mice 6, 10 and 28 days later were 2, 3 and 12 respectively; the corresponding figures for deaths of 20 g. mice were 1, 2 and 3. Thus, the newly weaned mice were clearly more susceptible than the older animals ($P < 0{\cdot}002$ on day 28), but apparently less susceptible than the newly weaned mice bred earlier at the Nuffield Institute (Expt. A, Table 5).

Comparison of the susceptibility of mice from seven different sources

Finally, it seemed of interest to investigate the range of susceptibility to *A. fumigatus*, strain AF_1, shown by mice from a number of sources in the south-east of England. In one experiment, SA mice were compared with SB, SC, SD and SE_1 mice by inoculating various dilutions of spore suspension prepared from *A. fumigatus* strain AF_1. In a second experiment, carried out 14 weeks later, SA mice were compared with the specific pathogen free SE_2 and SF mice. The details of experimental design and the results are given in Table 6.

Expt. 1 revealed no clear differences between mice from five different sources in terms either of mortality 3 weeks after infection, or of active lesions in survivors killed at 3 weeks. Slight differences nevertheless occurred: for example, on the fourth day after inoculation, deaths produced in groups of eight animals by the 1/5, 1/10 and 1/40 inocula were respectively, 6, 4 and 1 in SD mice, but only 1, 0 and 0 in SE_1 mice and 2, 0 and 0 in SA mice. Expt. 2 revealed no differences in the susceptibility of SA and SE_2 mice, but the SF animals were clearly less resistant as judged by mortality 33 days after infection ($P < 0{\cdot}005$ at the 1/10 dose level). This difference in susceptibility was much less dramatic than that resulting from the natural increase in resistance of the SA mice (see Table 2). The greater susceptibility of SF mice is unlikely to have been related to their specific pathogen free status, since the more resistant SE_2 mice were also specific pathogen free. In general, $1{\cdot}4 \times 10^6$ spores produced low mortality in the experiments.

Table 6. *Comparison of the susceptibility of mice from seven different sources to* Aspergillus fumigatus, *strain* AF_1

Dilutions of Brown's no. 19 spore suspension (strain AF_1)	Deaths in mice from seven different sources							
	Expt. 1: results recorded 21 days after inoculation					Expt. 2: results recorded 33 days after inoculation		
	SA	SB	SC	SD	SE_1	SA	SE_2	SF
1/5	6/8 (2)	7/8 (1)	8/8	8/8	5/8 (3)	11/12 (1)	10/12 (2)	6/6
1/10	5/8 (3)	5/8 (3)	8/8	5/8 (3)	5/8 (3)	4/12 (5)	6/12 (6)	12/12
1/20	2/8 (2)	N	N	N	N	3/12 (4)	1/12 (9)	5/12 (6)
1/40	0/8 (5)	1/8 (6)	3/8 (4)	3/8 (5)	2/8 (4)	1/12 (8)	2/12 (9)	6/12 (5)
1/80	1/8 (4)	1/8 (4)	1/8 (6)	5/8 (2)	1/8 (4)	3/12 (3)	3/12 (4)	1/12 (8)
1/160	N	N	N	N	N	0/12 (3)	0/12 (8)	3/12 (5)
1/640	1/10 (4)	1/10 (1)	0/10 (0)	0/10 (3)	0/10 (1)	N	N	N

Figures in parentheses indicate number of survivors showing active *A. fumigatus* lesions on slaughter.

In both Expts 1 and 2, the 1/40 doses contained $1{\cdot}4 \times 10^6$ spores.

N = not done.

DISCUSSION

The use of mice provided a sensitive system for studying both experimental and natural resistance. Slight differences in susceptibility were revealed only by variations in survival time, but greater differences gave rise in addition to differences in infection rate. Challenge doses within an optimum range showed that sub-lethal doses of spores given intravenously could produce resistance of mice to *A. fumigatus*. Although this resistance was stronger when the initial dose was large enough to produce pathogenic effects in a proportion of animals, it also occurred after a dose too small to result in established infections.

Repeated observations over a period of 34 months revealed a single, very striking decrease in dose–response of the SA mice to *A. fumigatus*, strain AF_1. This was shown by evidence of a necessarily indirect or circumstantial nature to be due not to any decrease in virulence of strain AF_1, but to an increase in resistance of the mice, resulting from environmental and not genetical factors. Although the experiments of Scholer (1959) were carried out under slightly different conditions, it is interesting that the dose response which he observed resembled that initially shown by the SA mice. It is strange that, after the resistance of the SA mice suddenly increased, a search for highly susceptible mice from a considerable number of other sources in the south-east of England was unsuccessful, although minor differences in susceptibility between mice of different origin were observed. It is also interesting that, before the autumn of 1970, mice bred by supplier A were highly susceptible even after being kept for as long as 7 weeks at the Nuffield Institute between purchase and inoculation; yet subsequently, SA mice bred at the Institute appeared to possess susceptibility of a degree which was intermediate, though clearly greater than that of mice of the same stock bred by

supplier A. These circumstances indicate that, after the autumn of 1970, environmental resistance-producing factors may have been operating simultaneously, though to varying degrees, on a number of premises housing laboratory mice in south-east England. Unfortunately, mice from breeders other than supplier A were not examined before the autumn of 1970. In view of Scholer's (1959) findings concerning dose-response, it seems justified to suggest that the environmental factors which brought about the increase in resistance of SA mice to strain AF_1 also produced resistance to at least some, if not all, of the 4 additional strains used.

There is no proof as to which of the many possible environmental factors was responsible for the increase in resistance of the SA mice, but the following points may be pertinent. *A. fumigatus* is a ubiquitous organism. The concentration of fungal spores in the air is known to vary greatly both from place to place and from time to time (Hyde, Richards & Williams, 1956; Baruah, 1961; Noble & Clayton, 1963) and certain materials such as hay, particularly if mouldy, can provide abundant sources of fungal spores, many of which are of the genus *Aspergillus* (Gregory & Lacey, 1963*a*, *b*; Lacey & Lacey, 1964). Using an experimental system which was admittedly highly artificial, the present study showed that previous experience of the organism could result in a degree of resistance. Though not proved, it seems probable that the sudden increase in the resistance of the SA mice was due to a change in the degree of environmental contact with *A. fumigatus* or related fungi, living or dead, or to their metabolites. Such contact might have occurred either by inhalation or by ingestion in foodstuffs, and further research along these lines is indicated. The difference in susceptibility of mice of the same stock bred on different premises may have had a similar basis. Mice immediately after weaning at the age of 3 weeks were more susceptible than young adults of the same stock bred on the same premises, but the susceptibility of newly weaned mice appeared to be by no means uniform under all conditions. The lower resistance of 3-week-old mice may have been due to lack of specific immunity or to physiological factors.

It seems likely that environmental factors such as those which influenced the susceptibility of mice to artificial infection also operate in relation to other mammals and birds. This might have an important bearing on the occurrence and pathogenesis of the natural diseases produced by *A. fumigatus*.

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Serological detection of enterotoxin in foods implicated in staphylococcal food poisoning

By R. J. GILBERT, ANTONNETTE A. WIENEKE, JANICE LANSER* and MAGDA ŠIMKOVIČOVÁ†

Food Hygiene Laboratory, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT

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SUMMARY

Two methods are described for the extraction of enterotoxin from foods incriminated in incidents of staphylococcal food poisoning. Enterotoxin was detected serologically in 12 of 24 food samples from 20 separate incidents: eight samples contained enterotoxin A, three contained D and one both A and B. The amount of enterotoxin in nine foods, based on 100% recovery, varied from 0·02 to 0·09 μg./g.

Data are also given on the numbers of *Staphylococcus aureus* isolated from samples of food from 39 food poisoning incidents. Colony counts varied between $7{\cdot}5 \times 10^5$ and 9×10^9/g. with a median value of 7×10^7/g.

INTRODUCTION

In the past the laboratory investigation of staphylococcal food poisoning has been concerned with the isolation of *Staphylococcus aureus* from suspected foods, from faecal and vomit specimens, and from the hands and nose of suspected food handlers; phage-typing has enabled the strains to be correlated. Since the preparation of purified enterotoxins and specific antisera it has been possible to determine the type of enterotoxin produced by the strains isolated (Casman, Bennett, Dorsey & Issa, 1967; Šimkovičová & Gilbert, 1971). Although the results from such tests provide good evidence that staphylococcal enterotoxin caused an outbreak, the demonstration of enterotoxin in the suspected food itself is even better proof. Such detection is especially important when cooking or other treatment has killed the organisms but left the enterotoxin still active; also, in some foods, e.g. cheese, the organisms may have died during storage.

The demonstration of enterotoxin in food using the monkey feeding test or even the intraperitoneal kitten test is impracticable for routine procedures owing to the cost involved and, furthermore, they are not sufficiently sensitive to detect the amount of enterotoxin ordinarily encountered in foods from outbreaks (Bergdoll, 1969). The kitten test, in particular, has been criticized by various workers (Fulton, 1943; Casman *et al.* 1967; Bergdoll, 1970). However, various workers in

* Present address: Department of Pathology, Royal Alexandra Hospital for Children, Camperdown, New South Wales, Australia.

† Present address: Krajska hygienicko-epidemiologicka stanica, Trnavska cesta 60, Bratislava, Czechoslovakia.

the U.S.A. (Casman & Bennett, 1965; Hall, Angelotti & Lewis, 1965; Casman, 1967; Zehren & Zehren, 1968) have described *in vitro* tests for detecting enterotoxin in food.

This paper describes our experience with an *in vitro* technique for the detection of staphylococcal enterotoxin in food.

MATERIALS AND METHODS

Foods and cultures

Twenty-four food samples from 20 separate food poisoning incidents in England and Wales, where there was good clinical and bacteriological evidence that staphylococcal food poisoning had occurred, were received during 1969–71. Cultures of *Staph. aureus* from victims were also submitted on most occasions.

The foods used in experiments where measured amounts of enterotoxin were added were ham from a freshly opened can, cooked, peeled prawns and freshly cooked chicken.

Control enterotoxins and antisera

Enterotoxins A, B, C and D, all partially purified, and their specific antisera were supplied by Professor M. S. Bergdoll (A, B and C) and Dr E. P. Casman (D). The enterotoxins and antisera of A, B and C were diluted in 0·02 M phosphate buffer in saline, pH 7·4, containing 0·02 % thiomersal, and those of D were diluted in Difco Brain-Heart Infusion (BHI) broth in saline. Towards the end of the work 10 % of BHI broth was added to the diluents used for enterotoxins A, B and C and their antisera to increase the sensitivity of the gel-diffusion test (Casman, Bennett, Dorsey & Stone, 1969).

Enumeration of Staph. aureus

Counts of *Staph. aureus* were made on phenolphthalein diphosphate agar containing polymyxin (PPAP) (Hobbs, Kendall & Gilbert, 1968) using a modified Miles & Misra (1938) technique with incubation for 48 hr. at 37° C. Selected colonies were tested for coagulase production in 10 % plasma broth.

Information on counts of *Staph. aureus* in foods from a further 19 food poisoning incidents were obtained from Hobbs (1955) and from unpublished data from several Public Health Laboratories. Various selective media including PPAP had been used for counts of *Staph. aureus*.

Production of enterotoxin by cultures of Staph. aureus

Cultures were examined for their ability to produce enterotoxins A, B, C and D. Enterotoxin was produced by means of a sac-culture technique and detected serologically by a slide gel double-diffusion method (Šimkovičová & Gilbert, 1971).

Extraction of enterotoxin from food

Method 1. The food, usually 50 g. was homogenized in an Atomix beaker and then rehomogenized at half speed with 200 ml. of 0·2 M-NaCl. The slurry was centrifuged at 2000 *g* for 30 min. and the supernatant retained. The sediment was

re-extracted with 100 ml. of 0·2 M-NaCl and centrifuged. The two extracts were pooled and then vigorously shaken with at least two separate one-fourth volumes of chloroform: the chloroform layers were drawn off and discarded. The extract was reduced to a volume of about 20 ml. by dialysis against 30% (w/w) polyethylene glycol (PEG) Carbowax 20 M, (Union Carbide Co. Ltd., Hythe, Southampton) and centrifuged at 40,000 *g* for 1 hr. at 4° C. The supernatant fluid was concentrated by dialysis against PEG to approximately 0·4 – 1 ml. and examined for the presence of enterotoxin by gel-diffusion.

Method 2 (modified from Casman, 1967). The food, usually 50 g., was homogenized and extracted with 200 ml. of 0·02 M phosphate buffer in saline, pH 7·4. The pH was checked and adjusted to about pH 7·0–7·4. The slurry was centrifuged at 2000 *g* for 30 min. and the supernatant retained. The sediment was re-extracted with 100 ml. of buffer-saline and centrifuged. The two extracts were pooled and after vigorous shaking with chloroform the chloroform layer was discarded and the extract concentrated to dryness in PEG. The residue was taken up in 20 ml. of 0·01 M phosphate buffer, pH 7·4, and shaken again with chloroform. After removing the chloroform layer the extract was diluted with 40 vol. of 0·005 M phosphate buffer, pH 5·7. The pH was checked and if necessary adjusted to pH 5·7. The diluted extract was allowed to percolate at room temperature through a column of 1 g. of Whatman carboxymethylcellulose (CM 32, H. Reeve Angel and Co. Ltd., London, E.C. 4) equilibrated at pH 5·7 in 0·005 M phosphate buffer. After washing the column with the same buffer, any toxin adsorbed was eluted with 100 ml. of 0·2 M phosphate buffer, pH 7·4. The eluate was concentrated to dryness in PEG, re-suspended in 0·2 ml. of 0·02 M phosphate buffer in saline, pH 7·4, containing 10% BHI broth and 0·02% thiomersal, and examined for the presence of enterotoxin by gel-diffusion.

Slide gel double-diffusion test for enterotoxin

Slight modifications were made to the method described by Šimkovičová & Gilbert (1971). Two test areas were prepared on an agar-coated slide between three parallel double layers of tape set 2 cm. apart. Each area was filled with 0·2 ml. of an agarose-phosphate buffer-saline mixture and covered with a Perspex matrix with four funnel-shaped wells surrounding a fifth central well. Three of the outer wells were filled with dilutions of food extract or culture filtrate, the fourth outer well with reference enterotoxin and the central well with antiserum of the corresponding type. Slides were incubated in moist chambers at room temperature for 2 days (culture filtrates) or 5 days (food extracts). The staining procedure used to enhance precipitin lines was also modified. Slides were immersed in 0·1% thiazine red in 1% glacial acetic acid for 1–2 min (food extracts) or 10 min (culture filtrates). The presence of one or more of the enterotoxins A, B, C and D in a culture filtrate or food extract was verified by the coalescence of its precipitation line with the reference line of the corresponding type.

An optimal precipitin line mid-way between the two reactant wells required 2·5 μg./ml. of enterotoxins A, B or C and dilutions of 1/25 of antiserum A, or 1/75 of antisera B and C: a faint precipitin line was formed with 0·6 μg./ml. of toxin. The

reaction for enterotoxin D was optimal with 1/20 dilutions of toxin and antiserum.

When BHI broth was included in the diluent a control line required 1 μg./ml. of enterotoxins A, B, or C and 1/50 dilution of antiserum A, or 1/100 dilutions of antisera B and C: a faint precipitin line was formed with 0·25 μg./ml. of toxin.

Recovery of enterotoxin from foods

Measured amounts of enterotoxin were added to separate 50 g. samples of canned ham, cooked prawns and cooked chicken. Method 1 was used to extract enterotoxins A (20 μg.), B (100 μg.) and C (20 μg.) from ham and prawns, and enterotoxins A (15 μg.), B (75 μg.) and C (15 μg.) from samples of chicken. Method 2 was used to extract enterotoxin A from samples of chicken (0·25 and 1 μg.) and prawns (0·25, 1, 5 and 10 μg.).

RESULTS

Recovery of enterotoxin added to foods

The estimated recovery of enterotoxins A, B or C from samples of ham, chicken and prawns, using method 1 for the extraction, varied between 20 and 55 % of the quantity added with a mean value of 33 %. When method 2 was used for extracting enterotoxin A from samples of chicken and prawns the estimated recovery was 20 %. Method 2 was more satisfactory than method 1 for the quantitative estimation in food because the final concentrated extract contained less non-specific components. As a result precipitin lines in gel-diffusion tests were more distinct with less non-specific precipitation.

Detection of enterotoxin in foods implicated in food poisoning

Two methods were used for the extraction of enterotoxin from foods implicated in food poisoning. Enterotoxin was detected in nine of 17 foods using method 1 for the extraction of toxin, and in three of seven foods from the more recent outbreaks using method 2: enterotoxins A, D and A and B together were detected in foods from eight, three and one incidents respectively (Table 1). In each instance *Staph. aureus* was present in large numbers, from 5×10^6 to 2×10^9/g. of food, and the enterotoxin present was the same as that produced by growth of the organisms in sac-cultures: strains isolated from patients also produced the same enterotoxin. In one outbreak, however, strains isolated from the food and from patients produced both enterotoxins C and D, but only enterotoxin D was detected in the food. Subsequent tests have shown that strains from three of the incidents, where enterotoxin A was detected in the food, also produce enterotoxin D. The amount of enterotoxin A or A and B in nine foods, based on 100 % recovery, varied from 0·02 to 0·09 μg./g.

Enterotoxin was not detected in 12 foods. Cultures from eight of these foods and from specimens of patients produced enterotoxins A or C. However, in two incidents where *Staph. aureus* was isolated at concentrations of 2×10^6/g. and from 9×10^6 to 9×10^9/g. (3 foods) none of the cultures produced enterotoxins A, B, C or D.

Table 1. *Detection of enterotoxin in foods implicated in staphylococcal food poisoning in England and Wales* (1969–1971)

Extraction method used (see text)	Incident	No. of persons ill / No. at risk	Place	Food	Count of *Staph. aureus*/g.	Enterotoxin in food: Type	Enterotoxin in food: Amount*(μg./g.)
1	1	1/1	Home	Prawns (canned)	40×10^6	A and B	A – 0·03 B – 0·03
	2	30/120	Dog show	Cold chicken	55×10^6	A	0·02
	3	21/350	Mental hospital	Cold chicken	150×10^6	A	0·04
	4	21/82	Convalescent home	Trifle	150×10^6	A	0·05
	5	38/48	Coach outing	Tongue and beef	200×10^6	A	0·04
	6	4/4	Hotel	Ham	250×10^6	A	0·05
	7	35/35	Coach outing	Ham	450×10^6	A	0·08
	8	2/2	Home	Cold chicken	200×10^6	D	†
	9	50/?	Canteen	Ham	1000×10^6	D	†
2	10	4/?	Restaurant	Prawns‡ (frozen)	6×10^6	A	0·02
	11	2/2	Home	Prawns (canned)	5×10^6	A	0·09
	12	7/7	Home	Veal, ham and egg pie	2000×10^6	D	†

* Based on 100% recovery.

† Not calculated because concentration of reference enterotoxin unknown.

‡ After thawing, the prawns were eaten several hours later in the form of a prawn cocktail.

Table 2. *Counts of* Staphylococcus aureus *in foods implicated in* 39 *incidents of staphylococcal food poisoning in England and Wales*

	Count of *Staph. aureus*/g.			
	$7{\cdot}5 \times 10^5 - 9{\cdot}9 \times 10^6$	$10^7 - 9{\cdot}9 \times 10^7$	$10^8 - 9{\cdot}9 \times 10^8$	$> 10^9$
No. of incidents	8	15	9	7
% of incidents	21	38	23	18

Counts of Staphylococcus aureus implicated in food poisoning

Table 2 summarizes the plate counts of *Staph. aureus* in foods implicated in 39 incidents where there was good clinical and bacteriological evidence that staphylococcal food poisoning had occurred. Counts varied between $7{\cdot}5 \times 10^5$ and 9×10^9/g. with a median value of 7×10^7/g.

DISCUSSION

Enterotoxin was detected in 12 foods from 20 incidents of staphylococcal food poisoning. However, the technique used on most occasions (method 1) to extract the toxin was not satisfactory because the final extract contained various soluble constituents of the food which interfered with the gel-diffusion test and was often viscid. Method 2 was more satisfactory in both respects and the final product could be concentrated to a smaller volume. Although method 2 is now used routinely in this laboratory for the extraction of enterotoxin from food a negative result in the gel-diffusion test cannot be interpreted as 'absence of enterotoxin'.

Most food samples submitted from outbreaks weighed *ca.* 50–60 g. However, in one incident where enterotoxin A was detected in the food (Table 1, incident 11) the sample tested weighed only 17 g.

Although human volunteers have been used for the detection of enterotoxin in food and in culture filtrates of *Staph. aureus*, little has been published on the amount of enterotoxin required to cause illness in man. However, such volunteer experiments have now been carried out in the U.S.A. and they indicate that the estimated illness dose (ID50) for adults fed highly purified enterotoxins A, B or C is between 0·14 and 0·19 μg./kg. (Dr D. A. Kautter, personal communication); thus for a man weighing 70 kg. the ID50 is about 10–13 μg. These results are similar to those of Raj & Bergdoll (1969) who reported that a dose of 20–25 μg. of pure enterotoxin B caused typical symptoms of staphylococcal food poisoning in three volunteers. In contrast, Bergdoll (1969, 1970) has reported that 1 μg. of enterotoxin A or less may cause illness in sensitive individuals. By assuming a direct correlation between growth and enterotoxin production and by disregarding any differences in abilities of culture media and certain foods to support the production of enterotoxin, Casman & Bennett (1965) estimated that the concentration of enterotoxin A in food from various outbreaks was from 0·01 to 0·4 μg./g.: thus if 100 g. of food was consumed the dose was from 1 to 4 μg. There is a wide variation in the sensi-

tivity of individuals to staphylococcal enterotoxin (Dack, 1956). Consideration of all the evidence available suggests that 1 μg. or less of enterotoxin may cause illness in sensitive individuals.

In the present work the total amount of enterotoxin A or A and B was estimated to be from 0·02 to 0·09 μg./g. of food. If we assume that 50–100 g. of food was consumed and that no enterotoxin was produced in the time-interval between consumption of the food and testing for toxin in the Food Hygiene Laboratory, then the dosage was from 1 to 9 μg. In all the incidents recorded in Table 1 the victims suffered symptoms typical of staphylococcal food poisoning, and several were admitted to hospital. In one incident (Table 1, no. 2), fourteen persons were admitted five of whom required intravenous fluid therapy.

Recent reports indicate that the biosynthesis of enterotoxin A may be quite distinct from that of enterotoxin B. Enterotoxin A production occurs mainly during the exponential phase of growth (Markus & Silverman, 1970). In contrast, 95% of enterotoxin B is synthesized and released during the late exponential and early stationary phases of growth (Markus & Silverman, 1969; Morse, Mah & Dobrogosz, 1969). These results may be one of the reasons why enterotoxin B is implicated infrequently in staphylococcal food poisoning.

Except for data by Hobbs (1955) and Casman & Bennett (1965), little information has been published on the numbers of staphylococci present in foods implicated in outbreaks of staphylococcal food poisoning. The results shown in Table 2 indicate that large numbers of staphylococci, usually > 1 million/g., must be present before there is a sufficient concentration of enterotoxin to cause symptoms. During the time-interval between consumption of contaminated food and investigation of the incident, the numbers of staphylococci in the food may have increased or decreased. Nevertheless, we believe that the counts obtained are of importance as well as the phage-typing results and will continue to be so until procedures for the detection of staphylococcal enterotoxin in food are simplified.

We are indebted to Professor M. S. Bergdoll, Food Research Institute, University of Wisconsin, U.S.A., for providing enterotoxins A, B and C and their antisera, and to the late Dr E. P. Casman, Food and Drug Administration, Washington D.C., U.S.A., for providing enterotoxin D and its antiserum. We are also grateful to the many Directors of Public Health Laboratories for sending us the food samples and other information and to Dr Betty C. Hobbs for her advice and encouragement.

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Toxoplasma antibodies in the sera of immigrants to the United Kingdom from Asia and East Africa

By J. S. DODGE

Department of Preventive and Social Medicine, Medical School, University of Otago, Dunedin, New Zealand

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SUMMARY

Sera from 203 Asian immigrants to the United Kingdom were examined for toxoplasma antibodies. The antibody titres were not significantly different from those found in the indigenous population although there was some indication that a higher domestic animal–human contact in Asia was associated with a higher positivity rate.

INTRODUCTION

Ludlam, Wong & Field (1969) noted the low positivity rate for toxoplasma antibodies in sera from Hong Kong, and quoted papers indicating a low rate generally in the Far East. Blood samples were being collected from Asian immigrants (Indians and Pakistanis) who were attending a health screening clinic in Bradford, Yorkshire. A series of serum samples from this group were examined for toxoplasma antibodies by the Sabin–Fieldman dye test. The patients whose sera were so examined did not differ from the patients not tested for those antibodies; the group examined in this series comprised two separate consecutive groups of patients, each of about 100, attending the screening clinic.

RESULTS

A total of 203 sera were examined by the Sabin–Fieldman dye test, 123 from men and 80 from women. Results are shown in Table 1. Twenty-nine of the men (23·6 %) and 26 of the women (32·5 %) gave a positive titre of over 1/8. Two young men aged between 16–25 years had a positive titre of 1/512. Four women aged between 16 and 35 had a positive titre of 1/512 or over; two of them had a titre of 1/1024 and one titre of 1/2048. No clinical details of these four women are to hand.

Although there is no significant difference between the sexes in the total results for all ages, there is a significant difference in the 26–45 age group where there is a higher rate in women. If the natural sources of the infection are domestic animals, this difference is probably the result of a closer human–domestic animal contact in the housewife as compared to the male member of the household. There was a higher incidence of positive reactions in men who had been farmers before migration compared to those who had been in other occupations. (27 % compared to 20 %): this difference was not large enough to be significant at the 0·05 % level ($P = 0{\cdot}3$).

Table 1. *Asian immigrants: examination of sera for toxoplasma antibodies*

(Percentages given in parentheses.)

Age	Sex	No. examined	Negative	Positive in titre: 1/8 and above	1/32 and above	1/128 and above
11–15	M	8	5 (62·5)	3 (37·5)	2 (25·0)	1 (12·5)
	F	1	1 (100·0)	0 (–)	0 (—)	0 (—)
	Total	9	6 (66·7)	3 (33·3)	2 (22·2)	1 (11·1)
16–25	M	43	36 (83·7)	7 (16·3)	5 (11·6)	4 (9·3)
	F	36	28 (77·8)	8 (22·2)	7 (19·4)	6 (16·7)
	Total	79	64 (81·0)	15 (19·0)	12 (15·2)	10 (12·7)
26–45	M	53	41 (77·4)	12 (22·6)	7 (13·2)	2 (3·8)
	F	34	19 (55·9)	15 (44·1)	9 (26·5)	5 (14·7)
	Total	87	60 (69·0)	27 (31·0)	16 (18·4)	7 (8·0)
46+	M	19	12 (63·2)	7 (36·8)	5 (26·3)	2 (10·5)
	F	9	6 (66·7)	3 (33·3)	2 (22·2)	0 (–)
	Total	28	18 (64·3)	10 (35·7)	7 (25·0)	2 (7·1)
Total	M	123	94 (76·4)	29 (23·6)	19 (15·4)	9 (7·3)
	F	80	54 (67·5)	26 (32·5)	18 (22·5)	11 (13·7)
	Total	203	148 (72·9)	55 (27·1)	37 (18·2)	20 (9·9)

Table 2. *Asian immigrants: both sexes; toxoplasma antibody rates according to country of last residence*

(Percentages given in parentheses.)

Country of last residence	No. examined	No. positive at a titre of: 1/8 and above	1/16 and above
Azad Kashmir	68	20 (29·4)	15 (22·1)
West Pakistan	38	4 (10·5)	4 (10·5)
East Pakistan	22	6 (27·3)	5 (22·7)
India	57	15 (26·3)	13 (22·8)
East Africa	17	10 (58·8)	9 (52·9)
Other	1	0	0
Total	203	55 (27·1)	46 (22·7)

Table 2 shows the variation in the antibody rates when grouped according to the immigrants' country of origin. There is some difference between these rates, and the rate in those from East Africa is significantly higher than the combined rate in those from the Indian sub-continent. This difference is not significantly affected by differences in the age/sex distribution in these groups.

In Britain 21 % of blood donors aged 21–30 gave a positive titre of 1/16 or more; in the age group 31–40 the figure is 30 %, and for 41–50, 40 % (Fleck 1969). The rates from this group of immigrants examined are illustrated in Fig. 1, grouped to compare with the U.K. rates: they are somewhat lower than the latter rates but are within the range of rates found in nine areas in England and Wales by Fleck (1969).

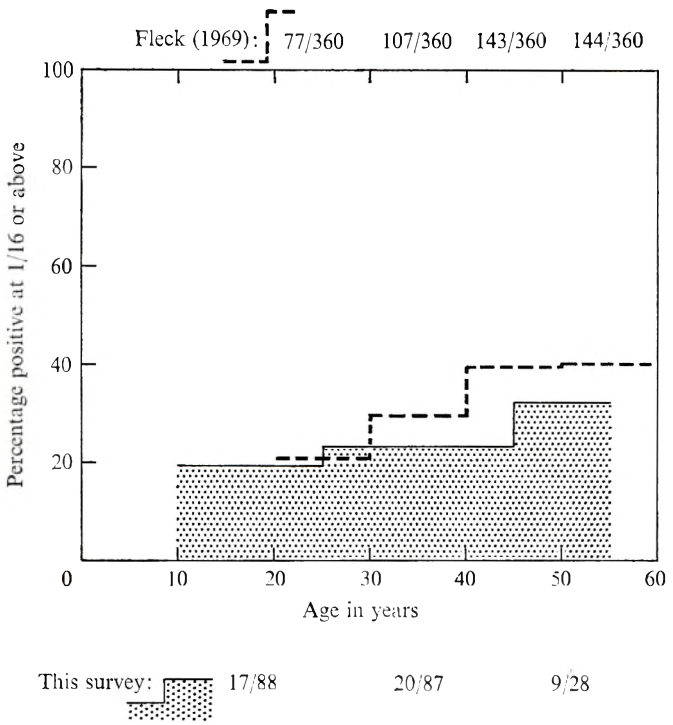

Fig. 1. Dye test on sera from Asian immigrants in Bradford, England.

It is considered that the results obtained in this survey indicate that toxoplasmosis would not be a significant imported disease in immigrants of Asian origin from India, Pakistan or East Africa and that no special examination of this group of the population is required in this respect.

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The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses

By D. HOBSON and R. L. CURRY
Department of Medical Microbiology, University of Liverpool
A. S. BEARE
M.R.C. Common Cold Research Unit, Salisbury
and A. WARD-GARDNER
Medical Department, Esso Refinery, Fawley

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SUMMARY

The intranasal inoculation of volunteers with living partially attenuated strains of influenza A and B viruses offers a new opportunity to determine the protective effect of serum haemagglutin-inhibiting antibody against a strictly homologous virus, under conditions where the time and dosage of the infective challenge can be controlled, the scoring of proven infections can be more precise and higher rates of infection can be achieved than in most natural epidemics.

In 1032 adult volunteers, whose serum HI antibody titre was determined immediately before virus challenge, there was a consistent inverse quantitative relationship between the HI titre and the likelihood of infection. The PD 50 (50 % protective dose) of HI antibody was 1/18–1/36, but an unusual finding was that volunteers with no detectable pre-challenge antibody often seem to be less susceptible to infection than those with pre-challenge antibody in low titre.

In one group of volunteers challenged with an influenza B strain there was no evidence that pre-challenge antibody titres against viral neuraminidase had any significant protective effect against challenge infection.

INTRODUCTION

Within recent years it has become increasingly apparent that infection or vaccination of man with influenza viruses can induce quite separate antibodies against two distinct virus proteins, the haemagglutinin (HA) and neuraminidase (Nase), and that antibodies of each specificity may be found both in the IgG class of immunoglobulins in the bloodstream and in the IgA class in nasal secretions. The frequency of induction, the titres and the duration of each type of antibody in each location can vary quite independently (Schild & Newman, 1969; Downie, 1970). It is still not clear which of these antibodies or which combination of antibodies is essential for protection against natural infective risk in man. In recent small-scale volunteer studies it has been shown that nasal

antibodies against the HA antigen (Downie & Stuart-Harris, 1970) and serum antibody against the Nase antigen (Slepushkin *et al.* 1971) may play some part in protection against experimental challenge infection with influenza viruses, whereas for the past 20 years it has been customary to regard serum antibody against the HA antigen as the essential protective agent, and in fact all commercially available influenza vaccines are designed and assessed on their ability to produce high sustained titres of haemagglutinin-inhibiting (HI) antibody.

It can readily be shown in the laboratory that the HI titre of a serum corresponds closely with its neutralizing activity against the infectivity of the homologous virus for chick embryo or tissue cultures (Hoyle, 1968). Nevertheless, surprisingly conflicting data have been reported on the protective effect of circulating HI antibody in man (e.g. Bashe, Stegmuller, Leonida & Greenwald, 1964; Hoyle, 1968; Maynard *et al.* 1968). Many of the discrepancies are undoubtedly due to the inherent difficulty of studying small groups of people exposed to natural infective risk. The time of onset of an epidemic after the HI titres of a population have been recorded is unpredictable; the epidemic strain of virus may show antigenic drift from the strain used for HI titrations; rates of infection vary from epidemic to epidemic, and are often below 5%; the exact virus challenge dose cannot be calculated, but must certainly be extremely variable from person to person.

Since serum HI antibody titrations are by far the simplest and still the most widely used method for epidemiological surveys and for estimating the efficacy of influenza vaccines in man, it seemed worth while to re-examine the relationship between HI titres and protection against influenza in a large number of adult volunteers, under conditions where the exact timing and dosage of infection by a strictly homologous virus strain could be carefully controlled, and where the total score of infections could be expected to be much higher and more easily proven than under field conditions in natural outbreaks.

In various clinical trials under the auspices of the Medical Research Council over 1000 volunteers have been given a deliberate intranasal challenge with known laboratory strains of influenza A and B virus. The rate of infection has been assessed by increases in the titre of homologous serum HI antibody and also, in several trials, by virus excretion studies after the challenge virus inoculation and by questionnaires about clinical signs and symptoms. The immediate purpose of these trials was to compare the infectivity of various virus strains, or to assess the efficiency of various influenza virus vaccines.

It now seems that a more detailed survey of previously unanalysed data from the whole series of trials would provide a unique opportunity to examine on a numerically larger scale than hitherto whether serum HI antibodies can still be regarded as a useful measure of the protection of a community against influenza, and would provide useful background data against which to evaluate recent claims that protection against influenza depends upon other antibodies of different specificity or distribution.

MATERIALS AND METHODS

Clinical trial procedures

The present data are derived from two types of trial: (*a*) challenge experiments in small groups of segregated volunteers in the Common Cold Unit, Salisbury, of the pattern previously described by Tyrrell (1963) and Beare *et al.* (1971); (*b*) larger-scale field trials in industrial workers who were divided randomly into groups some of which received various live or killed influenza vaccines, whilst others were given placebo materials or were left unvaccinated (see Beare *et al.* 1968, 1969). The challenge infections analysed in the present paper were instituted 2–3 weeks after any schedule of vaccination was completed. Serum samples were taken before and 2 weeks after inoculation of the challenge virus, and nasal swabs were taken for virus isolation studies wherever possible 48 hr. after challenge.

The challenge infection was with chick embryo-grown pools of either B/Eng/13/65, or with the pre-Hong Kong A2 influenza virus strains A2/Leningrad/4/62 or A2/Eng/501/68, or with A2/Hong Kong/1/68 or strains related to it, e.g. A2/Eng/878/68. A small number of the Salisbury volunteers were inoculated with genetic recombinant strains of influenza A containing both the HA and Nase antigens of Hong Kong virus (Beare *et al.* 1971). The infective challenge dose was administered by nasal spray or droplets in doses of 10^5–10^6 EID 50 per volunteer, as described previously (Beare *et al.* 1969). A positive challenge was scored in any volunteer who showed a fourfold or greater rise in serum HI titre against the homologous virus, or from whom virus was isolated in chick embryos or in monkey kidney tissue culture.

Serological procedures

HI antibody was titrated in cholera filtrate-treated sera by the World Health Organization (1953) technique, with 4 HA units of virus incubated 1 hr. at room temperature with serum dilutions before adding 0·5 % fowl erythrocytes.

Serum antineuraminidase (NI) antibody was investigated in only one group of volunteers challenged with B/Eng/13/65. The Nase assay was the modification of Warren's method described by Webster & Laver (1967) using fetuin as the substrate. Assays of NI antibody were similar to those described by Schild & Newman (1969) but with the following modifications; (*a*) the time of interaction of Nase and fetuin was 18 hr. at 37° C.; (*b*) viral Nase and serum were incubated for 1 hr. at 37° C. during enzyme inhibition; (*c*) the source of Nase was B/Lee which had been treated by 0·5 % sodium dodecyl sulphate (Laver, 1963) to disrupt the virion and separate the HA and Nase components. This procedure was necessary to ensure that HI antibody would not produce non-specific inhibition of the enzyme by steric hindrance, as may happen on the intact virus particle (Easterday, Laver, Pereira & Schild, 1969). Titres of NI antibody were expressed as that dilution of the volunteer's serum which reduced the activity of neuraminidase to 50 % of its value in controls with normal rabbit serum.

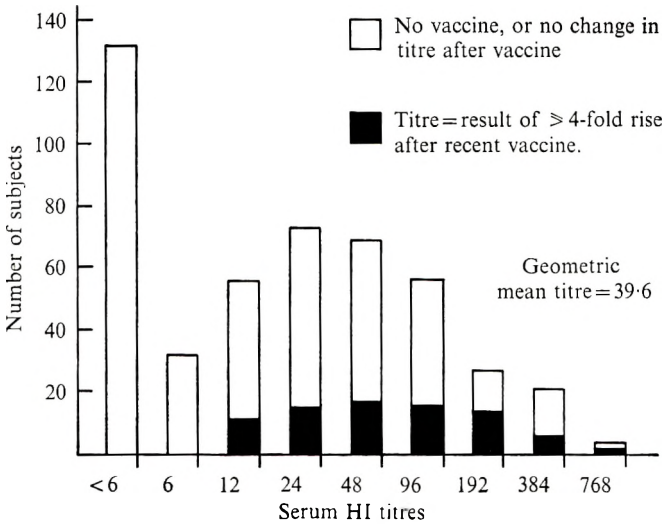

Fig. 1. The distribution of serum HI antibody titres to B/Eng/13/65 virus in 462 volunteers before homologous challenge infection.

RESULTS

I. *Influenza B trials*

The results of trials in three large industrial groups were closely similar and have been combined. The distribution of homologous HI serum antibody titres in 462 volunteers immediately before challenge infection are shown in Fig. 1. About 29 % of the volunteers were without detectable HI antibody and the rest showed a Gaussian distribution of titres, with a geometric mean value of 1/39·6. Most of the volunteers had not previously been given vaccine, and their titres presumably represent residual antibody from natural infection in earlier years. However, as shown in Fig. 1, in about 17 % of volunteers the antibody titre at the time of challenge was the result of a fourfold or greater increase due to recent vaccination. This vaccine-enhanced group will later be discussed separately, because of the possibility that newly induced HI antibody might be of a different class or avidity from 'old' antibody, and because a recent antigenic stimulus might have resulted in antibodies, additional to serum HI antibody, which might not have persisted in the main group with 'old' HI antibody.

The number of infections in persons with no or low titres of antibody was greater than in those with high HI titres (Fig. 2), but it was difficult to see whether there was any consistent trend, because of the varying totals of people at each particular titre. When the results were recalculated (Fig. 3) in terms of the infection rate per cent of volunteers at each pre-vaccination antibody titre it was apparent that the likelihood of infection diminished progressively with increasing antibody titre. The PD50 of HI antibody (i.e. the titre at which the infection rate is reduced to half the maximum observed rate) was approximately 1/18, though it may be noted for later discussion that the infection rate in those with no detectable antibody was in fact very little higher than in those with the lowest detectable titre (1/6). When the group of people with 'old' antibody were considered separately from

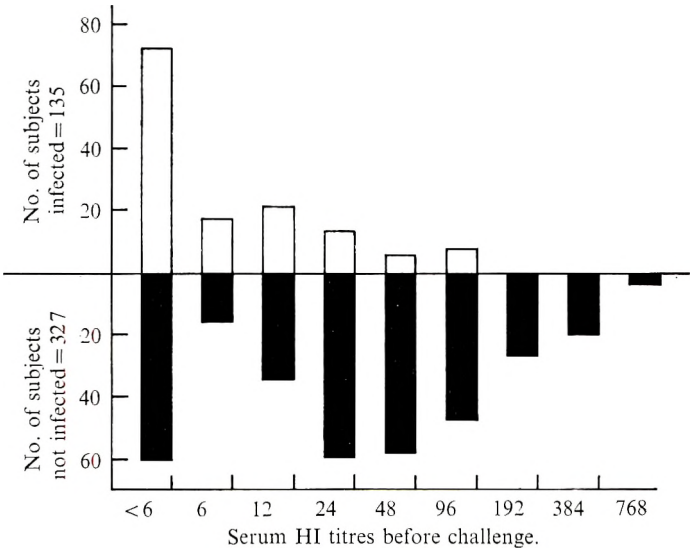

Fig. 2. The incidence of infection after challenge with B/Eng/13/65 virus in volunteers with differing pre-challenge titres of serum HI antibody

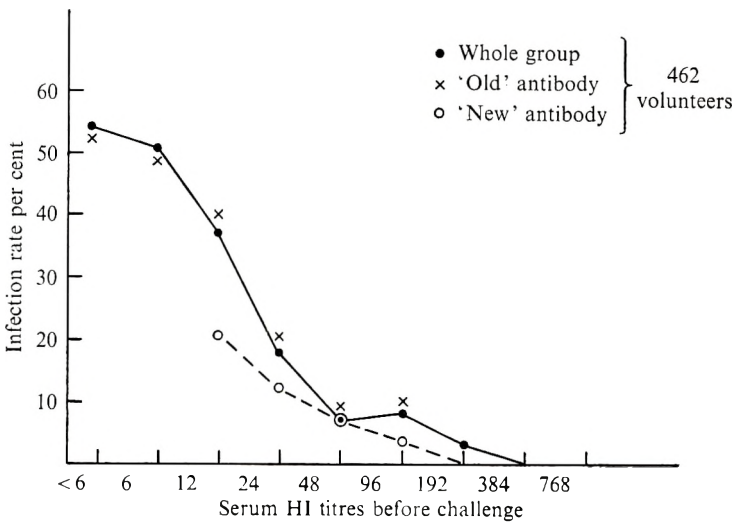

Fig. 3. The infection rates of volunteers challenged with B/Eng/13/65 virus, in relation to their pre-challenge titres of serum HI antibody.

those with 'new' antibody it appeared that both types of antibody, titre for titre, indicate similar degrees of protection against challenge.

The results suggest that serum HI antibody confers protection, or alternatively that it is a good indirect quantitative indicator of some other factor such as NI antibody which is itself protective.

In one group of industrial volunteers the distribution of antibody against the neuraminidase of influenza type B viruses was compared with that of HI antibody to B/Eng/13/65. There was little or no correlation between HI and NI titres in individual sera, and many of those without detectable HI antibody against

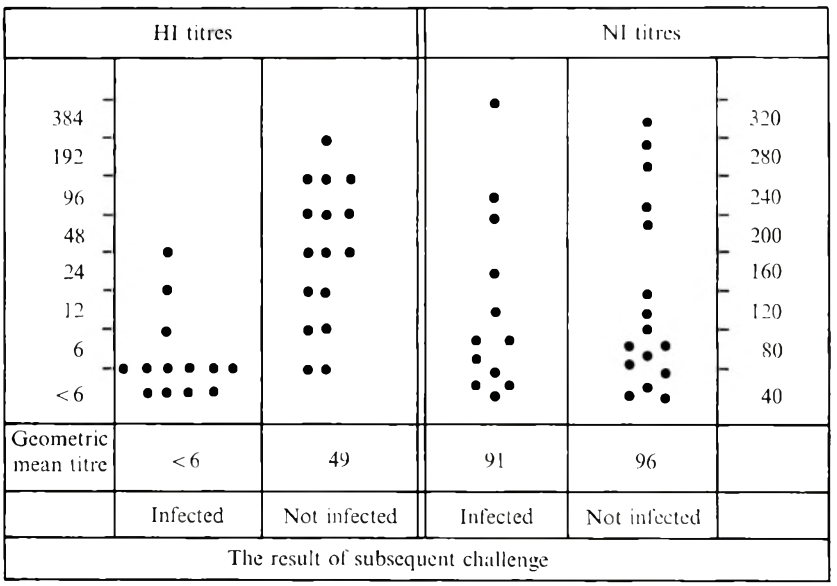

Fig. 4. The distribution of HI and NI serum antibodies in 29 volunteers before challenge infection with B/Eng/13/65 virus.

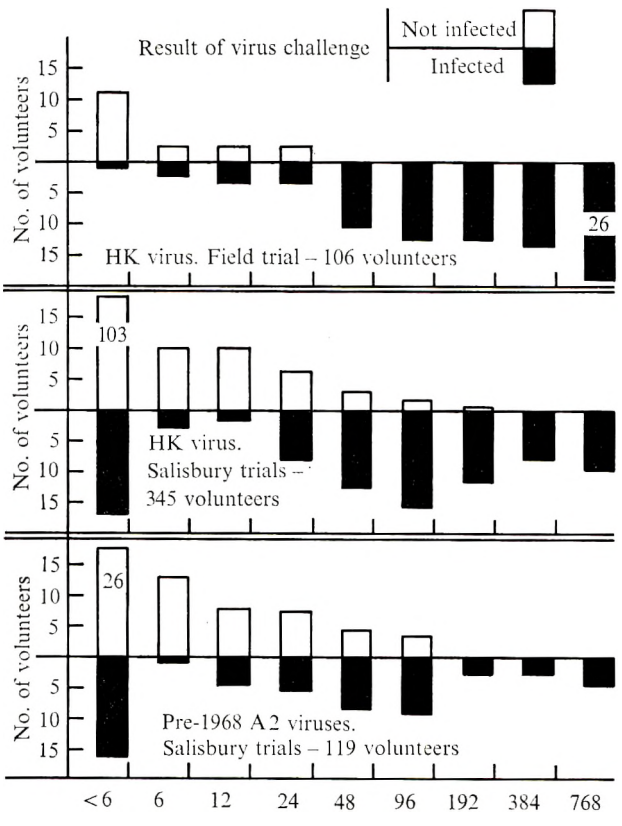

Fig. 5. The effect of challenge infection with A2 viruses in volunteers with differing pre-challenge titres of homologous serum HI antibody.

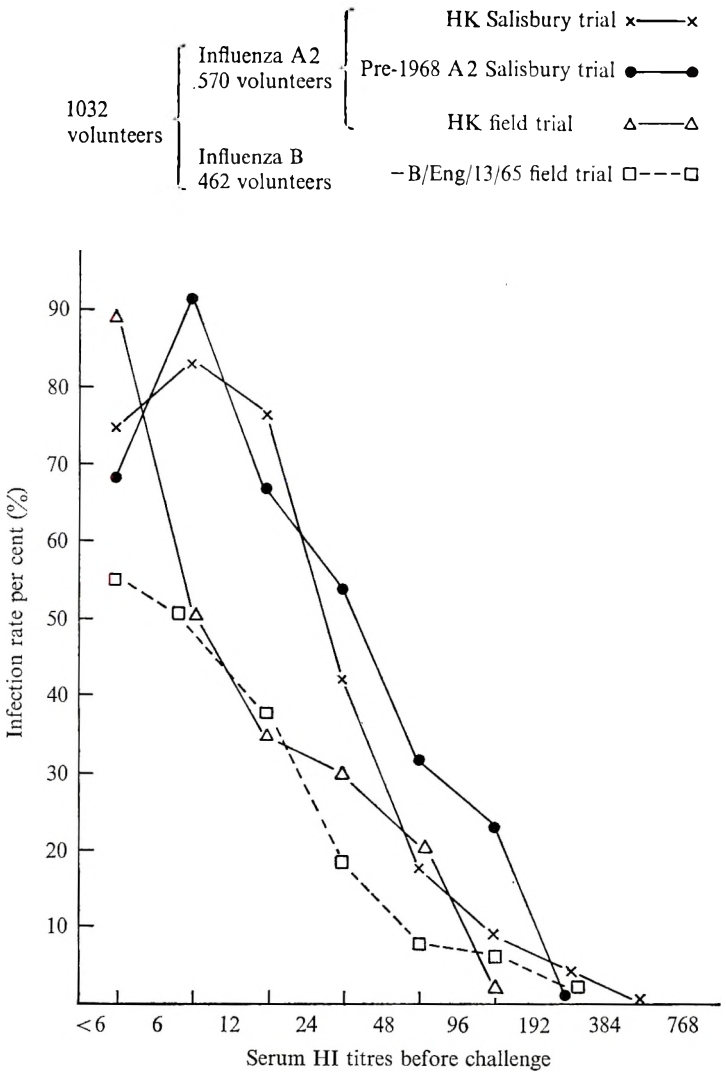

Fig. 6. The infection rate of volunteers challenged with various influenza viruses, in relation to their pre-challenge titres of homologous serum HI antibody.

B/Eng/13/65 had substantial NI activity against the type-common enzyme. It is probable that repeated previous natural infection of these adult volunteers with a variety of foregoing type B viruses had maintained antibody to the common enzyme, without inducing HI antibody to the distinctive HA antigen of the more recent B/Eng/13/65. The serum NI titre was not apparently correlated with protection against challenge infection in this small group of volunteers (Fig. 4), whereas HI titres were lower in those who became infected after challenge than in those who did not.

II. *Influenza A trials*

A total of 570 volunteers were studied, comprising two industrial populations challenged with A2/Hong Kong/1/68 and Salisbury volunteers challenged with either pre-Hong Kong or Hong Kong-like strains of influenza A2 virus. The dis-

tribution of homologous HI titres immediately before challenge is shown in Fig. 5, and the infection rate per cent of volunteers in terms of their initial antibody level is shown separately for each class of trial in Fig. 6. Only one group of industrial volunteers had previously received influenza vaccines, and the small number with a significant induction of fresh antibody before challenge is too small to merit separate discussion.

An unexpected anomalous finding in two of the three groups shown in Fig. 6 was that the rate of infection in those with no detectable antibody was noticeably less than in those with antibody at the lowest detectable level (1/6). However, in those subjects with antibody before challenge there was a progressive fall in infection rate with increasing serum HI titres, as seen earlier in the influenza B trials. The PD50 of serum HI antibody again appeared to be low, of the order of 1/18–1/36, both with the Hong Kong-like viruses against which antibodies could only have been induced in the preceding twelve months or so, and with the earlier A2 strains with which the community had had repeated immunogenic experiences over several years.

DISCUSSION

The results of challenge infection of 1032 adult volunteers with influenza A2 or B viruses show that the distribution of homologous HI antibodies in the community immediately before challenge reflects the distribution of subsequent infections closely and consistently. However, observational data of this type can obviously not confirm that a quantitative relationship between antibody and resistance to infection is a causal relationship. In many cases the HI antibody is only part of a wider immune response to each of the distinct antigens of the influenza virus, and in the short term serum NI antibody and nasal NI and HI antibodies may persist alongside the more readily measured serum HI antibody. The apparent lack of correlation between HI and NI antibodies in the sera of one of the present groups is of interest, and the evidence suggests that the former is more closely correlated with infection than the latter. It is possible that NI antibody is of less importance in protection against influenza B virus infections than against influenza A as has been suggested by Slepushkin *et al.* (1971), but a much larger scale survey than the present study would be required to evaluate the inter-relationship of the two antibodies in protection against influenza.

The low titre of serum HI antibody which appears to give significant protection against infection (i.e. the PD50 titre) may suggest that other protective factors are also involved, but it is possible that the low PD50 merely reflects the essential requirement of the present trials that the challenge virus strains should be selected chick embryo-grown variants of somewhat attenuated virulence, likely to produce less persistent or clinically severe infections than parental wild-type strains. It is thus not possible to extrapolate from the present experimental procedures to forecast that similar low titres of serum HI antibody would protect against natural infection with highly virulent epidemic strains of virus. However, it is of interest that Meiklejohn, Kempe, Thaiman & Lennette (1952) calculated that in a natural influenza A1 epidemic in servicemen the percentage attack rate

in those with pre-epidemic homologous HI antibody titres of 1/16 was only 1·5% as compared with 18% in those without detectable antibody.

The low PD50 of antibody shown in the present trial suggests that the development of vaccines capable of inducing very high HI titres of 1/1000 or greater, which has been the aim of manufacturers in the past, may not be necessary as far as short-term protection is concerned, and may only be required, if at all, to ensure that the decline to subeffective titres after vaccination is postponed as long as possible. However, it is possible that naturally acquired serum HI antibody, as in the majority of the present volunteers, may be quantitatively different from that induced by vaccine or that the ratio of NI antibody and nasal antibodies to serum HI antibody is greater after natural infection than after vaccination.

An anomalous finding in the present study was the apparently lower susceptibility to challenge infection in those without serum HI antibody than in those with antibody at the minimum detectable level of 1/6. Further larger-scale investigations would be necessary to show whether this is merely a chance finding due to small numbers, or is peculiar to these particular attenuated virus strains. However, similar discrepancies have been found in field trials with live influenza vaccines (McDonald, Zuckermann, Beare & Tyrrell, 1962). A simple explanation could be that those without detectable antibody are a heterogeneous group comprising those who have never been infected and thus have no relevant clone of committed lymphocytes, together with those who have been immunized previously, but in whom the rate of production of specific immunoglobulins has fallen to levels undetectable in the HI test. In this case, it might be expected that the latter subgroup would respond to infection like those already showing antibody, i.e. by a rapid immunological recall and production of readily detectable titres of new antibody, whereas those in the truly unsensitized subgroup who become infected would show only a primary immune response, of insufficient size to be detected in the present test. There would thus be a difference in the precision of scoring the infection rates in the two subgroups. An earlier trial (Hobson, Lane, Beare & Chivers, 1964) with adjuvant inactivated influenza vaccine showed that the immunological response, in terms of the HI test, of a group of people who were all devoid of pre-vaccine HI antibody was greater in those with previous sensitization, detectable by a more sensitive tissue culture neutralization test, than in those who were absolutely inexperienced.

However, the limited virus isolation studies in the present trials suggest that antibody-negative volunteers show not merely a deficient response to infection, but a reduced rate of infection. Thus, the intriguing possibility exists that the fraction of the population which is found to be apparently unimmunized several years after a particular subtype of influenza has become prevalent in the community may be a naturally selected 'resistant' group rather than a random collection of people who have merely escaped influenza by chance.

It is well known that resistance to other human infective diseases, e.g. tuberculosis, may be determined by many genotypic and phenotypic factors, and in influenza itself it has been suggested (McDonald & Zuckerman, 1962; Potter, 1969) that the susceptibility of people with Blood Group A is lower than those of

Group O constitution. Similarly, there are many instances of genetically controlled differences of mice to many microbial infections (Allison, 1965), including influenza (Lindenmann, Lane & Hobson, 1963).

It would be of interest in future epidemiological surveys to determine whether those who continue to escape influenza in natural epidemics, despite an apparent lack of serum HI antibody, are different from the general population in certain specific ways. It is possible that this group may be incapable of making serum HI antibody whilst remaining immunologically competent to make antibody to viral neuraminidase, but it is equally possible that their relative resistance to infection may be due to non-immunological factors, such as a greater facility for interferon production or inhibitor-secretory activity, or because they may develop some form of cell-mediated immunity which may be more closely correlated with resistance than is their serological status.

It is a pleasure to express our gratitude to Professor Sir Charles Stuart-Harris, Dr D. A. J. Tyrrell and other members of the Influenza and other Respiratory Virus Vaccines Committee of the Medical Research Council for facilitating these studies and for their advice on their presentation.

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Identification of the immunoglobulin class active in the Rose Bengal plate test for bovine brucellosis

By M. J. CORBEL

Ministry of Agriculture, Fisheries and Food,
Central Veterinary Laboratory, Weybridge, Surrey

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SUMMARY

The antibodies active in the Rose Bengal plate test (RBPT) for bovine brucellosis have been studied. The results of fractionation experiments showed that RBPT activity was associated with fractions containing immunoglobulin of the IgG_1 class; other immunoglobulin classes were inactive in this respect although active in other tests. These results were confirmed by inhibition tests with specific antisera and by elution of the antibody from agglutinated RBPT antigen.

The major proportion of the serum complement-fixing activity was also present in the IgG_1 fraction and it is suggested that the RBPT and CF reactions are probably mediated by the same antibodies.

INTRODUCTION

The serum agglutination (SA) test using standardized antigen has been used extensively for the diagnosis of brucellosis in man and domestic animals. However, it suffers from the disadvantage that false positive reactions sometimes occur. These may be the result of cross-reactions between antigens of *Brucella* spp and unrelated organisms, for example *Yersinia enterocolitica* (Ahvonen, Jansson & Aho, 1969; Corbel & Cullen, 1970) or they may result from the presence of non-specific agglutinins distinct from antibodies, which are present in certain bovine sera (Hess, 1953*a*, *b*). In an attempt to differentiate specific brucella agglutinins from these non-specific factors, Rose & Roepke (1957) introduced a modification of the plate-agglutination test in which the antigen was buffered at pH 4·0 immediately before use. They observed that at this pH, agglutination of *Br. abortus* cells by the non-specific agglutinins of bovine serum was inhibited, whereas the activity of specific brucella antibodies was largely unaffected. Subsequent experience showed that the acid plate-agglutination test was of value as a supplemental test in screening field samples of serum for brucella-specific agglutinins (Lambert & Amerault, 1962*a*, *b*).

More recently, a modification of the acid plate-agglutination test, employing a suspension of *Br. abortus* cells stained with Rose Bengal dye and buffered at pH 3·65 was introduced by the United States Department of Agriculture, National Animal Diseases Laboratory, as the basis of a card test for bovine brucellosis. In field trials this test was found to be a more accurate indicator of infection than the SAT (Nicoletti, 1967).

Using the Rose Bengal antigen prepared according to USDA procedures as the basis of a plate test for examining serum samples, Morgan (1969) and Morgan, MacKinnon, Lawson & Cullen (1969) obtained results which correlated well with those of the complement-fixation (CF) test. Comparison of the results of the combined CF and SA tests, interpreted as laid down for the Brucellosis (Accredited Herds) Scheme, with those of the Rose Bengal plate test (RBPT) showed agreement for 90·8 % of sera. Later results (Davies, 1971) showed agreement for 97 % of sera. At present the RBPT is in routine use for screening field samples of cattle sera in connexion with the Brucellosis Incentives Scheme.

However, although the relationship between the results of the CF test and the RBPT suggested that similar antibodies may be involved in both tests, little specific information was available on this point. This study was undertaken, therefore, with the object of characterizing the antibodies involved in the RBPT and determining their relation to the results of other diagnostic tests for brucellosis.

MATERIALS AND METHODS

Sera used

Bovine sera. High titre serum was collected from cattle infected with virulent field strains of *Br. abortus*. For fractionation purposes batches of serum from three different animals were pooled. Serum was also obtained from cattle vaccinated with *Br. abortus* Strain 19 vaccine. Samples were collected at various intervals after vaccination.

Brucella-negative serum was obtained from bullocks kept under Brucella-free conditions.

All serum samples were membrane filtered and stored at − 20° C. until required.

Rabbit antisera. Rabbit antiserum to bovine serum proteins was produced by repeated subcutaneous and intramuscular injection of New Zealand white rabbits with bovine serum emulsified in Freund's complete adjuvant. The animals were exsanguinated after a course of injections extending over 4 months. Rabbit antiserum to bovine γ-globulin was produced by a similar process except that γ-globulin fractions prepared according to Keckwick (1940) were used as antigen.

Class-specific antisera to IgG_1, IgG_2 and IgM were prepared essentially according to Porter & Noakes (1970). They were rendered specific by absorption with foetal calf serum and heterologous immunoglobulin.

Serological tests

The procedures used for the SAT, Coombs antiglobulin, RBPT, quantitative RBPT (QRBPT) and immunodiffusion tests have been described or referred to previously (Corbel & Cullen, 1970). CF tests were done according to MacKinnon (1963). Rivanol and heat inactivation tests were performed as described by Morgan (1967).

Disulphide bond reduction tests

For the test, 0·2 ml. of serum was incubated with an equal volume of 0·02 M dithiothreitol (DTT; British Drug Houses, Poole) in phosphate buffered saline (PBS; 0·15 M-NaCl in 0·01 M phosphate pH 7·5) at 37° C. for 30 min. A volume of

0·6 ml. of 0·15 M-NaCl was then added to the mixture and serial doubling dilutions made in the same diluent using a unit volume of 0·5 ml. After addition of an equal volume of standard *Br. abortus* agglutination suspension to each tube, the tests were incubated and read as for the SA test (WHO Monograph 19, 1953).

Immunoglobulin inhibiton tests

Specific immunoglobulins were removed from bovine serum or serum fractions by addition of 2 vol. of class-specific antiglobulin serum followed by incubation for 16 hr. at 4° C. Precipitates were removed by centrifugation at 20,000*g* for 30 min. and the samples examined by immunoelectrophoresis. If necessary the process was repeated until the specific precipitin arc could no longer be detected.

Serum fractionation procedures

Density gradient ultracentrifugation. A modification of the method of Cowan & Trautman (1965) was used. Gradients were formed in 15 ml. polycarbonate centrifuge tubes (MSE, London) by layering 3 ml. volumes of 65 % saturated $NaNO_3$ over 5 ml. volumes of 75 % saturated KBr. Samples of 2 ml. of serum were layered above the gradient and residual space was filled with liquid paraffin. Centrifugation was performed at an average relative centrifugal force of 90,000*g* for 24 hr. at 12° C. Fractions of 0·5 ml. volume were collected by aspiration. These were freed of gradient salt by dialysis against PBS and concentrated by ultra-filtration (Selecta Ultra-thimbles; Schleicher u. Schull, Germany).

Gel filtration. Essentially the method of Flodin & Killander (1962) was used. A column of Sephadex G200 (Pharmacia, Uppsala) 850 mm. × 25 mm. diameter, equilibrated with Tris (hydroxymethyl amino methane)–HCl buffer (Tris buffer; 1 M-NaCl; 0·1 M Tris-HCl, pH 8·0; NaN_3 0·01 M) was used. Samples of up to 5 ml. volume of serum were applied and eluted by upward development with the equilibrating buffer at a flow rate of 25 ml. per hour. Effluent was monitored for absorption of ultraviolet light at 280 nm. wavelength by a Uvicord II detector (LKB Produkter, Bromma) and fractions of 5 ml. volume collected automatically by an Ultro-Rac fraction collector (LKB Produkter, Bromma). Before testing, fractions were dialysed and concentrated as described for density gradient centrifugation.

Ion-exchange chromatography

This was performed on 300 mm. × 10 mm. columns of QAE-Sephadex-A50 (Pharmacia, Uppsala) equilibrated with 0·1 M phosphate buffer, pH 7·0. Volumes of 2·0 ml. of serum dialysed to equilibrium with the starting buffer were applied to the column and fractions eluted sequentially with 0·1 M, 0·2 M, 0·3 M, 0·4 M and 1·0 M-NaCl buffered at pH 7·0 with 0·01 M phosphate. Fractions were treated as described for gel filtration.

Preparative zone electrophoresis

This was done using 200 mm. × 50 mm. × 5 mm. blocks of cellulose acetate gel (Cellogel; Chemetron, Milan) equilibrated with barbital buffer, 0·05 *I*, pH 8·6. The procedure recommended by the manufacturers was followed throughout.

Volumes of 0·2 ml. of serum were applied to each block. After electrophoresis, blocks were sectioned tranversely into 10 mm. segments and fractions expressed from the gel and concentrated by ultra-filtration.

Immuno-adsorption and elution of antibody

Rose Bengal plate test antigen (prepared according to USDA, National Animal Diseases Laboratory, Diagnostic Reagents Manual 65c) was washed by two cycles of centrifugation in lactate buffer, pH 3·65, and one cycle of centrifugation in 0·1 M formate-HCl buffer, pH 1·0, and resuspended to its original volume in lactate buffer, pH 3·65.

Equal volumes of this suspension and pooled high titre bovine anti-*Br. abortus* serum were mixed and incubated at 4° C. for 16 hr. The agglutinated cells were sedimented by centrifugation at 2000*g* for 20 min. and the supernatant discarded. The sediment was resuspended in lactate buffer at pH 3·65 to the original volume and centrifuged again at 5000*g* for 20 min. The supernatant was discarded and the deposit resuspended in PBS and washed twice by centrifugation in this medium. Antibodies were eluted by resuspension of washed agglutinated cells in 0·15 M-NaCl buffered at pH 1·0 with 0·1 M formate-HCl buffer. After standing for 4 hr. at 4° C. the cells were removed by centrifugation at 15,000*g* for 30 min. The supernatant was neutralized by addition of NaOH and concentrated by ultra-filtration.

To check for possible non-specific adsorption of serum proteins an identical experiment was performed in parallel but using negatively reacting bovine serum.

Analytical methods

Immunoglobulin concentrations were measured by radial immunodiffusion (Mancini, Carbonara & Heremans, 1965) using antisera specific for bovine IgG_1, IgG_2 and IgM globulins.

Immunoelectrophoresis was performed essentially according to Scheidegger (1955). To identify unknown components, the interrupted trough method (Wieme, 1965) and a combination of immunoelectrophoresis and immunodiffusion were used. In the latter case immunoelectrophoresis of defined antigens was performed in the normal manner except that before addition of antiserum to the peripheral troughs an additional antigen well was cut at a distance of 10 mm. from the centre of the estimated position of the precipitin arc corresponding to the known component. This well was then filled with a sample of the unknown component, antiserum was added to the peripheral troughs and diffusion allowed to proceed in the usual manner. In some tests the positions of the defined and undefined samples were reversed.

Disk electrophoresis was performed according to Davis (1964). Thin-layer gel filtration on Sephadex G200 Superfine was done according to Morris (1964). Results were recorded by making imprints of the wet gel on cellulose acetate membrane and staining with 0·002 % nigrosin in 2 % acetic acid.

Estimations of total protein concentration were made spectrophotometrically according to Cullen & Corbel (1970) using pure bovine IgG_2 globulin as standard.

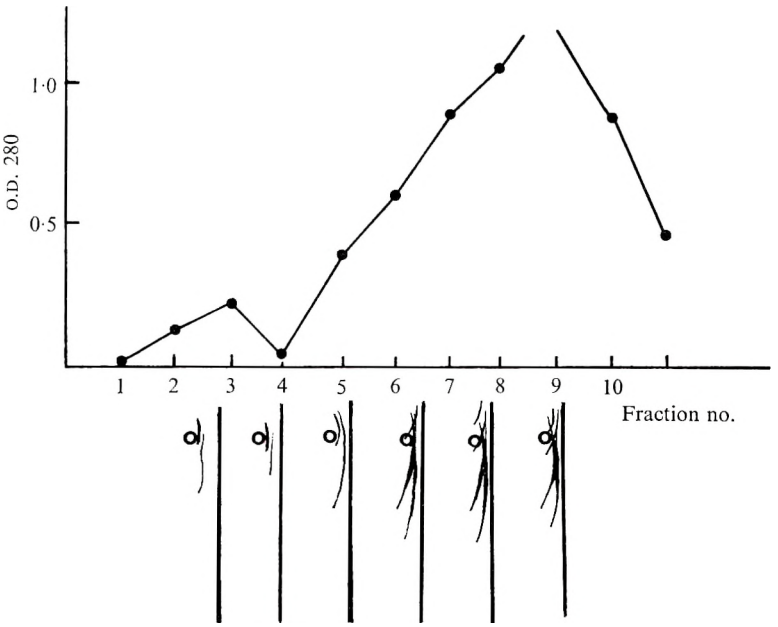

Fig. 1 Density gradient ultracentrifugation of bovine serum.

RESULTS

Fractionation of sera

Density gradient ultracentrifugation

Fractionation of high titre serum from infected cows produced the results shown in Fig. 1. The serological activities of the fractions are summarized in Table 1. Antibodies active in the SAT and antiglobulin tests were present in fast sedimenting and slowly sedimenting fractions and their distribution was related to that of the 19s and 7s immunoglobulins. The major part of the Coombs antibody was present in the 7s fractions. These also contained all detectable CF, RBPT and DTT-stable antibodies.

Fractionation of serum from cattle vaccinated with *Br. abortus* S19 vaccine produced results which varied with the interval after vaccination.

In the case of serum collected at 7 days after vaccination agglutinins were largely confined to the fast sedimenting fractions. Some Coombs antibody was however detected in both fast and slow fractions. No significant CF, RBPT or DTT-stable activity was detected in any fraction although the unfractionated serum had low titres of CF and RBPT antibodies (Table 1). When serum collected 22 days post-vaccination was fractionated by the same procedure the results obtained were very similar to those given by serum from infected animals. Again, agglutinins active in the SAT were present in both fast and slowly sedimenting fractions but the RBPT, CF and DTT-stable activities were confined to the slow fractions (Table 1). When serum collected from cattle vaccinated 3 years previously and having a low residual SAT and positive RBPT was fractionated in the same way, agglutinins were detected in fast sedimenting fractions only.

Table 1. *Serological activity of fractions separated from bovine antisera to* Br. abortus *by density gradient centrifugation*

Sample	Reciprocal titres				
	SAT	Coombs test	DTT test	QRBPT	CFT
Pooled high titre serum	2560	> 5120	1280	1024	4000
Fraction no.					
1	< 10	10	< 10	< 1	4
2	< 10	20	< 10	< 1	4
3	10	40	< 10	4	4
4	40	160	20	8	20
5	160	640	80	160	80
6	320	2560	320	160	400
7	640	2560	320	320	1000
8	640	2560	640	320	1000
9	320	2560	160	160	200
10	80	640	40	32	40
Serum 7 days after S19 vaccine	160	320	20	4	40
Fraction no.					
1	< 10	< 10	< 10	< 1	< 2
2	10	10	< 10	< 1	< 2
3	40	40	< 10	< 1	< 2
4	80	80	< 10	< 1	4
5	10	20	< 10	1	10
6	10	20	10	2	4
7	10	20	10	2	2
8	10	20	< 10	1	2
9	< 10	10	< 10	< 1	< 2
10	< 10	< 10	< 10	< 1	< 2
Serum 22 days after S19 vaccine	640	2560	320	64	200
Fraction no.					
1	< 10	< 10	< 10	< 1	< 2
2	< 10	10	< 10	< 1	< 2
3	< 10	20	< 10	< 1	< 2
4	40	80	10	1	10
5	40	160	20	4	20
6	40	160	20	3	20
7	80	320	40	8	40
8	40	160	20	4	10
9	10	40	< 10	1	2
10	< 10	10	< 10	< 1	< 2
Serum ca. 3 years after S19 vaccine	40	80	< 10	1	< 2
Fraction no.					
1	< 10	< 10	< 10	< 1	< 2
2	< 10	10	< 10	< 1	< 2
3	40	80	< 10	< 1	< 2
4	10	10	< 10	< 1	< 2
5	10	10	< 10	< 1	< 2
6	< 10	< 10	< 10	< 1	< 2
7	< 10	< 10	< 10	< 1	< 2
8	< 10	< 10	< 10	< 1	< 2
9	< 10	< 10	< 10	< 1	< 2
10	< 10	< 10	< 10	< 1	< 2

Table 2. *Fractionation of bovine sera by gel filtration on Sephadex G200*

Fraction no....	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
							(*a*) Serum from naturally infected cattle									
Absorption peak	0*	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3
SAT	2560	< 10	< 10	10	10	< 10	20	40	160	160	40	20	< 10	< 10	< 10	< 10
Coombs	> 5120	< 10	< 10	10	10	40	40	160	640	1280	640	160	10	< 10	< 10	< 10
DTT	1280	< 10	< 10	< 10	< 10	< 10	20	160	640	640	320	160	< 10	< 10	< 10	< 10
QRBPT	1024	< 1	< 1	< 1	< 1	< 1	4	16	32	32	8	8	2	< 1	< 1	< 1
CFT	4000	< 2	< 2	< 2	2	10	20	80	200	200	100	40	2	< 2	< 2	< 2
							(*b*) Serum: 7 days after S19 vaccination									
Absorption peak	0*	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3
SAT	160	< 10	20	40	20	10	10	< 10	< 10	10	< 10	< 10	< 10	< 10	< 10	< 10
Coombs	320	< 10	20	40	20	40	40	10	20	20	< 10	< 10	< 10	< 10	< 10	< 10
DTT	20	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
QRBPT	4	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
CFT	20	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
							(*c*) Serum: 22 days after S19 vaccination									
Absorption peak	0*	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3
SAT	640	< 10	< 10	< 10	< 10	10	20	40	80	80	80	80	< 10	< 10	< 10	< 10
Coombs	2560	< 10	10	20	40	40	80	320	640	640	320	320	< 10	< 10	< 10	< 10
DTT	320	< 10	< 10	< 10	< 10	< 10	10	20	40	40	40	40	< 10	< 10	< 10	< 10
QRBPT	64	< 1	< 1	< 1	1	1	2	8	16	16	16	4	< 1	< 1	< 1	< 1
CFT	200	< 2	< 2	< 2	4	4	10	20	20	40	40	40	< 2	< 2	< 2	< 2
							(*d*) Serum: 3+ years after S19 vaccination									
Absorption peak	0*	1	1	1	1	1	2	2	2	2	2	2	3	3	3	3
SAT	40	< 10	10	20	10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Coombs	80	< 10	10	20	10	10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
DTT	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
QRBPT	1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
CFT	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2

* Unfractionated serum.

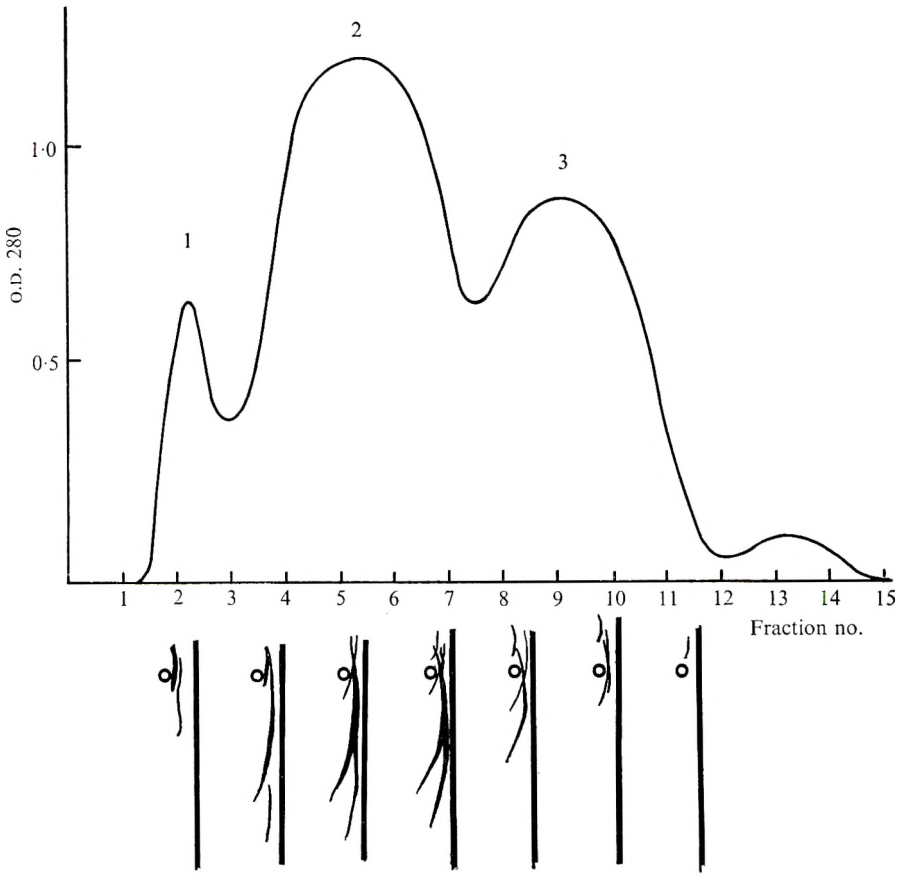

Fig. 2. Gel filtration of bovine serum on Sephadex G200.

No CF and RBPT activities were detected and the agglutinating activity was DTT-labile.

Gel filtration

Fractionation of high titre anti-*Br. abortus* serum from infected cattle produced an elution pattern as shown in Fig. 2. Antibody activity towards *Br. abortus* was present in fractions corresponding to absorption peaks 1 and 2. No antibody activity was detected in fractions corresponding to peak 3. Agglutinins active in the SAT were recovered from peak 1 and 2 fractions. Antibodies active in the DTT, Coombs and CF tests were restricted almost entirely to fractions corresponding to peak 2. Similarly, agglutinins active in the RBPT were only recovered from peak 2 fractions (Table 2).

Fractionation of serum samples from cattle vaccinated with *Br. abortus* S19 vaccine produced results varying with the time of collection of serum samples. In each case the elution profiles were similar to those shown in Fig. 2 but the antibody activities of the fractions varied. Serum collected 7 days after vaccination contained agglutinins restricted largely to peak 1 with only low titres of Coombs antibodies and no DTT-stable, CF or RBPT activity being detected in any fraction, although these activities were present to low titre in the unfrac-

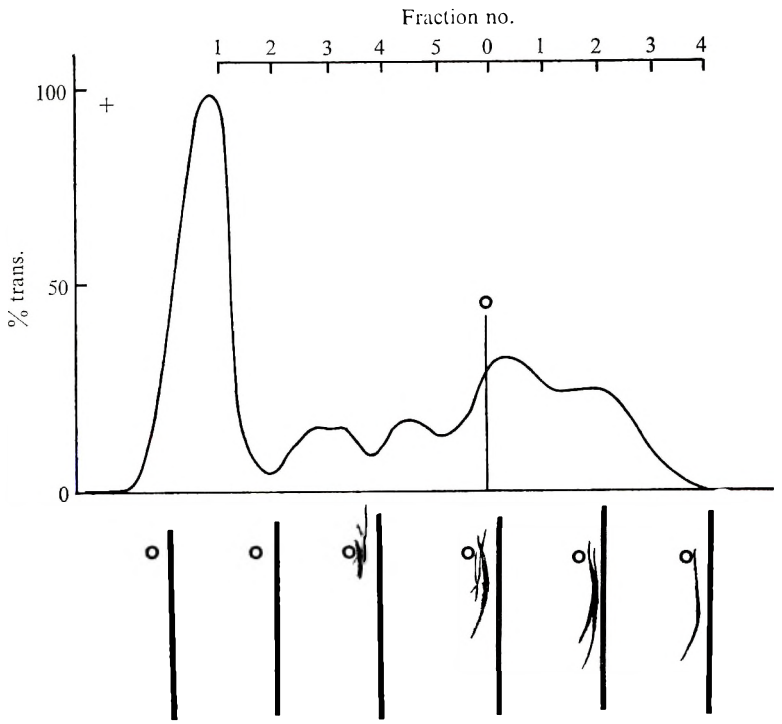

Fig. 3. Cellogel block electrophoresis of bovine serum.

tionated serum. Serum collected at 22 days produced a pattern similar to that of high titre serum from naturally infected animals. Agglutinins were present in fractions corresponding to peaks 1 and 2 but DTT stable agglutinins, CF and RBPT activity were restricted to peak 2 fractions. Some Coombs antibody was present in peak 1 fractions but the major part was in the peak 2 fractions (Table 2).

Serum with a low residual agglutinin titre pooled from animals vaccinated 3 years previously gave results similar to those of the 7 days serum. Agglutinins were restricted to peak 1 and the only other activity detected was Coombs antibody in low titre which was present in peak 1 and 2 fractions (Table 2). The RBPT was positive on undiluted serum but no activity was recovered in fractionated material.

A sample of negative serum fractionated as a control gave fractions inactive in all tests although the ultraviolet absorption profile was virtually identical with that of positive serum.

Preparative electrophoresis

Electrophoresis of high titre anti *Br. abortus* serum in cellulose acetate gel blocks produced the results shown in Fig. 3. Serological activity was present in fractions from the origin and cathodal to the origin. Agglutinating activity was present in γ_1 and γ_2 fractions. CF activity was restricted to the γ_1 fractions. DTT-stable antibodies and Coombs antibodies were present in γ_1 and γ_2 fractions but RBPT activity was restricted to the γ_1 region.

Table 3. *Serological activity of fractions separated from bovine antisera to* Br. abortus *by zone electrophoresis*

Sample	Distance from origin (mm)	SAT	Coombs	DTT	QRBPT	CFT
Pooled high titre bovine serum	Unfractionated	2560	> 5120	1280	1024	4000
Fraction no.						
1	+50	< 10	< 10	< 10	< 1	< 2
2	+40	< 10	< 10	< 10	< 1	< 2
3	+30	< 10	< 10	< 10	< 1	< 2
4	+20	10	20	< 10	1	< 2
5	+10	80	640	40	8	80
6	0	160	1280	80	32	200
7	−10	160	1280	160	32	200
8	−20	80	640	40	8	40
9	−30	10	640	< 10	< 1	< 2
10	−40	< 10	10	< 10	< 1	< 2
Serum 22 days after S19 vaccine	Unfractionated	640	2560	320	64	200
Fraction no.						
1	+50	< 10	< 10	< 10	< 1	< 2
2	+40	< 10	< 10	< 10	< 1	< 2
3	+30	< 10	< 10	< 10	< 1	< 2
4	+20	< 10	10	< 10	< 1	< 2
5	+10	40	160	20	4	20
6	0	160	1280	80	16	40
7	−10	80	640	80	2	4
8	−20	10	640	< 10	< 1	2
9	−30	< 10	320	< 10	< 1	< 2
10	−40	< 10	10	< 10	< 1	< 2

No activity was recovered on fractionation of low titre sera from animals vaccinated with *Br. abortus* strain 19. Fractionation of serum collected 22 days after vaccination produced results qualitatively similar to those obtained with serum from naturally infected animals (Table 3).

Ion-exchange chromatography

Fractionation of high titre serum samples produced the results illustrated in Fig. 4. Agglutinating antibodies with Coombs activity and stable to DTT, but with no CF or RBPT activity, were eluted in fractions corresponding to peak 1. Immunoelectrophoresis showed that these fractions contained almost entirely IgG_2 immunoglobulin (Fig. 4). Antibodies with RBPT, Coombs, CF and agglutinating activity stable to DTT were recovered in fractions corresponding to peak 2. Peaks 3–5 contained agglutinins with Coombs activity. Those from peak 5 were partially susceptible to degradation with DTT. With the exception of peak 3 no CF or RBPT activity was present in these fractions (Table 4). Immunoelectrophoresis showed that peak 2 contained, *inter alia*, IgG_1 immunoglobulin. The other fractions were more heterogeneous and IgM and possibly IgA were present (Figure 4).

Fractionation of low titre sera from vaccinated animals did not produce useful

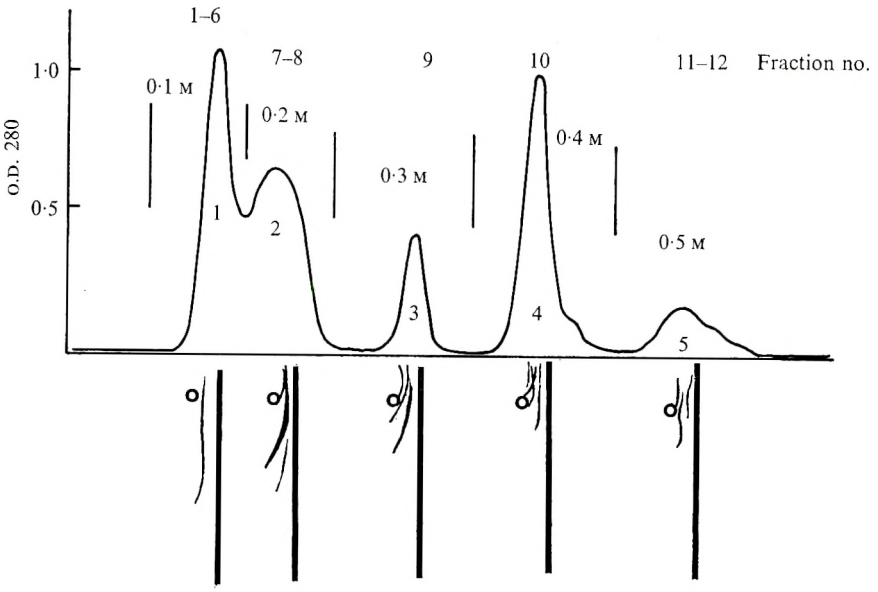

Fig. 4. Ion-exchange chromatography of bovine serum on QAE Sephadex

results. Fractionation of serum collected 22 days after vaccination produced results which were qualitatively identical with those obtained with high titre serum from infected animals (Table 4).

Examination of antibodies recovered by immuno-adsorption and elution

The eluates recovered from RBPT antigen by acid elution were examined for antibody activity in serological tests. The results summarized in Table 5 showed that eluates contained agglutinating, CF, RBPT, Coombs and DTT-stable antibodies. The RBPT and CF titres in relation to the SAT titres were higher than in the original serum. On diffusion against *Br. abortus* extracts (Corbel & Cullen, 1970) the eluates formed precipitin lines against the lipopolysaccharide component but showed only slight activity towards the intracellular antigens.

The eluates were characterized by disk electrophoresis, thin-layer gel filtration, immunodiffusion and immunoelectrophoresis. Disk electrophoresis followed by staining for protein, revealed a single major band in the γ-globulin region (Pl. 1; Fig. 1). Several faintly staining minor components of fast mobility were also detected but these were probably derived from the RBPT antigen. Thin-layer gel filtration demonstrated a single protein component with exclusion properties identical with those of bovine 7s globulin (Pl. 1; Fig. 2).

Immunoelectrophoresis of eluates showed a single precipitating component of γ_1 mobility. Precipitation occurred with antisera to bovine serum, bovine γ-globulin and specific antiserum to bovine IgG_1 globulin. No precipitation was produced by specific antisera to IgM or IgG_2 immunoglobulins nor by antiserum to bovine colostrum absorbed with bovine IgG_1 protein. Use of the interrupted trough technique showed fusion of the eluate precipitin arc with the IgG_1 arc of a bovine γ-globulin fraction containing IgG_1 and IgG_2 immunoglobulins (Pl. 1; Fig. 3).

Table 4. *Serological activity of fractions separated from bovine antisera to* Br. abortus *by ion-exchange chromatography on QAE-Sephadex*

Sample	Peak number	SAT	Coombs	DTT	QRBPT	CFT
Pooled high titre serum	Unfractionated	2560	5120	2560	1024	4000
Fraction no.						
1	1	20	1280	10	< 1	< 2
2	1	20	1280	10	< 1	< 2
3	1	20	1280	10	< 1	< 2
4	1	20	640	10	< 1	< 2
5	1	10	40	10	< 1	2
6		10	40	< 10	< 1	2
7	2	320	2560	160	32	200
8	2	640	5120	640	128	800
9	3	20	640	10	2	10
10	4	20	40	< 10	< 1	< 2
11	5	10	10	< 10	< 1	2
12	5	< 10	< 10	< 10	< 1	< 2
Serum 22 days after S19 vaccine	Unfractionated	640	2560	320	64	200
Fraction no.						
1	1	< 10	160	< 10	< 1	< 2
2	1	10	320	< 10	< 1	< 2
3	1	20	640	10	< 1	< 2
4	1	10	320	< 10	< 1	< 2
5	1	< 10	20	< 10	< 1	2
6		10	40	10	< 1	2
7	2	160	1280	80	16	200
8	2	160	1280	80	32	200
9	3	40	160	20	8	10
10	4	10	20	< 10	1	2
11	5	10	10	< 10	< 1	2
12	5	< 10	< 10	< 10	< 1	< 2

Table 5. *Serological activity and stability of immunoglobulin fractions eluted from agglutinated RBPT antigen*

Sample	SAT	Coombs test	DTT	Rivanol test	Heat* inactivation test	QRBPT	CFT
BS 1†	< 10	160	< 10	< 10	< 10	8	4
BS 2†	10	640	10	< 10	10	32	20
BS 2 (DTT reduced)	< 10	320	< 10	< 10	< 10	8	< 2
BS 2 (rivanol precipitated)	< 10	320	< 10	< 10	< 10	8	4
BS 2 (heated*)	10	320	< 10	< 10	10	32	10
BS 2 (papain digested‡)	< 10	< 10	< 10	< 10	< 10	< 1	ND

* SAT on serum heated at 65° C. for 15 min.

† Proportions of total IgG_1 globulin absorbable with *Br. abortus* cells were 17% for BS 1 and 23% for BS 2.

‡ Incubated at 37° C. for 1 hr. with 10 mg. per ml. papain + 0·001 M EDTA + 0·01 M DTT at pH 7·0.

Table 6. *Effect of selective removal of immunoglobulin classes on serological activity of bovine antisera to* Br. abortus

Sample	SAT	Coombs	DTT	QRBPT	CFT
IBS 1* + normal rabbit serum	2560	> 10,240	2560	1024	4000
IBS 1* + rabbit anti-bovine IgM	1280	> 10,240	640	1024	1000
IBS 1* + rabbit anti-bovine IgG_1	40	640	20	< 1	10
IBS 1* + rabbit anti-bovine IgG_2	640	2560	640	512	4000
IBS 1* + rabbit anti-bovine colostrum serum (IgG_1 absorbed)	640	2560	320	256	4000

* IBS 1 = high titre serum from naturally infected cow.

Combination of immunoelectrophoresis and immunodiffusion showed that the eluates contained a single precipitating component giving a reaction of identity with bovine IgG_1 globulin (Pl. 2; Fig. 1). Diffusion of eluate against antiserum to bovine IgG_1 globulins produced a reaction of identity with a bovine IgG_1 preparation (Pl. 2; Figure 2). The proportion of IgG_1 immunoglobulin in the eluate possessing specific antibody activity was determined by radial immunodiffusion titration of samples before and after absorption with RBPT antigen. As shown in Table 5, only a minor proportion of eluted immunoglobulin had antibody activity. Presumably most antibody activity was destroyed by denaturation under the conditions of elution.

Specific immunoglobulin inhibition tests

Treatment of sera with specific antisera to bovine IgM or IgG_2 had only a marginal effect on the quantitative RBPT activity, although other serological activities were reduced. Treatment with antiserum to IgG_1, with resultant removal of detectable IgG_1 from the serum, eliminated all quantitative RBPT activity (Table 6).

DISCUSSION

Although the non-specific agglutinins of bovine serum have been at least partially characterized as a heterogeneous group of fast sedimenting proteins (Rose, Roepke & Briggs, 1964), little direct information has been presented in relation to the nature of the specific agglutinins detected by the acid plate agglutination test or its modification – the RBPT.

It is clear from previous reports that both 7s and 19s immunoglobulins may be active in the SA test (Rose & Roepke, 1964; Tailyour & Cochrane, 1966). The results of the present gel filtration and ultracentrifugation experiments confirmed this and also showed that the 19s fraction had no activity in the RBPT. The density gradient ultracentrifugation results showed coincidence of maximum quantitative RBPT titre and 7s γ-globulin concentration. This was clearly confirmed by the gel filtration results in which all RBPT activity was confined to fractions corresponding to the second absorption peak. Further substantiation was given by the results of inactivation experiments. Treatments which destroy IgM activity, such as rivanol precipitation and reduction with thiols, left the quantitative RBPT titres virtually unaffected, although reducing the SA titre

of the serum. These results suggested that RBPT activity was a property of the 7s immunoglobulin fraction.

The presence of CF, Coombs and DTT-stable agglutinating activity in the same fractions also suggested that all or some of these properties might be activities of the same antibody molecules. This possibility was examined by further fractionation experiments.

Preparative zone electrophoresis showed that agglutinins active in the SA test were present in all fractions of γ and slow β mobility. However, RBPT and CF activities were confined to the γ fractions of high mobility. The slow γ fractions only contained reduction-stable agglutinins and Coombs antibodies. These results showed that the RBPT activity was confined to a subclass of the 7s immunoglobulins. Bovine serum has been shown to contain at least three classes of 7s immunoglobulins, IgG_1, IgG_2 and IgA, as well as the 19s IgM (Murphy, Osebold & Aalund, 1965; Aalund, 1968; Mach, Pahud & Isliker, 1969; Porter & Noakes, 1970). It seemed probable that the RBPT activity was restricted to a single class of 7s, electrophoretically fast γ globulin. In an attempt to resolve this, ion-exchange chromatography on the strong anion-exchange resin QAE-Sephadex A 50 was performed. This process fractionated the γ-globulins into distinct classes. IgG_2 globulin was eluted free of other serum proteins. This did not contain RBPT or CF activity but showed DTT-stable agglutinating and Coombs antibody activities. Fractions subsequently eluted were not homogeneous, but the only ones active in the RBPT were those containing IgG_1. These fractions also accounted for most of the CF activity recovered. This suggested that the immunoglobulin class responsible for RBPT activity was IgG_1.

This conclusion was strongly supported by the results of specific-inhibition tests. Clearly IgG_1 was essential for RBPT activity and its specific removal resulted in loss of activity. Removal of IgG_2 or IgM by specific antiserum had little effect on quantitative RBPT titres.

The immuno adsorption-elution experiments confirmed the results of the previous experiments. The only serum protein detected in the eluates had the immunochemical properties of IgG_1. Its serological activity, although much reduced relative to the original serum, strongly suggested that CF and RBPT activities are properties of the same antibody molecule.

It is also apparent from the results presented that RBPT and CF activities are not necessarily equivalent to reduction-stable agglutinating and Coombs activities.

These findings are significant from the diagnostic point of view. Thus, in surveys of large numbers of sera conducted at this laboratory, Morgan *et al.* (1969) and Davies (1971) found a high degree of consistency between the results of the CF test and those of the RBPT. In some cases a positive RBPT reaction was observed in the absence of detectable CF antibody but the reverse situation was rarely encountered. These observations are explicable on the basis of the greater sensitivity of the RBPT. In the present work tests on antibodies eluted from RBPT antigen showed that these reacted to a higher dilution in the quantitative RBPT than in the CF test.

At present the CF test is widely used for differentiating cattle with persisting agglutinins resulting from *Br. abortus* Strain 19 vaccination from those naturally infected with *Br. abortus*. The selection of this procedure is based on the observations that in vaccinated cattle persisting agglutinins are likely to be due to IgM antibodies, whereas in chronically infected animals they are predominantly of the IgG type and are associated with CF activity (Yuskovets, 1956; Anderson, Jenness, Brumfield & Gough, 1964; Jenness, Anderson & Gough, 1965; Schimmel & Erler, 1967). The results of the present work suggest that a quantitative adaptation of the RBPT might offer an alternative to the CF test for detecting IgG_1 antibodies. However, it should be noted that complete correlation between the results of RBPT and CF tests should not be expected. As shown by Corbel (1972) the RBPT detects only antibodies directed against the agglutinogens of *Br. abortus*, whereas the CF test may detect antibodies to both agglutinogenic and non-agglutinogenic antigens.

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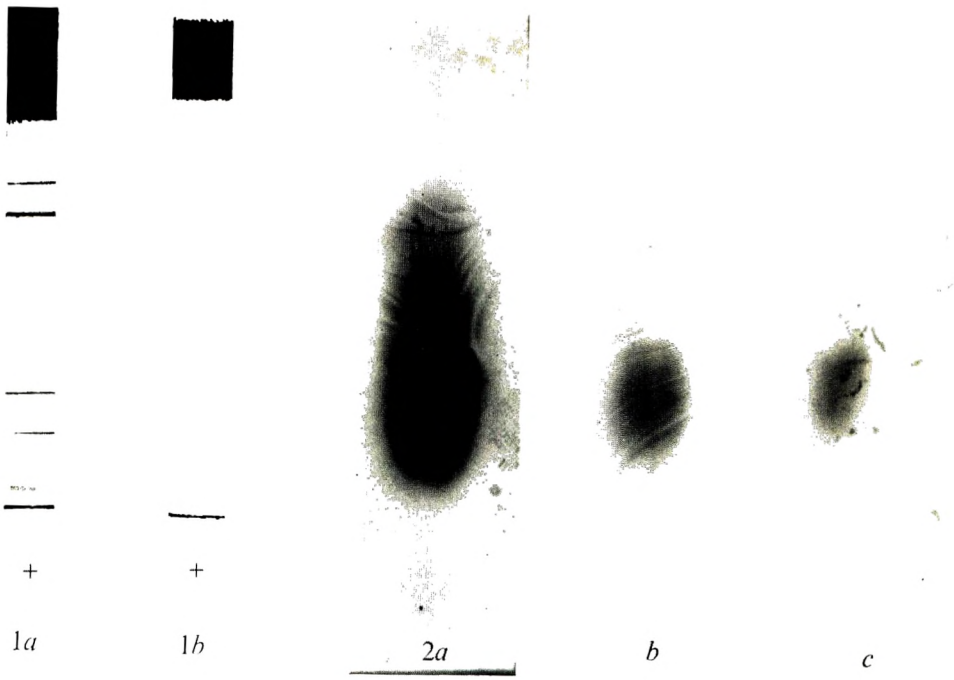
EXPLANATION OF PLATES

PLATE 1

Fig. 1. (*a*) Disk electrophoresis of the immunoglobulin fraction eluted from agglutinated RBPT antigen at pH 1·0. A single broad zone with an electrophoretic mobility identical with that of γ-globulin is apparent. Minor protein components, probably extracted from the *Br. abortus* cells, are also visible. (*b*) Disk electrophoresis of purified bovine IgG immunoglobulins. A single broad zone of low mobility is visible.

Fig. 2. Thin-layer gel filtration of (*a*) bovine serum, (*b*) fraction eluted from agglutinated RBPT antigen at pH 1·0, (*c*) purified bovine IgG immunoglobulins. Fraction (*b*) clearly contained components of molecular dimensions similar to IgG immunoglobulin.

Fig. 3. Immunoelectrophoresis of: (*a*) bovine γ-globulins; (*b*) fraction eluted at pH 1·0 from agglutinated RBPT antigens; (*c*) bovine serum; (*d*) and (*e*) interrupted trough method to show cross-reaction of eluted immunoglobulin fraction (*d*) with IgG_1 immunoglobulin in bovine γ-globulin fraction (*e*); (*f*) bovine γ-globulins. The antiserum used was rabbit anti-bovine γ-globulin serum.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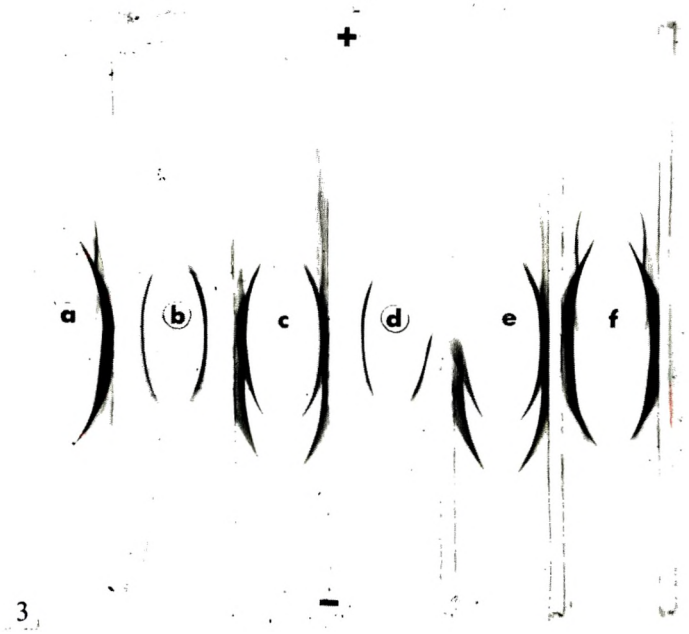
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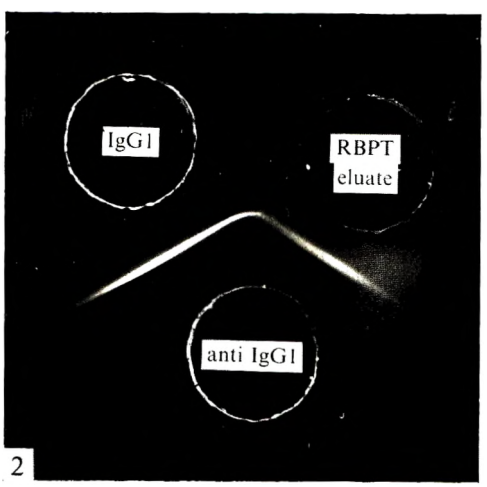
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PLATE 2

Fig. 1. Immunoelectrophoresis of: (*a*) bovine γ-globulins; (*b*) bovine serum; (*c*) and (*d*) combined immunoelectrophoresis and immunodiffusion. The upper well of (*c*) contained immunoglobulin eluted from agglutinated RBPT antigen at pH 1·0 and the lower well bovine γ-globulins. In (*d*) the positions of these reactants was reversed. In each case the eluted immunoglobulin has given a reaction of identity with the IgG_1 immunoglobulin arc. (*e*) Immunoglobulin eluted at pH 1·0 from agglutinated RBPT antigen, (*f*) bovine γ-globulins.

Fig. 2. Diffusion of immunoglobulin fraction eluted from agglutinated RBPT antigen and purified IgG_1 immunoglobulin against rabbit antiserum to bovine IgG_1 globulin. A reaction of identity was given by the two fractions.

Catalogue of the National Collection of Type Cultures – 1972

It is a pleasure to welcome the fifth edition of this Catalogue, the first full catalogue since 1936, although the gap has been partly filled by a *List of Species Maintained* (1948) and a *Catalogue of Species* (1958).

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