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Bacteriological evaluation of a laminar cross-flow tunnel for surgery under operational conditions

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(*Received* 18 *December* 1972)

SUMMARY

A transportable surgery cross-flow unit has been tested under 'operational conditions'. By the use of artificial aerosols and a volunteer surgical team, or dummies, it was found that, at an air velocity of 0·45 m./sec., a detectable transfer to above the table occurred only when quite highly concentrated aerosols (of more than $10^{3·6}$ bacteria/m.^{3} of air or more) existed underneath the table. The short disappearance time under these conditions and the quite stable flow pattern above the table found when a surgical team was working, standing along both sides of the table, make it unlikely that an aerosol of detectable concentration can develop during surgery, at this site. The chance that particles, liberated from the heads of the surgical team, settle on the table, was found to be strongly reduced when a cross-flow tunnel operated at an air velocity of 0·45 m./sec. The transfer from outside the unit to the inside was prevented by closing the upper part of the open front side.

INTRODUCTION

In recent years an increasing number of studies on the use of unidirectional flow (U.D.F.) in operating theatres has been reported in the literature. They often deal with the advantage(s) of cross-flow over down-flow or vice versa (Scott, Sanderson & Guthrie, 1971; Whyte & Shaw, 1971). Cross-flow seems to offer two practical advantages over down-flow: (1) little or no disturbance of the flow pattern by a standard operation lamp; (2) cross-flow units are easier to install in conventional operating theatres. They do not necessarily require major changes in the building. At relatively little cost, conventionally ventilated operating rooms can, if necessary, be changed into laminar flow ventilated ones. For this purpose, mobile U.D.F. cross-flow tunnels are on the market. It is claimed that these units considerably reduce the risk of airborne contamination when they are operated at an air velocity of 0·45 m./sec. Since the ventilation of a number of existing operating theatres requires improvement, particularly those for 'ortho-paedic', 'neuro'- and 'open heart' surgery, we considered it necessary to test such a cross-flow unit.

To obtain a good insight into the functioning of the unit under operational conditions, several parameters were studied:

(*a*) The transfer of bacterial aerosols from outside (upstream) the unit to the

inside. This should not occur, even during activities such as that of the 'circulating nurse'.

(*b*) The occurrence of transfer of bacterial aerosols from underneath as well as from behind the members of the surgical team to above the operation table.

(*c*) The disappearance time of bacterial aerosols, i.e. the time elapsing between aerosolization of bacteria and the moment that the 'last cell' of that aerosol is sampled, was determined above and underneath the table.

(*d*) The correlation between bacterial concentration/m.^{3} of air and the occurrence of fall-out above and underneath the table was investigated.

(*e*) The deviation from horizontal transport of aerosol particles in the cross-flow area. This was studied to obtain information on the fate of bacteria generated from the heads of the surgical team.

All tests were performed with bacterial aerosols of different concentrations and at three different air velocities: 0·45 m./sec.; 0·25 m./sec. and with the air flow off. In this way, a fuller insight could be obtained into the conditions under which transfers occurred.

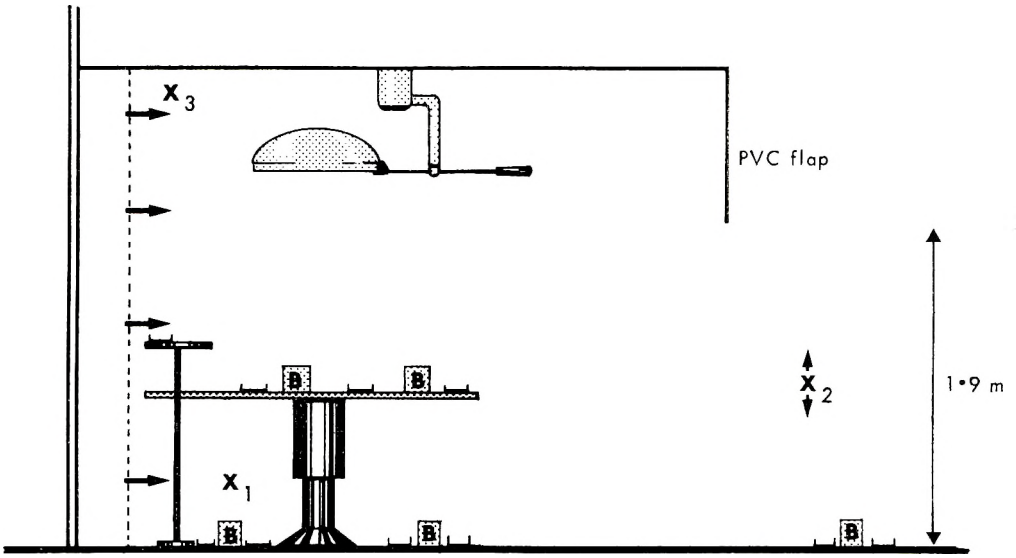
MATERIALS AND METHODS

Cross-flow unit

For our study an Enciramedic 'surgery isolator for sepsis control' was used, made under Envirco's licence by CEAG Schrip Reinraumtechnik in Germany. The size of the filter wall was 3·20 m. × 2·80 m. while the tunnel was 3·60 m. deep. This unit could be operated with an air velocity at the site of the filter wall of 0·25 and 0·45 m./sec. The flow pattern was not turbulence-free at an air velocity of 0·25 m./sec. This improved considerably when the unit was operated at 0·45 m./sec. A dummy operating table with standard dimensions was placed inside the unit near the filter wall (figure 1). During the first four experiments, the influence of 'surgical activity' by a team of four volunteers imitating surgical activity around the table was studied. For this purpose, the results were compared with those obtained with a dummy team. The dummy team consisted of four puppets of human size. Since no significant differences were found when a human team of four persons was standing around the table or with the immobile dummy team, the remaining experiments were performed with the dummy team. Because some influence on the transfers was noticed when an individual walked inside the enclosure during the experiments, this activity was continued in the experiments with the dummy team around the table. To prevent the occurrence of transfer of bacterial aerosols from outside the enclosure, a P.V.C. flap closing the upper part of the open front side was found necessary (Fig. 1).

Bacterial aerosols

Escherichia coli was used in this study to obtain an adequate decay rate of the aerosolized bacteria (Brown, 1954). In each experiment, three overnight broth cultures of *E. coli* were suspended in water. The suspensions consisted of 10^5, 10^7, and 10^9 bacteria per ml. A spray which aerosolized approximately 1 ml. of



B= Bourdillon slit sampler

X= site of aerosolization of E.coli in the various experiments :

X_1 = transfer from underneath the table

X_2 = transfer from outside

X_3 = vertical displacement

⌴ settle plate (3 per slit sampler)

Fig. 1. Schematic drawing of the test situation. The surgical team is omitted from the drawing for the sake of simplicity.

suspension per second was used. In the resulting aerosols, *E. coli* had a decay rate of 0·5–1 % per minute. The experiments were repeated 6 times.

The *E. coli* particles were smaller in size (90 % of the single particles ranged between 0·6 and 3·0 μm. in diameter) than the particles contaminated with bacteria that are generated by human individuals (Noble, Lidwell & Kingston, 1963; Whyte, 1968). The apparent settling velocity underneath the table was estimated according to Foord & Lidwell (1972) and was found to vary between 15 and 29 cm./min.

Aerosolization was performed at several sites (Fig. 1):

(1) Underneath the table to determine the transfer to the air above the table and the fraction that sedimented. Spraying was performed for 20 sec.

(2) Behind the surgeons along both long sides of the table in the direction of the flow. Spraying was performed for 10 sec. at each side.

(3) Outside the unit before the open front side to determine the transfer of cells to the inside of the enclosure. Aerosolization was continued for 15 sec. in each experiment.

(4) Directly in front of the filter plenum near the upper edge. This was necessary in order to study the vertical deviation of aerosolized *E. coli* particles.

All experiments were performed six times so that significant results could be obtained.

Sampling

The four locations of the Bourdillon type 'slit samplers' and the sedimentation plates in the various experiments are shown in Fig. 1. The slit samplers were used to determine the concentration of bacteria in the air after aerosolization as well as the disappearance time. The latter was calculated from the size of the segment of the agar plates which showed *E. coli* colonies after incubation. The rotation speed of the plate during sampling was 3° per sec. The slit samplers were operated at an air sampling volume of 30 l./min. In all experiments, the sampling time was 2 min. After each experiment, the room was ventilated for at least 2 min. by switching on the cross-flow at a 'high velocity' (0·45 m./sec.).

Sampling was performed on Endo agar (DIFCO). By use of this culture medium, the counting of colonies in experiments in which the flow was off, was not hampered by colonies of staphylococci or other airborne bacterial species.

Temperature and humidity. These were not controlled and varied slightly. The temperature varied between 20 and 22° C.; the relative humidity, between 51 and 55 %.

Air velocity. The air velocity was determined with a Wilh. Lambrecht K 6 (Gottingen) hot wire flow meter (type 641 N). During surgical activity inside the enclosure, the air velocity was determined in front of the filter wall as well as at a distance of 2·5 m. at nine different points located 1 m. from each other.

RESULTS

The flow pattern above the table appeared to be quite stable when the cross-flow tunnel, operated at 0·45 m./sec., was in use. In a cross-section through the room at a distance of 2·5 m. from the filter wall (just behind the operating table) the air velocities were measured at nine different points. Greater variations were found when the unit was operated at 50 % of the normal speed than when at full speed (0·45 m./sec.) operation (Table 1). At the site of the filter wall, the air velocity distribution was more homogeneous and varied within the tolerances specified by U.S. Federal Standard, 209 A. Transfer from outside the enclosure was detected only with an aerosol of over 10,000 bact./m.^{3} of air. It could virtually be eliminated with a plastic flap in the open front side extending to 1·9 m. above the floor. This reduced the opening and, therefore, increased the air velocity in the opening to 0·66 $\pm$ 0·05 m./sec.

In order to be able to realize the goals outlined in the introduction, it was found necessary to challenge the system with relatively highly concentrated bacterial aerosols. This was particularly necessary to determine the occurrence of a 'transfer' from underneath to above the table and to investigate whether, under surgical (activity) conditions, a transfer could occur from the operating theatre environment into the enclosure.

With the flow switched off, only convection currents and air movements induced

Table 1. *Air velocity (m./sec.) at nine points 1 m. apart, all at 2·5 m. from the filter wall*

Point no.	Half blower capacity		Full blower capacity	
	Flap open	Flap shut	Flap open	Flap shut
1	0·26–0·28	0·26–0·28	0·42–0·44	0·40–0·42
2	0·24–0·26	0·20–0·22	0·38–0·40	0·30–0·32
3	0·26–0·28	0·24–0·26	0·48–0·50	0·40–0·42
4	0·18–0·30	0·24–0·26	0·20–0·30	0·38–0·42
5	0·28–0·30	0·22–0·24	0·46–0·50	0·48–0·50
6	0·22–0·26	0·24–0·26	0·40–0·42	0·46–0·48
7	0·05–0·15	0·16–0·20	0·10–0·30	0·20–0·30
8	0·26–0·30	0·28–0·30	0·53–0·55	0·52–0·54
9	0·18–0·32	0·24–0·26	0·38–0·50	0·52–0·54

The nine points were distributed in a vertical plane as follows:

1 2 3
4 5 6
7 8 9

Table 2. *Transfer and settling of Escherchia coli aerosols inside a cross-flow tunnel operated at different air velocities*

Air velocity (m./sec.)	Mean log bact. (count/m.^{3}) (S.D.)		Transfer index	Average bact. fall-out/m.^{2} above the table in 2 min.	Settling† velocity (cm./min.)
	Underneath the table	Above the table*			
0	2·8 (0·1)	1·9 (0·1)	0·125	1·5	0·93
	3·8 (0·3)	2·6 (0·2)	0·062	5·0	0·62
	4·2 (0·2)	3·1 (0·3)	0·083	16·0	0·61
0·25	2·5 (0·2)	—	—	0	—
	3·7 (0·2)	1·2 (0·1)	0·003	0·8	2·5
	4·4 (0·2)	2·2 (0·2)	0·006	76	2·4
0·45	2·7 (0·2)	—	—	0	—
	3·6 (0·3)	—	—	0	—
	4·3 (0·3)	1·5 (0·2)	0·0015	4·0	6·2
	5·0 (—)	2·5 (—)	0·0031	3·3	5·5

* Mean concentration of two slit samplers (see Fig. 1).
† According to the formula of Foord & Lidwell (1972).

by 'surgical activity' were found. These occasionally resulted in upward-directed turbulences, as were seen during smoke tests. The area directly above the table may have been reached, however, in two different ways: (1) as a result of convection streams and movements from the surgical team; and (2) by air coming from more remote places inside the enclosure. Any person walking inside the enclosure during the test may have contributed to the latter. Under cross-flow ventilation conditions, a different mechanism was presumably responsible for the transfer from underneath the table. Aerosol spread to all sides within the enclosure, as was seen when the flow was off, did not occur, and convection streams induced by the surgical team were no longer found. Upward-directed air movements also

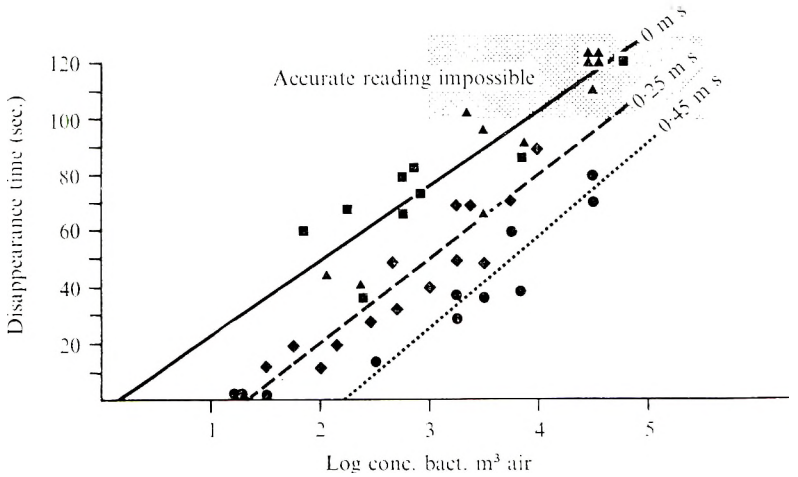


Fig. 2. Disappearance time of several aerosols at air velocities above and underneath the table.

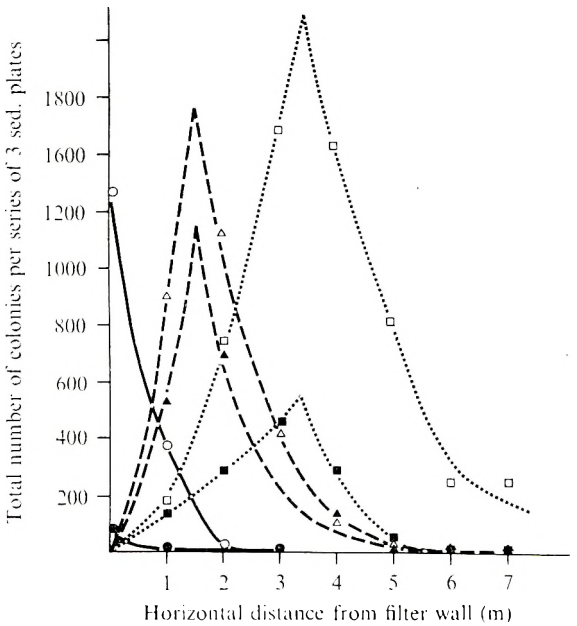


Fig. 3. Sampling on table level at 1 m. intervals (horizontal distance) from the filter wall following aerosolization 1·7 m. above the table directly in front of the filter wall.

occurred under these circumstances, but now owing to disturbances in the laminar flow pattern. These occasional upward-directed air turbulences were made visible by smoke tests and were seen at various places around the table 'down stream' from the members of the surgical team.

The results of the bacterial aerosolization are presented in Table 2. The bacterial counts per m.³ of air which were found in a total of 18 different experiments performed with each air velocity are presented in three classes as the mean log value

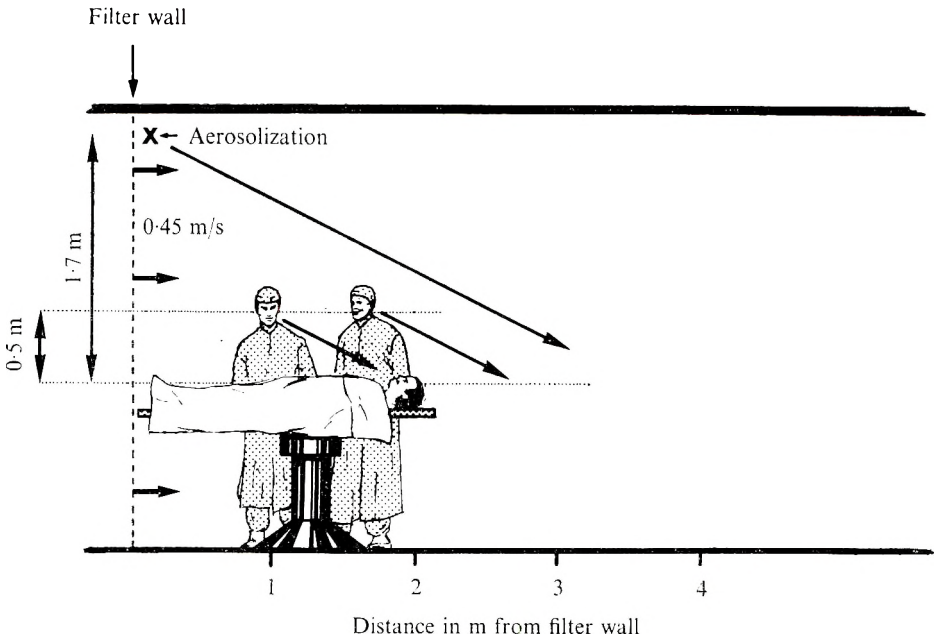


Fig. 4. Diagram showing the vertical displacement of aerosolized bacteria in a cross-flow of 0·45 m./sec.

(and S.D.) of five to seven observations. When the unit was operated at 0·45 m./sec., a few extra experiments were performed. In one of these a very dense aerosol of 10^5 cells/m.^{3} of air was achieved underneath the table (Table 2). The Transfer Index (Lidwell, 1960) calculated from these data shows some fluctuation at each air velocity, but decreased significantly in value at increasing air velocity. A less favourable observation was that the sedimentation of the *E. coli* particles increased considerably when the flow was increased (Table 2). The transfer index of experimental aerosols generated behind the members of the team when standing along both long sides of the table to above the table was zero, even when suspensions of 10^7 bacteria/ml. were aerosolized.

The disappearance time of aerosols above and underneath the table should be as short as possible. This has become an important requirement, since we have indicated that settling above the table is adversely influenced by higher (cross-flow) air velocities (Table 2). The disappearance time was found to be greatly reduced at increasing air velocities (Fig. 2). For an aerosol of about 500 bacteria per m.^{3} of air, the average disappearance time was found to be 16 sec. at an air velocity of 0·45 m./sec., 42 sec. at 0·25 m./sec., and 72 sec. when the flow was switched off.

The site of settling of bacteria shed from the heads of the operating team was approximated by aerosolization of bacteria at 1·70 m. above the table. The experimental aerosol was made as high as possible above the table in order to assure that the 'peak settling' at various air velocities occurred at well separated sites. No bacteria sedimented on the table from the heads of the team under operation conditions. This was also found in a previous study (Van der Waaij,

unpublished data). Inconclusive results were obtained by aerosolization of bacteria at 50 cm. above the table (approximate distance of the heads of the surgical team above the table). However, by following aerosolization of highly concentrated suspensions from high above the table (1·70 m.), an area could be found 'down stream' where peak settling on the table occurred (Fig. 3). The estimated area of 'peak settling' of bacteria generated at 50 cm. above the table is shown in Fig. 4.

DISCUSSION

The results of the present study indicate that, during surgery, inside a cross-flow tunnel operated at an air velocity of 0·45 m./sec., quite highly concentrated bacterial aerosols are required underneath the table to accomplish a transfer of bacterial aerosol particles to above the table. At an air velocity of 0·45 m./sec., an aerosol of $10^{3·6}$ bacteria per m.^{3} underneath the table did not result in a measurable transfer (Table 2). It should be realized, however, that the time that the aerosol existed underneath the table was short. An aerosol of 10^3 bacteria/m.^{3} of air remained only a little longer than the time during which it was generated, namely, 30 sec. (Fig. 2). The members of a surgical team will shed most bacteria from the lower half of their body (May & Pomeroy, 1973) and consequently underneath the table. After having worn a surgical gown for 4 hr., an individual may disperse quite a number of *Staphylococcus aureus* cells which can result in a concentrated aerosol (of up to 1000 cells/m.^{3} of air in an unventilated enclosure of 30 m.^{3}) according to Blowers, Hill & Howell (1973). When it is taken into consideration that the particles used in our study were smaller and had a lower settling velocity than the bacterially contaminated particles shed by humans, we can assume that our test situation was less favourable for the prevention of a transfer than is the case when these larger particles must be transferred. Secondly, the rapid disappearance time, which is also favourably influenced (shortened) by a higher settling velocity, will prevent the formation of concentrated aerosols such as Blowers described. This means that, even in the extreme case in which the surgical team consists exclusively of *S. aureus* dispersers and an aerosol of 10^3 cells/m.^{3} could persist for a short time underneath the table, the chance of a transfer to above the table is small. Bacteria dispersed inside the enclosure behind the team (for example, by a circulating nurse) will only be transferred to above the table when the source moves upstream from the table and at the level above the surface of the table. Bacteria liberated by the surgeons above the table are apparently the only ones that may contaminate the drapes or the wound. We have found that the settling velocity was increased about sixfold when the air velocity was increased from zero (unit switched off) to 0·45 m./sec. Particles dispersed 50 cm above the table will, according to our findings, land on the drapes about 1 m. down stream. This means that particles shed from the heads of the team members will generally not settle on the surgical linen but further down stream (Fig. 4). This indicates that good shielding of the nose, mouth, and hair is indicated not only in a down-flow set-up, but also under cross-flow conditions. An important difference between the two remains, however, that, in down-flow,

particles liberated from the upper half of the body have, owing to the strongly increased settling velocity (van der Waaij & van der Wal, 1973), an increased chance of landing on the table. Under cross-flow conditions, this may only occur when the particles are liberated at a level of less than 50 cm. above the table by individuals standing along the long side of the table and particularly those at the foot end. The more down stream the surgeons are standing near the 'head end' of the table, the safer is the situation (Fig. 4).

Transfer from outside the enclosure could easily be prevented by reducing the opening in the front side of the cross-flow tunnel so that the air velocity through the opening was increased. It can be concluded, therefore, that cross-flow ventilation of surgical rooms can provide excellent protection from airborne contamination of the wound, instruments, and drapes covering the patient, provided the following points are taken into consideration:

(1) No source of contamination may exist upstream from the table.

(2) Adequate surgical clothing, particularly the use of a good face mask and cap, is necessary.

(3) Bending over the patient to a level less than 50 cm. is potentially dangerous.

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REFERENCES

Blowers, R., Hill, J. & Howell, A. (1973). Shedding of *Staphylococcus aureus* by human carriers. In *Airborne Transmission and Airborne Infection* (ed. J. F. Ph. Hers & K. C. Winkler), p. 432. Utrecht: Oosthoek Publishing Co.

Brown, A. D. (1954). The survival of airborne micro-organisms. Effects of temperature. *Australian Journal of Biological Sciences* **7**, 444.

Foord, N. & Lidwell, O. M. (1972). The control by ventilation of airborne bacterial transfer between hospital patients and its assessment by means of a particle tracer. *Journal of Hygiene* **70**, 279.

Lidwell, O. M. (1960). The evaluation of ventilation. *Journal of Hygiene* **58**, 297.

May, K. R. & Pomeroy, N. P. (1973). Bacterial dispersion from the body surface. In *Airborne Transmission and Airborne Infection* (ed. J. F. Ph. Hers & K. C. Winkler), p. 426. Utrecht: Oosthoek Publishing Co.

Noble, W. C., Lidwell, O. M. & Kingston, D. (1963). The size distribution of airborne particles carrying microorganisms. *Journal of Hygiene* **61**, 385.

Scott, C. C., Sanderson, J. T. & Guthrie, T. D. (1971). Choice of ventilation system for operating theatres. *Lancet* i, 1288.

Waaij, D. van der & Wal, J. F. van der (1973). 'Laminar' airflow ventilation: crossflow or downflow? In *Airborne Transmission and Airborne Infection* (ed. J. F. Ph. Hers & K. C. Winkler), p. 584. Utrecht: Oosthoek Publishing Co.

Whyte, W. (1968). Bacteriological aspects of air-conditioning plants. *Journal of Hygiene* **66**, 567.

Whyte, W. & Shaw, B. H. (1971). An experimental down/cross flow operating room. *Contamination Control* **10**, 6.

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Experimental *Salmonella* infection in calves. 1. The effect of stress factors on the carrier state

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SUMMARY

Four calves were infected with $2{\cdot}4 \times 10^8$ *S. dublin* and penned separately. Fluctuations in environmental temperature did not affect the symptoms of the disease or the duration of excretion. Faecal samples were superior to rectal swabs for detecting excretors.

After transport stress, *Salmonella* was isolated from several organs at slaughter in an excreting calf, and uninoculated control calves became cross-infected. During slaughter, carcasses became contaminated on their surfaces. From one contaminated carcass *Salmonella* was recovered after chilled storage at 0° C. for 1 week and also after freezing at −20° C. for 1 month.

INTRODUCTION

Salmonellosis is one of the most important types of food poisoning. In 1968 it accounted for 73% of all reported incidents in England and Wales (Vernon, 1970). Being a zoonosis, the main vehicle seems to be meat and meat products. The organisms have been found in all species of animals used for meat production, but veal has been incriminated in the most serious outbreaks of salmonellosis in this country (Anderson, 1968). Calves suffering from a clinical *Salmonella* infection may give rise to outbreaks of food poisoning if they are slaughtered and the meat used for consumption. However, healthy, latently infected animals give more cause for concern, because they are extremely difficult to detect. Nottingham & Urselmann (1961) found *Salmonella* after slaughter in 13% of 1100 apparently healthy calves, while Edel, Guinée & Kampelmacher (1969) found 22·7% of 1000 calves examined at slaughter positive for *Salmonella*. On the farm, Rankin, Taylor & Burrows (1969) found the incidence of *Salmonella* excretion to be approxi-

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mately 1 %, and there is evidence that there is a build-up of infection in calves during transport and time in holding pens. Anderson, Galbraith & Taylor (1961) found that 0·5 % of the calves in the market were infected with *Salmonella* and calves kept at dealers' premises for only a few hours before slaughter had an infection rate of 0·6 %. However, calves kept in lairage for 2–5 days before slaughter showed an infection rate of 35·6 % at slaughter.

Stress factors appear to increase the susceptibility of calves to salmonella infections (Buxton, 1960; Gibson, 1965). Therefore, a study has been made of some of the factors which in practice may contribute to this increase, such as temperature fluctuations and transport stress. Additionally, the effect of slaughter operations on cross-contamination of carcasses has been investigated.

Most published work is based upon infections with *S. typhimurium*. Because calfhood infections with *S. dublin* closely resemble those with *S. typhimurium* (Gibson, 1961), and *S. dublin* is the most important serotype in cattle (Sojka & Field, 1970), this serotype was selected for the experimental infection of young calves.

MATERIALS AND METHODS

Animals

Four Friesian calves (B47, B49, 440 and 719), 2 weeks old, were kept in pens. Before the experiment started, faecal samples were examined daily for 1 week to exclude the possibility of a natural infection. Towards the end of the experiment, which lasted for 6 weeks, 2 new Friesian calves, 2 weeks old, were introduced to study the effect of cross-contamination.

Housing

The calves were kept in separate pens throughout the experiment. Two of the calves (B47 and 719) were maintained at a temperature of 14–16° C. The other two were stressed by reducing the daytime temperature to between −2° and +5° C. by opening the doors and turning off the heating. During the night, the temperature was maintained at 14–16° C.

S. dublin

The four calves were inoculated orally with $2{\cdot}4 \times 10^8$ viable cells of *S. dublin*. The strain (06/8 B_2), kindly supplied by Dr A. H. Linton, University of Bristol, had been isolated from an outbreak of salmonellosis in calves and was resistant to neomycin, tetracycline and ampicillin.

Bacteriological examinations

Ante-mortem samples. Daily, 1 g. of faeces taken from a sample removed from the rectum of each calf, using a polythene sleeve, was suspended in water to give a 10 % solution, and tenfold dilutions were made. Counts of *S. dublin* were made on bismuth sulphite agar (WB) and brilliant green agar (BG) by spreading duplicate 0·02 ml. drops from 20 SW stainless steel cannulae (Astell Ltd, Brownhill Road, Catford, London S.W. 6) on the surface of the plating media and counting

characteristic colonies after incubation for 48 hr. at 37° C. At the same time, enrichment cultures of 1 g. of faeces were made in selenite broth (SB) (Stokes & Osborne's modification, 1955) at 37° C. Plating from the enrichment medium was made on WB and BG after 24 and 48 hr., and the plates were incubated at 37° C. for 48 hr. After 48 hr. typical colonies were identified serologically and examined for antibiotic resistance. During the first period of the experiment rectal swabs were also taken but were discontinued because they were found to give far less consistent results than faecal samples.

Post-mortem samples. After captive bolt stunning, calves were killed by exsanguination and skinned and dressed following normal slaughterhouse practice. Samples of muscles, organs and viscera were taken with aseptic precautions. All but intestinal samples were placed in water at 100° C. for 6 sec. (Kampelmacher, Guinée & Janssen, 1964). The samples were then cut in small pieces with sterile scissors and placed in SB, approximately 10 g./100 ml. enrichment broth.

Before spraying, the surfaces of the carcasses were sampled using cotton-wool swabs, *ca.* 15 × 10 cm., moistened in peptone water. One swab was used on the outside and one on the inside of each carcass. Sterile plastic gloves were used during the handling of the swabs which, after use, were kept inside the gloves until they were put into enrichment broth (Kitchell, Ingram & Hudson, 1973).

Serology

Agglutination tests were made using antigens prepared from two strains of *S. dublin*, the vaccine strain (Smith, 1965) and a field strain.

Preparation of 'O' antigen. 0·1 ml. quantities of an overnight culture at 37° C. were spread on several nutrient agar plates, incubated at 37° C. and the culture harvested using approximately 5 ml. of saline per plate. After adding 10 times the amount of absolute alcohol, the pooled suspension was heated in a water bath at 53 ± 1° C. for 2 hr., centrifuged at 1000 rev./min. for ½ hr. and the deposit re-suspended in saline with a trace of merthiolate as a preservative. The antigen suspension was standardized using a nephelometer and a standard 'O' suspension (Wellcome Research Laboratories, Beckenham, Kent, BR3 3BS).

Preparation of 'H' antigen. 1 ml. of an overnight nutrient broth culture was added to 100 ml. of nutrient broth in a 250 ml. flask and incubated at 37° C. in an orbital incubator. After overnight incubation, formalin was added to give a final concentration of 2 %. After standing overnight, the suspension was centrifuged (1000 rev./min. for ½ hr.) and the deposit was re-suspended in 1 % formalin in saline. The antigen was standardized using a nephelometer and a standard 'H' suspension (Wellcome Research Laboratories, Beckenham, Kent, BR3 3BS).

RESULTS

Before the experiment started, sera from the four calves were negative for 'H' and 'O' agglutinins, and none of the animals were found to harbour *Salmonella*. Within 24 hr. of inoculation, all the calves showed symptoms of salmonellosis, with temperatures rising to 104–106° F. on the fourth day. High numbers of

Table 1. *Excretion of* S. dublin *in faeces of calves after oral infection with a single dose of* $2{\cdot}4 \times 10^8$ *cells*

(Counts are numbers per g. of faeces.)

No. of days after inoculation	Calves kept under fluctuating temperature conditions		Calves kept at constant temperature	
	B49	440	719	B47
2	$1{\cdot}6 \times 10^6$	$1{\cdot}7 \times 10^6$	$1{\cdot}6 \times 10^6$	$7{\cdot}2 \times 10^4$
3	$1{\cdot}0 \times 10^4$	$6{\cdot}0 \times 10^3$	$3{\cdot}2 \times 10^4$	$1{\cdot}0 \times 10^4$
4	$8{\cdot}0 \times 10^5$	$1{\cdot}8 \times 10^4$	$9{\cdot}6 \times 10^4$	$3{\cdot}8 \times 10^5$
5	$4{\cdot}0 \times 10^4$	$8{\cdot}0 \times 10^3$	$4{\cdot}0 \times 10^4$	$6{\cdot}0 \times 10^6$
6	$1{\cdot}0 \times 10^6$	$< 10^3$	$8{\cdot}0 \times 10^3$	$7{\cdot}6 \times 10^5$
7	$< 10^3$	$< 10^3$	$< 10^3$	$< 10^3$
10	$< 10^3$	$< 10^3$	$< 10^3$	$< 10^3$
14	$< 10^3$	—	$< 10^3$	$< 10^3$
21	$< 10^3$	—	$< 10^3$	—
28	$< 10^3$	—	—	—
35	$< 10^3$	—	—	—
42	$< 10^3$	—	—	—
49	—	—	—	—

salmonellas were excreted for 5–6 days (Table 1) and low numbers ($< 10^3$/g. faeces), which could be detected only after enrichment, for 10–42 days at which time the animals were slaughtered. The symptoms were no more severe in the stressed calves than in the unstressed.

Table 2 lists the 'O' and 'H' agglutinin titres in the sera of the calves and shows that the calf excreting the organisms throughout the experiment (B49) also had the highest titres. 'H' titres seem a more reliable means of assessing infection than 'O' titres.

When faecal samples from three of the four inoculated calves were negative for *S. dublin* (42 days), the animals were transported for 7 hr. in a trailer together with two uninfected control calves, but separated from them by a double partition. The following day all six calves were slaughtered. Faecal samples were taken 2 hr. before slaughter, and *S. dublin* was demonstrated in the excretor (B49) and the two control calves. *S. dublin* was isolated from either the inner or outer surface or both of five of the six carcasses; the exception was an inoculated animal (719) (Table 3). The persistent excretor (B49) was positive for *S. dublin* from several sites, but the other inoculated calves were negative except for the surfaces of two and a liver sample from another. The organisms were demonstrated in abomasal contents and on the surface of the carcasses of the two control calves.

After slaughter one half of each carcass was hung in a chill room, where they were cooled to 0° C. at a relative humidity of 91 % and stored for one week. During storage they lost 6·6–12 % of their weight and the surfaces became very dry. Swabs taken after 1 week showed no *Salmonella* except on B49. This carcass and one other were frozen to −20° C. and tested again after 1 month. B49 was still positive, the organisms being isolated from swabs both from the outside and the inside of the carcass, while the other was negative.

Table 2. *'O' and 'H' agglutinin titres in sera of calves during the experiment*

No. of days after inoculation	B49				440				719				B47			
	MH	AH	MO	AO	MH	AH	MO	AO	MH	AH	MO	AO	MH	AH	MO	AO
0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10	1/40	—	1/40	—	—	—	—	—	—	—	—	—	1/160	1/40	—	—
15	—	—	1/40	1/40	—	—	1/40	—	—	—	—	—	1/80	1/80	—	—
20	1/320	1/320	1/80	1/160	1/40	1/40	—	1/80	1/40	1/40	—	1/80	1/320	1/320	—	1/320
28	1/320	1/320	1/160	1/80	1/80	1/40	—	—	1/40	1/40	—	—	1/160	1/320	—	—
35	1/640	1/640	1/40	1/20	1/160	1/160	—	1/40	—	1/20	—	—	1/160	1/160	—	—

MH = 'H' – antigen-suspension from 'Mellovax' Vaccine strain.
MO = 'O' – antigen-suspension from 'Mellovax' Vaccine strain.
AH = 'H' – antigen-suspension from field strain.
AO = 'O' – antigen-suspension from field strain.

Table 3. *Isolations of* S. dublin *from post-mortem samples*

	Inoculated calves				Control calves	
Organ (samples)	B49	440	719	B47	1217	1218
Swab from outside of carcass	+	–	–	–	–	+
Swab from inside of carcass	+	+	–	+	+	+
Lung	+	–	–	–	–	–
Liver	+	+	–	–	–	–
Spleen	+	–	–	–	–	–
Gall bladder	+	–	–	–	–	–
Small intestine	–	–	–	–	–	–
Colon	+	–	–	–	–	–
Kidney	–	–	–	–	–	–
Mandibular lymph nodes	–	–	–	–	–	–
Mesenteric lymph nodes	–	–	–	–	–	–
Prescapular lymph nodes	–	–	–	–	–	–
Crural lymph nodes	–	–	–	–	–	–
Muscle from forelimb	–	–	–	–	–	–
Muscle from hindlimb	–	–	–	–	–	–
Abomasal contents	–	–	–	–	+	+

DISCUSSION

All four inoculated calves showed symptoms of salmonellosis for 1 week, with fever, diarrhoea and dullness, but no loss of appetite. Fluctuations in environmental temperature did not seem to affect them but this could be explained by the fact that otherwise conditions of management were excellent, and the calves were penned singly.

Three of the calves ceased to excrete *Salmonella* 2–4 weeks after infection, but one continued to excrete the organisms until slaughtered at 6 weeks without showing symptoms of disease. After transporting this animal, *S. dublin* was recovered from lungs, liver and spleen at slaughter. The presence of the organism in lungs and spleen suggests that the infection could have been re-activated by the stress of transport typical of that to which very young calves are commonly subjected when moved from one part of the country to another.

The other three experimentally infected calves showed no symptoms of re-infection after stress and bacteriological examination of their organs and viscera for *Salmonella* gave negative results except for a single liver sample (calf 440). As one calf in the group was excreting *Salmonella*, they would all have been subjected to cross-infection, but having already passed through a stage of infection, they were apparently resistant to re-infection. Faecal samples positive for *Salmonella* obtained the next day from the two uninoculated control calves indicated that cross-contamination had occurred, despite the control calves being separated from the inoculated calves by a double partition. The fact that at post-mortem examination organisms were only detected in abomasal contents indicated that invasion had not, however, taken place. It is difficult to explain how cross-infection occurred. Had it been by contaminated droplets one would have expected initial lung infection with generalization and there was no evidence

of this. One can only assume that some faecal splashing had occurred, giving opportunity for the control calves to lick infected material.

During slaughter all the calves except one (719) became contaminated on the surface of the carcasses although handled under hygienic conditions. This demonstrated that when one calf is infected at slaughter, several carcasses may become contaminated on their surfaces, and confirms the findings of Sandbu (1960) who examined calves slaughtered a few weeks after salmonellosis due to *S. typhimurium*. Although none were found to be excreting *S. typhimurium* at slaughter, the organism was isolated from several organs and the viscera and also from the surface of the carcasses. Once there, the survival and growth of *Salmonella* will be decided by the conditions under which the meat is handled and stored.

The survival and growth of salmonellas on carcass meat will also depend upon how many are present initially on the surface. Theile (1970) found that less than 50 *S. dublin*/cm.^{2} would not survive cooling at 4° C. for more than 5 days. Greater numbers would survive up to 23 days, but they would not grow at that temperature. Four of our calves had probably a light contamination only, and no salmonellas were recovered after 7 days at 0° C. However, from calf B49 which was heavily infected, salmonellas were readily recovered after 7 days at 0° C. and also after a further month at −20° C. The rate at which *Salmonella* as superficial contaminants died during prolonged chilled or frozen storage has yet to be determined.

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REFERENCES

ANDERSON, E. S. (1968). Drug resistance in *Salmonella typhimurium* and its implications. *British Medical Journal* iii, 333.

ANDERSON, E. S., GALBRAITH, N. S. & TAYLOR, C. E. D. (1961). An outbreak of human infection due to *Salmonella typhimurium* phage-type 20a associated with infection in calves. *Lancet* i, 854.

BUXTON, J. C. (1960). Salmonellosis in calves. *Veterinary Record* **72**, 640.

EDEL, W., GUINÉE, P. A. M. & KAMPELMACHER, E. H. (1969). Studies about the occurrence of salmonellae in calves after slaughter. *Tijdschrift voor Diergeneeskunde* **94**, 1170.

GIBSON, E. A. (1961). Salmonellosis in calves. *Veterinary Record* **73**, 1284.

GIBSON, E. A. (1965). *Salmonella* infection in cattle. *Journal of Dairy Research* **32**, 97.

KAMPELMACHER, E. H., GUINÉE, P. A. M. & JANSSEN, L. M. (1964). Studies on the use of surface decontamination of *Salmonella*-contaminated mesenteric lymph nodes by boiling water. *Tijdschrift voor Diergeneeskunde* **89**, 504.

KITCHELL, A. G., INGRAM, G. C. & HUDSON, W. R. (1973). Microbiological sampling in abbattoirs. In *Society for applied Bacteriology Technical Series* **7**. *Sampling – Microbiological Monitoring of the Environment* (ed. R. G. Board and D. W. Lovelock). London: Academic Press.

NOTTINGHAM, P. M. & URSELMANN, A. J. (1961). *Salmonella* infection in calves and other animals. *New Zealand Journal of Agricultural Research* **4**, 449.

RANKIN, J. D., TAYLOR, R. J. & BURROWS, M. (1969). *Salmonella* infection in young calves. *Veterinary Record* **87**, 582.

SANDBU, P. (1960). Den hygieniske betydning av latente salmonellainfeksjoner hos slaktedyr. *Nordisk Veterinarmedicin* **12**, 29.

SMITH, H. W. (1965). The immunization of mice, calves and pigs against *Salmonella dublin* and *Salmonella cholera-suis* infections. *Journal of Hygiene* **63**, 117.

SOJKA, W. J. & FIELD, H. I. (1970). Salmonellosis in England and Wales 1958–1967. *Veterinary Bulletin* **40**, 515.

STOKES, J. L. & OSBORNE, W. W. (1955). A selenite brilliant green medium for the isolation of *Salmonella*. *Applied Microbiology* **3**, 217.

THEILE, H. (1970). Tanasitat von Salmonellen bein Kuhlhaus-temperaturen. Veterinary Dissertation, Free University of Berlin.

VERNON, E. (1970). Food poisoning and salmonella infection in England and Wales, 1968. *Public Health, London* **84**, 238.

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Experimental *Salmonella* infection in calves. 2. Virulence and the spread of infection

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SUMMARY

Calves with a serum 'H' titre of 1/160 developed a chronic form of salmonellosis after infection with *S. dublin*. Their growth rate was severely retarded by this illness. An aerogenic strain of *S. dublin* was found to be much more virulent than an anaerogenic strain. Faecal samples were superior to mouth swabs in detecting animals carrying *S. dublin*.

During transport two uninoculated calves became infected and after transport stress, latent carriers of *S. dublin* with faecal samples negative for *Salmonella* for 5 weeks, restarted excretion. At slaughter *Salmonella* were isolated from viscera and organs and from the surface of five of six carcasses.

INTRODUCTION

It is generally accepted that *Salmonella* infections are commonly transmitted via the mouth and the alimentary system (Gibson, 1965). Infected droplets may also play a part in the spread of animal salmonellosis (Taylor, 1965). Tannock & Smith (1971*a*, *b*) induced a carrier state in mice and sheep by intranasal inoculations. This fact, and the report of possible airborne transmission of *S. abony* in a hospital ward (Stankovic, Barac, Rkman & Lupi, 1971), support the possibility of airborne spread of infection.

The virulence of the bacteria is also important in the spread of salmonellosis. Walton & Lewis (1971) observed that anaerogenic strains of *S. dublin* are less virulent for mice than aerogenic strains.

Experimental salmonellosis was studied in four calves penned together, two inoculated with an aerogenic strain of *S. dublin* and two with an anaerogenic strain. The effects of transport stress on latent carriers was also studied, together with cross-contamination of uninoculated controls.

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MATERIALS AND METHODS

Animals

Four Guernsey calves, 1 week old, were purchased from two different farms. None of them had been vaccinated against *Salmonella*. Faecal samples were examined daily for 1 week to exclude the possibility of a natural infection. Although the farms had no history of *Salmonella* infections, all four calves had a serum 'H' agglutinin titre of 1/160 against *S. dublin*. In spite of this, it was decided to use the calves and study the effect of this possible protection on an experimental infection. All four calves were penned together and maintained at a temperature of 14–16° C. After inoculation they were kept until faecal samples had been negative for *Salmonella* for at least 5 weeks. Towards the end of the experiment two new calves of the same breed were introduced to study the effect of cross-contamination.

Bacteriology and serology

The four calves were inoculated orally with *S. dublin* at an age of 2 weeks. Animals 410 and 411 were inoculated with $1{\cdot}2 \times 10^8$ viable cells of an anaerogenic strain (W) of *S. dublin* (WM 0023, of bovine origin, kindly provided by Dr J. Walton, University of Liverpool) resistant to nalidixic acid. Calves 412 and 561 were inoculated with $1{\cdot}2 \times 10^8$ viable cells of an aerogenic strain (L) of *S. dublin* (06/8 B2, kindly provided by Dr A. H. Linton, University of Bristol), isolated from an outbreak of salmonellosis in calves and resistant to neomycin, tetracycline and ampicillin. Faecal samples taken as described in the previous paper and, from the 5th day, mouth swabs, were taken daily and examined bacteriologically by the methods described by Grønstøl, Osborne & Pethiyagoda (1974), except that tetrathionate broth (Muller-Kaufmann modification) (TB) was used in parallel with selenite broth (SB) for enrichment. When blood samples were taken for serology, the blood clot was examined for *Salmonella* by incubation in brain, heart infusion broth and subculturing on bismuth sulphite agar and brilliant green agar.

Because of the high 'H' agglutinin titres in the sera of the calves faecal samples from the dams were examined bacteriologically for evidence of *Salmonella* infection, but they were all negative.

Post-mortem samples and sera were examined using the materials and methods described by Grønstøl *et al.* (1974).

RESULTS AND DISCUSSION

After inoculation, all four calves developed symptoms of the chronic form of salmonellosis. Temperatures did not rise above 103° F. except in calf 410, where the temperature reached 105° F. the third day after inoculation. They all excreted small numbers of *Salmonella* in their faeces, rarely exceeding 10^3 bacteria/g. Diarrhoea persisted for more than 2 weeks in a more or less severe form and, despite feeding reasonably well, the calves put on little weight and were severely under weight at slaughter.

Table 1. *Changes in the 'H'-agglutinin titres in the sera of calves following oral infection with* $1{\cdot}2 \times 10^8$ *cells of* S. dublin

Days after inoculation	Inoculated calves				Control calves	
	410	411	412	561	1261	1262
0	1/160	1/160	1/160	1/160	.	.
2	1/160	1/80	1/320	1/160	.	.
13	1/320	1/160	1/320	1/160	.	.
19	1/160	1/160	1/160	1/320	.	.
26	1/320	1/80	1/160	1/40	.	.
35	1/160	1/80	1/160	1/80	.	.
40	1/20	0	– *	.	.	1/80
63	1/20	0	0	1/160	0	0

* Not examined.

Table 2. *'H'-agglutinin titres in the dams of experimental calves*

Mother of calf no.					
Inoculated calves				Control calves	
410	411	412	561	1261	1262
1/80	1/160	1/160	1/80	0	1/160

The development of chronic salmonellosis rather than an acute form was probably caused by the calves being protected to some degree by humoral antibodies. The 'H' titres throughout the experiment are shown in Table 1. Because of the high 'H' titres in the calves at the start of the experiment, sera from their dams were examined approximately $2\frac{1}{2}$ months after parturition (Table 2).

Rankin & Taylor (1970) found a fourfold increase of antibodies in the udder compared with serum, and the antibodies could readily be transferred to calves through colostrum during the first hours after birth. They also found that calves having a serum 'H' titre of 1/128 or more did not develop a bacteraemia when challenged with 10^7 cells of *S. typhimurium*, although they developed severe diarrhoea. This agrees with our findings for *S. dublin* and implies that if calves with passive immunity become infected, they are more likely to contract chronic salmonellosis, which will considerably retard their growth rate.

For 3 days after inoculation, both the anaerogenic (W) and the aerogenic (L) strains were isolated from faeces of all four animals on one or more occasions, and on the third day strain L was isolated from the blood of calf 410, which had been inoculated with strain W. After the third day, only strain L was isolated from all four calves. More faecal samples than mouth swabs were found to be positive for *Salmonella* (Table 3). Faecal samples from calves 410 and 411, infected with strain W, were negative for *Salmonella* after 1 week, but the other two continued to show the presence of the organism for 18 and 29 days respectively. Calves 410 and 411 did, however, show positive mouth swabs on days 18 (410) and 25, 28 and 29 (411) respectively, suggesting they had been licking material contaminated by calves 412 and 561.

Table 3. *Recovery of* S. dublin *from faecal samples and mouth swabs of calves after oral infection by* $1{\cdot}2 \times 10^8$ *cells of* S. dublin

	Calves inoculated with anaerogenic strain W				Calves inoculated with aerogenic strain L			
	410		411		412		561	
Days after inoculation	Mouth swab	Faeces	Mouth swab	Faeces	Mouth swab	Faeces	Mouth swab	Faeces
1	.	W & L	.	W & L	.	–	.	W & L
2	.	–	.	–	.	W & L	–	W & L
3	.	W & L	.	W & L	.	W & L	.	–
4	.	–	.	L	.	L	.	–
5	–	L	–	–	L	L	–	–
6	–	L	–	L	L	L	–	–
7	–	–	–	–	L	–	–	–
8	–	–	–	–	L	L	L	L
9	–	–	–	–	L	L	L	L
10	–	–	–	–	L	L	–	L
11	–	–	–	–	L	L	–	L
12	–	–	–	–	L	L	L	L
13	–	–	–	–	L	L	–	L
14	–	–	–	–	–	L	–	–
15	–	–	–	–	L	L	–	L
16	–	–	–	–	L	L	–	–
17	–	–	–	–	–	L	–	–
18	L	–	–	–	–	L	–	L
19	–	–	–	–	–	L	–	–
20	–	–	–	–	–	L	–	–
21	–	–	–	–	–	L	–	–
22	–	–	–	–	–	L	–	–
23	–	–	–	–	–	L	–	–
24	–	–	–	–	–	L	–	–
25	–	–	L	–	–	L	–	–
26	–	–	–	–	–	L	–	–
27	–	–	–	–	–	L	–	–
28	–	–	L	–	–	L	–	–
29	–	–	L	–	–	L	–	–

There was a great difference in the virulence of the two strains of *S. dublin*, i.e. in their ability to invade, multiply and persist within an animal. The only blood sample positive for *S. dublin* came from calf 410 on the third day after inoculation, when its temperature rose to 105° F., and strain L was isolated in spite of the calf being inoculated with strain W.

Because the calves excreted a mixture of the two strains on the day after inoculation, it is assumed that cross-contamination occurred, although the possibility of contamination of the anus of one calf by the faeces of another cannot be completely ruled out. Strain L persisted much longer than strain W in all four calves; beyond the third day after inoculation, only strain L was isolated from faecal samples, mouth swabs and post-mortem samples. Considering only strain L, calves 412 and 561 were infected with $1{\cdot}2 \times 10^8$ cells, whereas calves 410 and 411 were infected by cross-contamination. Although it is impossible to assess the

Table 4. *Isolations of* S. dublin *from post-mortem samples of calves infected orally with* $1{\cdot}2 \times 10^8$ *cells* S. dublin

	Inoculated calves				Control calves	
Sample	410	411	412	561	1261	1262
Swab of outside of carcass	+	−	+	+	+	+
Swab of inside of carcass	−	−	−	−	−	−
Muscle from forelimb	−	−	−	−	−	−
Muscle from hindlimb	−	−	−	−	−	−
Pool of head lymph nodes	+	−	−	−	−	−
Mesenteric lymph nodes	−	−	−	−	+	+
Hepatic lymph nodes	−	−	−	−	−	−
Parotid salivary gland	+	−	−	−	−	−
Liver	−	−	−	−	−	−
Spleen	−	−	−	−	−	−
Lung	−	−	−	−	−	−
Caecum	−	+	+	+	+	+
Small intestine	+	−	−	+	−	+
Gall bladder	−	−	+	+	−	−

cross-infective dose, it is likely to have been smaller than the inoculum of 10^8 cells. The difference in the duration of excretion in the two sets of calves (Table 3) may reflect the difference in size of infective doses, as reported by de Jong & Ekdahl (1965).

Samples of feed, water and flies from the pen were examined bacteriologically during the experiment, but all were negative for *Salmonella*.

To test the effect of stress during transport, 5 weeks after the last occasion on which *Salmonella* had been demonstrated in faecal samples the calves were transported for 7 hr. together with two uninoculated calves, but separated from them by a double partition. The following day, three of four inoculated calves and both uninoculated control calves were positive for *Salmonella* from faecal samples.

Thirty-six hours after transportation all six calves were slaughtered and examined bacteriologically (Table 4). All calves except 411 became contaminated on the surface of their carcasses during the slaughter operations. Salmonellas were also found in the intestines, the mesenteric lymph nodes, or the gall bladder of one or more animals; caecum samples from all but calf 410 were positive. In calf 410 salmonellas were isolated from the parotid salivary gland and from a pool of lymph nodes from the head. This calf had been eager to lick the other calves and possibly for that reason carried *Salmonella* in these organs, although mouth swabs were positive on only one occasion. After refrigerated storage of the carcasses at 4° C. for 3 days, none was positive for *Salmonella* on the surface.

The finding of *Salmonella* in viscera and organs of all the calves and in the faeces of three out of four indicated that at least one, but probably more than one, of the four inoculated calves were latent carriers of strain L, and the stress of transport restarted its excretion. In contrast, in an earlier experiment with inoculated calves, all but one of which had cleared the infection before being moved (Grønstøl *et al.* 1974), transport did not restart excretion. In that experiment, the

calves produced an acute response to infection, whereas in the current one they developed chronic salmonellosis. Though agglutination tests failed to confirm the possibility, the first group of calves may have developed a higher titre of immunizing antibody.

In general, the results clearly confirm the greater virulence of aerogenic strains in meat animals as well as mice (Walton & Lewis, 1971); a fact explaining the failure of up to 10^{10} cells of Walton's anaerogenic strain (W) to kill sheep or cause extensive cross-infection (Kitchell & Leach, 1973).

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REFERENCES

DE JONG, H. & EKDAHL, M. O. (1965). Salmonellosis in calves – the effect of dose rate and other factors on transmission. *New Zealand Veterinary Journal* **13**, 59.

GIBSON, E. A. (1965). *Salmonella* infection in cattle. *Journal of Dairy Research* **32**, 97.

GRØNSTØL, H., OSBORNE, A. D. & PETHIYAGODA, S. (1974). Experimental *Salmonella* infection in calves. 1. The effect of stress factors on the carrier state. *Journal of Hygiene* **72**, 155.

KITCHELL, A. G. & LEACH, T. M. (1973). Observations on the artificial infection and natural cross-infection of housed sheep with *Salmonella dublin* and its subsequent transmission to meat. *M.R.I. Memorandum* no. 4. Langford: Meat Research Institute.

RANKIN, J. D. & TAYLOR, R. J. (1970). An attempt passively to immunize calves against *Salmonella* infection by vaccination of their dams. *Veterinary Record* **86**, 254.

STANKOVIC, M., BARAC, V., RKMAN, D. & LUPI, D. (1971). Possible airborne transmission of *S. abony* in a hospital ward. U.S. Department of Health, Education and Welfare/Public Health Service. *Salmonella Surveillance*. Report no. 109, 6.

TANNOCK, G. W. & SMITH, J. M. B. (1971*a*). A *Salmonella* carrier state involving the upper respiratory tract of mice. *Journal of Infectious Diseases* **123**, 502.

TANNOCK, G. W. & SMITH, J. M. B. (1971*b*). A *Salmonella* carrier state of sheep following intranasal inoculation. *Research in Veterinary Science* **12**, 371.

TAYLOR, J. (1965). Modern life and salmonellosis. *Proceedings of the Royal Society of Medicine* **58**, 167.

WALTON, J. R. & LEWIS, L. E. (1971). Preliminary studies of some biochemical properties of enteric and genital isolates of *Salmonella dublin* and a possible correlation with reduced virulence. *Veterinary Record* **89**, 112.

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A virological study of post-vaccinal encephalitis

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SUMMARY

In the province of Voyvodina 18 cases of post-vaccinal encephalitis have been recorded, three of which were fatal.

The estimated morbidity rate was 0·6 per 10^5 after revaccination, and 3·9 per 10^5 after primary vaccination. The virological studies described, as well as other findings referred to in this report, are consistent with the possibility of an auto-immune aetiology of the post-vaccinal encephalitis cases observed.

INTRODUCTION

During the last smallpox outbreak in Yugoslavia, in the province of Voyvodina, 91 % of the total population of 1,950,268 was successfully vaccinated during April and May 1972. According to the official data (Vuković, Miškov & Mudrić, 1973) 60,896 persons missed vaccination; in 50,392 persons vaccination was contra-indicated; in 68,212 vaccination failed; 1,770,750 persons were successfully vaccinated, and the remaining 18 persons developed post-vaccinal encephalitis.

Of the 18 encephalitis cases 11 (0·6 per 10^5 vaccinees) occurred after revaccination and seven cases (3·9 per 10^5 vaccinees) after primary vaccination. Details of these 18 cases are shown in Table 1. From the nine surviving patients a total of 16 serum samples were collected (at the 3rd to 17th day of illness, i.e. at the 14th to 29th day after vaccination), and from five of these patients six CSF samples were taken.

The 18 encephalitis cases were all admitted to hospital. The clinical findings, treatment and results have been fully reported by Orovačanec, Mudrić, Vučković & Vuković (1973).

The origin of the samples tested, and some data relating to the history of survivors donating samples of CSF and serum, are shown in Table 2. In the three patients who died the onset of illness occurred on the 11th to the 19th day after vaccination, and the patients died on the 1st or 2nd day after the onset of manifest illness.

CF titres in the serum samples ranged from 1/4 to 1/32 and the samples of CSF were negative even in dilution 1/1. The results of serological studies will be fully discussed elsewhere; here we present only the results of our attempts to recover virus from the samples of CSF.

MATERIALS AND METHODS

Before the addition of antibiotics, samples of undiluted CSF and serum were seeded on blood agar plates and into thioglycollate and plain broth media. After 48 hr. incubation all media remained sterile. Samples of CSF intended for inoculation were preserved with penicillin 1000 units/ml. and streptomycin 2 mg./ml. and stored at $-70°$ C. for 1–14 days.

Seed virus was prepared from 96 hr. old, once frozen and thawed cultures of vaccinia virus in primary rabbit kidney cells.

Media and diluents contained penicillin 100 units/ml. and streptomycin 100 μg./ml. Maintenance medium was medium 199 (pH 7·4–7·6) with 5 % calf serum and antibiotics. Growth medium was medium 199 (pH 7·2–7·3) with 20 % calf serum and antibiotics.

If not stated otherwise, inocula were both prepared and diluted with the maintenance medium.

Inoculation of eggs

Before inoculation, eggs were incubated at 38–39° C. Chorioallantoic membranes (CAM) of 12-day-old chick embryos (CE) were seeded with 0·2 ml. of inoculum per CAM, incubated at 36° C and candled twice daily.

Sluggish and dead CE within 24 hr. after inoculation were discarded. Two days after inoculation dead CE were harvested, and 3 days after inoculation both dead and live CE were harvested and the number of typical pocks was counted on each CAM. The sensitivity of the procedure used for isolation of vaccinia virus was tested by inoculation of CAM with tenfold dilutions of seed virus. The LD50 of the seed virus was a dilution of 10^{-5}, and a 10^{-7} dilution of the same suspension produced 5 to 15 pocks per CAM, with no death of embryos until the 72nd hour of incubation.

Primary cultures of trypsinized kidney cells

These were prepared by distribution of 1 ml. volumes of freshly made suspensions into tubes which were incubated without rolling. Cell cultures were kept in growth medium for 4–6 days at 37° C, with one fluid change after 48 hr. Confluent monolayers of cells were inoculated by adding 0·2 ml. inoculum to each tube.

The sensitivity of RK cell cultures for propagation of vaccinia virus was tested by inoculation of the cultures with dilutions of seed virus. Depending upon the dilution of the inoculum, CPE appeared within 24 hr. to 4 days after infection with vaccinia virus. Rounded-up cells of darker appearance were scattered throughout the field, first single and later aggregated. As the CPE spread, degenerated cells fell off the glass, leaving only small isolated aggregations of rounded cells. At the 4th day of incubation a $10^{-5·3}$ dilution of the seed virus induced CPE in 50 % of the tubes.

Herpes simplex virus gave an ID50 mostly in dilutions of $10^{-6·3}$. It is known that primary cultures of RK cells are sensitive also for other members of the herpes group, as well as for simian foamy virus and for some arboviruses. It

Table 1. *Post-vaccinal encephalitis in Voyvodina*

Age group (years)	Number of cases after: Primary vaccination	Revaccination	Total cases	Fatal cases
5–14	2 (1 S+F)*	3 (1 S+F)	5	1
20–68	5 (2 S+F)	8 (1 S+F) (4S)	13	2
All ages	7	11	18	3

* The figures in parentheses indicate numbers of patients from whom specimens were taken for examination. S+F = serum and cerebrospinal fluid; S = serum only.

should be noted that in cases of herpes simplex encephalitis the chances are especially poor for the isolation of the virus from lumbar CSF (MacCallum, 1969).

Inoculation of baby mice

Inoculation of 1-day-old sucklings with the seed virus (0·01 ml. cerebrally or 0·05 ml. peritoneally), produced pronounced runting, decreased motility, tremor, cyanosis and death of all animals within 2–6 days.

For detection of vaccinia virus, the inoculation of suckling mice proved to be inferior to the inoculation of cell cultures or CAM of embryos. Moreover, 10-day-old sucklings, or older animals, proved to be significantly less sensitive to cerebral or peritoneal infection with vaccinia than 1- or 2-day-old sucklings.

In our laboratory herpes simplex virus has been easily recovered by cerebral inoculation of suckling or adult mice with vesicular fluid of herpetic eruptions or with 10^{-5} dilutions of herpes simplex virus-infected tissue culture fluids. It is known that baby mice are a sensitive host also for the isolation of Coxsackie A and B, LCM, rabies and of many strains of arboviruses.

RESULTS AND CONCLUSIONS

Six samples of undiluted CSF were inoculated onto CAM. Each sample was inoculated onto six membranes, and after 72 hr. incubation the membranes were searched for pocks. Suspensions of membranes previously inoculated with single CSF were pooled, and 20% suspensions of the six membrane pools were sub-inoculated, each onto six membranes.

No suspect lesions could be detected on any of the 72 membranes inoculated.

Three CSF were inoculated in dilutions 1/4, each into four tubes. Samples of the other three CSF were inoculated undiluted, into four tubes each.

After allowing 2 hr. for viral adsorption, the growth medium was replaced by maintenance medium and the cultures were incubated at 35° C.

After 4 days no CPE could be observed in any of the 24 tubes. Two of each group of four tubes seeded with single fluids were frozen to minus 70° C., and the other two tubes were observed for a further 3 days. None showed signs of CPE.

Pairs of frozen cultures, seeded with the same CSF, were thawed, pooled, and 0·2 ml. of the undiluted pools were subinoculated, each into a new group of four cell cultures, in an attempt to recover virus through blind passage. After 8 days

Table 2. *Origin of the material tested*

Designation of samples (patients)	Illness		CS fluid drawn at day		CF titres	
	Onset at p.v. day	Duration in days	Of illness	P.v.	In CS fluid	In serum**
1 (1)*	11	33	3	14	< 1/1	1/16
2 (2)	12	32	5	17	< 1/1	1/16
3 (3)	8	15	6	14	< 1/1	1/32
4 (4)	12	15	7	19	< 1/1	1/32
5 (5)	7	26	15	22	< 1/1	1/4
6 (1)*	11	33	17	28	< 1/1	1/8
Average	10	20	11	19	< 1/1	1/14

P.v. = post-vaccination.
* Two samples obtained from the same patient.
** Drawn on the same day as the CS fluid.

no signs of CPE could be detected in any of the second series of 24 cell cultures. Thus, from none of the six CSF could either vaccinia or other viruses be recovered.

Each sample of the six CSF was inoculated into four suckling mice. Three groups of 1- to 5-day-old animals were inoculated with samples of three fluids diluted 1/4, both cerebrally (0·01 ml. per mouse) and peritoneally (0·05 ml. per mouse). Each of these sucklings received thus 0·06 ml. of CSF. Undiluted samples of the other three fluids were inoculated cerebrally into groups of 9-day-old mice (0·03 ml. per mouse). None of the mice showed signs of infection. Four to six baby mice from each of the six inoculated litters were left uninoculated to serve as controls.

Thus, from none of the CSF samples could either vaccinia or other viruses be recovered.

DISCUSSION

No virus could be recovered from any of the acute phase CSF specimens and this is consistent with the possibility of allergic encephalomyelitis.

Our patients had not been skin-tested with brain material, but the histopathological findings of Dožić (personal communication) on fatal cases in Belgrade, were suggestive of allergic encephalomyelitis and this is the currently accepted view in post-vaccinal encephalitis (Dixon, 1962).

REFERENCES

DIXON, C. W. (1962). *Smallpox*. London: J. and A. Churchill Ltd.

MACCALLUM, F. O. (1969). Herpes simplex virus encephalitis. In Whitty, C. W. M., Hughes, J. T. & MacCallum, F. O., *Virus Diseases and the Nervous System*. Oxford and Edinburgh: Blackwell Science Publications.

OROVAČANEC, K., MUDRIĆ, V., VUČKOVIĆ, S. & VUKOVIĆ, B. (1973). Neuropsihijatrijske komplikacije u vezi sa antivariolicnom vakcinacijom. *Medicinski Pregled* (in the Press).

VUKOVIĆ, B., MIŠKOV, D. & MUDRIĆ, V. (1973). Postvakcinalne komplikacije u Vojvodini posle masovne vakcinacije protiv velikih boginja 1972 godine. Jugoslovenski simpozijum o varioli Primošten 1972. *Zbornik radova* (in the Press).

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Skin reactivity and antibody response following vaccination against smallpox

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SUMMARY

Revaccination between 2 and 5 years after the last vaccination induced higher complement-fixation titres than revaccination 7–63 years after the last vaccination. The highest CF titres were reached during the 3rd to the 5th week after vaccination.

Fifty-two serum samples, taken 137 or more days after a successful vaccination, all showed CF titres lower than 1/2.

In a group of successfully vaccinated persons with no post-vaccinal complications, 65 out of 66 had a successful 'take' after one or two subsequent vaccinations.

Sera of persons receiving primary vaccination showed significantly lower CF titres than sera of revaccinated persons. Cases of post-vaccinal encephalitis showed greater antibody response than uncomplicated successfully vaccinated cases in both the primary vaccination and revaccination groups. The antibody response in revaccinated persons with post-vaccinal encephalitis was greater than that in the group of successfully revaccinated cases without encephalitis.

A group of 26 naturally hyporeactive persons completely failed to 'take' even after vaccination repeated between 3 and 10 times (average 5 times per person). The frequency of seropositives in this group, and the height of their CF titres, were significantly lower than in the group of successfully vaccinated persons. These findings support the view that untreated persons who repeatedly fail to 'take' with the vaccine must not be considered immune.

In vaccinated persons treated with immunoglobulin (with or without simultaneous treatment with Marboran) antibody production was apparently diminished. Treatment of patients with Marboran significantly lowers the capacity of the vaccine to 'take'.

INTRODUCTION

At the very beginning of the smallpox outbreak in Yugoslavia conflicting opinions emerged regarding several problems.

In Voyvodina, the following two rules were adopted by most of the vaccinators during the vaccination campaign in 1972: (1) Any person who had been successfully vaccinated within the last 3 years who failed to 'take' with four successive revaccinations was considered to be protected. (2) If vaccination failed in primary contacts or persons at high risk of being contacts, such persons were vaccinated

repeatedly until the vaccine 'took' or until the end of the campaign. Generally, repeated failures to 'take' with a vaccine of proven potency, after introduction by a well-controlled technique, have been ascribed to: (1) a solid, specific, cellular or humoral immunity of the host; (2) a natural 'skin immunity factor' or an increased local production of interferon in an otherwise receptive organism, or (3) some other defence mechanisms of the skin.

Cases of post-vaccinal encephalitis were treated conservatively by some, though the majority advocated the use of antivaccinal immunoglobulin.

All contacts were vaccinated and most were also treated with immunoglobulin. Most of the contacts were also treated with Marboran, but some physicians did not use this drug.

As a result of these differences of opinion, both theoretical and practical, we studied the appearance of skin reactivity and antibody response to vaccination, as well as the correlation of these findings with the vaccination status, in different categories of people.

MATERIALS AND METHODS

Serum donors

The vaccine was introduced by two parallel scratches. A total of 193 serum samples, collected from 186 individuals, were tested by complement-fixation (CF) test. The age of these persons varied from 5 to 68 years, the mean age being 39 years. Of the 186 persons tested 166 (89 %) had been vaccinated 2–68 years (average about 30 years) before 1972, and 20 (11 %) of them were first vaccinated in 1972.

According to the case-histories, they were divided into five groups.

Group 1. Vaccinated successfully April 1972, with no complications (66 persons).

Group 2. Not vaccinated in 1972, but a history of vaccination 3–63 years earlier (47 persons).

Group 3. Vaccinated repeatedly without success. 'Naturally hyporeactive' (28 persons).

Group 4. Vaccinated successfully and given 500–700 units of immunoglobulin (36 persons). Some of these received Marboran (26) or cortisone (2) as well.

Group 5. Vaccinated successfully and developed post-vaccinal encephalitis (9 persons).

In group 3, three of the hyporeactive persons had failed with their previous vaccinations, attempted 2, 9 and 20 years ago respectively. Sixteen others in this group carried marks of successful previous vaccinations. In group 5, sixteen serum samples were collected from the nine cases, and from five of these cases six samples of cerebrospinal fluid were obtained. Results with these CSF samples are described in a previous paper (Terzin, Mašić, Vuković & Mudrić, 1974). The onset of encephalitis took place 7–16 days (average 11·6 days) after vaccination. The duration of illness varied from 5 to 33 days (average 17 days).

Sera were preserved by sodium azide (final concentration 0·08 %), stored at 4° C and inactivated at 57° C for 30 min. No serum-sample with anticomplementary activity (even in dilution 1/1) was used for these studies.

Complement-fixation (CF) technique

This was a modification of Hoyle's technique, described earlier (Terzin *et al.* 1954), with overnight fixation at 4° C. Titres are expressed as dilutions of serum before addition of other reagents (i.e. the initial dilution and not the final).

The antigen used was the supernatant obtained by centrifuging at 1500 rev./min. for 3 min. the homogenized smallpox vaccine, i.e. vaccinia, produced by the Tashkent Research Institute for Vaccines and Sera. Its anticomplementary activity was less than 1/2. In checkerboard titration with a rabbit antiserum this antigen had a maximum titre of 1/32–1/64 so that working dilutions of 1/10 or 1/20 were selected as containing about 3 antigen units/unit volume. This antigen showed no cross-reactions with antisera against *Toxoplasma gondii*, *Mycoplasma pneumoniae* and *Rickettsia burneti*.

The statistical significance of the differences observed between frequencies of positive reactors has been evaluated by the chi-square test, and that of the differences between mean titres by the *t*-test (Chambers, 1952; Diem, 1960).

RESULTS AND DISCUSSION

Skin reactivity to vaccination

Table 1 shows the multiplicity of vaccinations applied in order to obtain a successful take, as well as the statistical significance of the findings listed.

Among the 66 successfully vaccinated persons, an unequivocal reaction occurred after one vaccination in 54 (81 %) and after two vaccinations in a further 11 (17 %). The last person required a third vaccination for a positive result. This is in contrast to the group of naturally hyporeactive persons in whom repeated vaccinations failed completely, as well as the group of persons treated with Marboran, who showed significantly inferior reactivity to vaccination (in both cases, at a significance level of $P < 0{\cdot}0001$), than the group of successfully vaccinated persons.

Twenty-six of the 28 naturally hyporeactive persons failed entirely to 'take' the vaccine, even after 3–10 times (average 4·5 times) repeated vaccinations per individual.

Most (77 %) of the patients treated simultaneously with Ig and Marboran had to be vaccinated 3–6 times (average 3·4 times), and the rest (23 %) twice subsequently, in order to attain a positive reaction. However, the group treated with Ig alone proved to be as responsive to vaccination as the untreated, successfully vaccinated, group of persons. It appears that treatment with Marboran supresses an adequate propagation of vaccinia virus, thus making the vaccine behave as one of diminished potency.

Both in the group of successfully vaccinated persons, with no post-vaccinal complications, and in the group of vaccinees displaying post-vaccinal encephalitis, the multiplicity of effective vaccinations was the same, amounting on the average to about 1·1. In about 83 % of the cases belonging to these two groups, the vaccine produced unequivocal takes at its first application.

Table 1. *Types of reactivity to vaccination in* 1972

Groups of vaccinees		No. of persons with multiplicity of vaccination						Mean no. of vaccinations per person (significance of difference)
		1 ×	2 ×	3 ×	4 ×	5 - 10 ×	Total	
Successfully vaccinated (v.)	Primary	5	1	—	—	—	6	1·17 ($P > 0·2$)
	Revaccinated	49	10	1	—	—	60	1·20 (null hypothesis)
Hyporeactive to revaccination		1	1	2	16*	8	28*	4·48 ($P < 0·0001$)
Revaccinated persons treated with	Anti-vacc. Ig	7	—	—	1	—	8	1·38 ($P > 0·2$)
	Cortisone and anti-vacc. Ig	2	—	—	—	—	2	1·0 ($P > 0·2$)
	Marboran and anti-vacc. Ig	—	6	14	1	5*	26*	3·38 ($P < 0·0001$)
Post-vaccinal encephalitis	Primary	3	—	—	—	—	3	1·11 ($P > 0·2$)
	Revaccinated	5	1	—	—	—	6	—
Totals		72	19	17	18	13	139	—

* One of these persons was receiving primary vaccination.

Table 2. *Complement-fixing antibody response in groups of vaccinated persons*

Groups of vaccinated persons		Successfully vaccinated			Hyporeactive to vaccination		Post-vaccinal encephalitis			Treated with immunoglobulin	
		13–33*	34–136*	137–163*	13–33*	85–121*	13–33*	34–136*	140*	13–33*	40–121*
Revaccinated 2–5 years after last vaccination	Pos/tstd†	2/2	2/2	—	—	0/4	—	—	—	—	5/15
	(%)	—	—	—	—	—	—	—	—	—	(33)
	g.m.t.‡	22·6	11·3	—	—	1·7	—	—	—	—	3·0
	Range	16–32	8–16	—	—	< 2–4	—	—	—	—	< 2–16
Revaccinated 7–63 years after last vaccination	Pos/tstd†	12/17	10/35	0/4	0/4	0/19	5/6	3/3	—	1/2	2/19
	(%)	(71)	(29)	—	—	—	(83)	—	—	—	(11)
	g.m.t.‡	10·2	3·1	1·7	1·7	1·4	22·6	16·0	—	5·7	2·2
	Range	< 2–128	< 2–32	< 2–2	< 2–4	< 2–4	4–64	8–32	—	4–8	< 2–16
Primary vaccination	Pos/tstd†	0/2	0/4	—	0/1	—	5/5	1/1	0/1	—	—
	(%)	—	—	—	—	—	(100)	—	—	—	—
	g.m.t.‡	1·0	1·7	—	1·0	—	13·9	8·0	1·0	—	—
	Range	< 2	< 2–2	—	< 2	—	8–32	8	< 2	—	—
No. of serum samples tested		21	41	4	5	23	11	4	1	2	34

* Days after the last vaccination in 1972 when the serum was tested.
† Pos/tstd = number of sera positive at > 1/4/number of sera tested.
‡ g.m.t. = geometric mean titre.

Table 3. *Statistical evaluation of some serological results*

Vaccination status of serum donors in 1972	Sera drawn at day following vaccination in 1972	Frequency of positive sera* (percentage)	Significance of differences between frequencies observed		
Not vaccinated; last vaccination 3–63 yr. ago	—	0/47 (0)	n.h.	< 0·0001	0·005
Successfully revaccinated (7–63 yr. after last vaccination)	13–33	12/17 (71)	< 0·0001	n.h.	0·02
	34–136	10/35 (29)	0·005	0·02	n.h.
Hyporeactive to revaccination (7–63 yr. after last vaccination)	13–33	0/4 (0)	> 0·2	0·05	—
	85–121	0/19 (0)	> 0·2	—	0·05
Encephalitis following revaccination in 1972; last vaccination 7–63 yr. ago	13–33	5/6 (83)	0·0001	> 0·2	—
	34–136	3/3 (100)	0·005	—	0·05
Revaccinated persons treated with Ig; last vaccination 7–63 yr. ago	40–121	2/19 (11)	> 0·2	—	0·2

* CF titre > 1/4.
n.h. = null hypothesis

Results of CF tests

Some data related to the antibody response of vaccinated persons and the statistical evaluation of some findings are shown in Tables 2 and 3. The frequencies of positive serum samples and/or the heights of the mean titres, observed in the various groups of vaccinees, seem to allow for the following conclusions:

CF titres reach their highest levels during the 3rd to 5th week after primary or revaccinations, subsequently falling.

After the 136th day after vaccination in 1972, no serum was found with titre higher than 1/2 (see Table 2). This finding was supported also by the fact that 47 serum specimens, drawn from patients unvaccinated in 1972, all showed CF titres lower than 1/2.

These findings agree with those of McCarthy, Downie & Bradley (1958) which were also confirmed by others.

Sera drawn from groups of persons revaccinated 2–5 years (average 3·7 years) after the last vaccination, showed suggestively higher CF titres than sera drawn from persons revaccinated 7–63 years after the last vaccination, provided that the samples were drawn at comparable intervals after revaccination in 1972 (see Table 2).

Sera drawn after primary vaccination show significantly lower CF titres than sera drawn after revaccination, with the exception of serum samples drawn from cases of post-vaccinal encephalitis.

The geometric mean of the CF titres (1/16), in sera of three patients with p.v.

encephalitis, proved to be significantly higher than the mean titre (1/3·1) found in sera of successfully vaccinated uncomplicated cases (see Table 2).

In patients with p.v. encephalitis even primary vaccination produced an antibody response comparable with, if not greater than, that shown by uncomplicated revaccinees (see Table 2).

In primovaccinees who developed p.v. encephalitis the antibody response was significantly greater than in successfully primovaccinated persons without post-vaccinal encephalitis.

In the group of naturally hyporeactive persons the frequency of positives was significantly lower than in the group of successfully revaccinated persons. Only four of the 19 hyporeactive cases (21 %) showed a titre of 1/4, and none showed titres higher than 1/4. In the group of successfully vaccinated persons (bled at comparable intervals after vaccination), 20 of the 35 cases (57 %) showed titres of 1/4 or higher. These findings are in accordance with the well-known rule, that failure to take the vaccine must not be regarded as evidence of immunity, usually supported by examples of smallpox cases, some fatal, appearing shortly after failure to take repeated vaccinations (van Rooyen & Rhodes, 1948; Dixon, 1962; Downie, 1965, and others).

The group of 36 patients treated with immunoglobulin alone, or simultaneously with Marboran, showed a suggestively weaker antibody response than the group of successfully vaccinated and otherwise not treated persons. No difference in antibody response could be detected between those treated with Marboran plus immunoglobulin, and those treated with immunoglobulin alone or with immunoglobulin and cortisone combined. The small number of vaccinees given combinations of immunoglobulin with Marboran or cortisone did not permit statistical comparisons.

The frequencies of positives shown in Table 2 were calculated by adoption of antibody titres > 1/4 (i.e. 1/8 or higher) as positive. When adopting as positive antibody titres > 1/2 (i.e. 1/4 or higher), instead of > 1/4, we find an increase in the frequency of positives, but no significant alteration of the differences revealed between the various groups of persons. For example, the group of persons revaccinated successfully in 1972, 6–63 years after the last vaccination, and bled at 13–33 days, 34–136 days and 137–163 days after revaccination in 1972, showed 70·6, 28·6 and 0 % of positives at a > 1/4 titre respectively. However, the frequency of positives at a titre > 1/2, in the same three groups of vaccinees, amounted to 88·3, 57·2 and 0 % respectively.

In order to minimize the risk of getting non-specific cross-reactions from any non-vaccinal antibodies that may have been present we took as 'positive' a titre of 1/8 or greater

REFERENCES

Chambers, E. G. (1952). *Statistical Calculations for Beginners.* Cambridge University Press.

Diem, K. (1960). Mathematische und statistische Tabellen. In *Documenta Geigy, Wissenschaftliche Tabellen.* Basle: J. R. Geigy A.G.

Dixon, C. W. (1962). *Smallpox.* London: J. and A. Churchill Ltd.

Downie, A. W. (1965). Poxvirus group. In Horsfall, Jr. F. L. and Tam, I.: *Viral and Rickettsial Infections of Man.* Philadelphia, Toronto: J. B. Lippincott Co.

McCARTHY, K., DOWNIE, A. W. & BRADLEY, W. H. (1958). The antibody response in man following infection with viruses of the pox group. II. Antibody response following vaccination. *Journal of Hygiene* **56**, 466–78.

TERZIN, A. L., BORDJOŠKI, M. N., MILOVANOVIĆ, M. V., STOJKOVIĆ, LJ. V. & DIMIĆ, M. M. (1954). Some viral, rickettsial and leptospiral infections diagnosed in Serbia. *Journal of Hygiene* **52**, 129–50.

TERZIN, A. L., MAŠIĆ, M., VUKOVIĆ, B. & MUDRIĆ, V. (1974). A virological study of post-vaccinal encephalitis. *Journal of Hygiene* **72**, 169–72.

VAN ROOYEN, C. E. & RHODES, A. J. (1948). *Virus Diseases of Man*. New York: Thomas Nelson and Sons.

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Isolation of a virus responsible for an outbreak of acute haemorrhagic conjunctivitis in Morocco

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SUMMARY

An epidemic of acute haemorrhagic conjunctivitis occurred in Morocco in 1970–1. It was caused by an enterovirus which appeared to be a new antigenic type similar to a virus isolated in South East Asia during the same period.

INTRODUCTION

In December 1970 and January 1971 an epidemic of acute haemorrhagic conjunctivitis (A.H.C.) occurred in several towns in Morocco (Bourdieu, 1972). Because no bacteria could be isolated from conjunctival secretions, these secretions were stored and sent frozen at $-20°$ C. from Rabat to the WHO Regional Centre for Enteroviruses in Lyon, France. The study of this material led to the isolation of a virus in the conditions described below.

MATERIALS AND METHODS

Clinical material

We obtained 100 conjunctival scrapings and swabs from patients with A.H.C. in Morocco within a few days of the onset of illness.

Cell cultures

Primary cultures of cynomolgus monkey kidney cells were prepared in tubes and incubated without rolling at 36° C. Eagle's minimum essential medium and lactalbumin-Earle's medium, each with 10% calf serum, were used as growth media. The maintenance medium was Parker's 199.

Human embryo fibroblasts (WI 38) and HeLa cells were grown at 36° C. without rolling in Eagle's basal essential medium with 10% calf serum. The maintenance medium was Parker's 199 with 2% foetal calf serum.

RESULTS

Ten strains, R5, R6, R7, R11, R20, R39, R73, R86, R87 and R98, were isolated on HeLa cells. The CPE was comparable with that caused by agents of the Picornavirus group. Attempts to isolate viruses on other cells were all unsuccessful.

The ten strains belonged to the same antigenic type, as shown by neutralization tests using an antiserum prepared in a monkey (*M. irus*) by inoculation with strain R6.

Further tests on the strains revealed the following characters:

(i) Infected cells showed an eosinophilic intracytoplasmic inclusion similar to the typical Picornavirus inclusion.

(ii) In the acid stability test infectivity was not diminished at pH 3·0.

(iii) Electron microscopic examination showed a characteristic Picornavirus structure with a diameter of 30 nm. and no envelope.

(iv) The virus was not neutralized by antisera against any of the following viruses: poliovirus types 1–3, Coxsackie A types 1–24, Coxsackie B types 1–6, echovirus types 1–33.

(v) One of the strains (R39), when inoculated into baby mice subcutaneously, provoked a slight temporary flaccid paralysis at the first passage but no symptoms at the second passage. All the other nine strains failed to produce any flaccid paralysis after two passages.

(vi) Monkey antiserum prepared against strain R6 has been tested against prototype enterovirus strains. Up to the present time this serum has failed to neutralize poliovirus types 1–3, Coxsackie A types 1–24, Coxsackie B types 1–6 and echovirus types 1–33.

(vii) Cross-neutralization tests between the 'A' Japanese strain (Kono *et al.* 1972) and the Moroccan R6 virus, using sera prepared in monkeys against the Japanese strain in Tokyo and the Moroccan strain in Lyon, show that these two viruses are of the same antigenic type.

DISCUSSION

Outbreaks of epidemic haemorrhagic conjunctivitis occurred in 1969 in Ghana (Chatterjee, Quarcoopome & Apenteng, 1970*a*, *b*) and in Nigeria (Parrott, 1971). No virus was grown in these cases, but the disease, which was called 'Apollo 11 disease', was clinically and epidemiologically similar to that later described in South East Asia and Japan.

A cytopathic agent was isolated from cases of epidemic haemorrhagic conjunctivitis in Singapore (Lim & Yin-Murphy, 1971). Preliminary evidence from the laboratory investigations of the virus strains grown from conjunctival swabs suggested that the agent was not an adenovirus. Later (Yin-Murphy, 1972) the laboratory findings suggested that the virus was a Picornavirus, not neutralized by 42 available enterovirus antisera, and it was referred to as 'Singapore Epidemic Conjunctivitis (1970) Virus'.

At a WHO Regional Seminar in Manila in December 1971, it appeared that

Singapore, Indonesia, Malaysia, Cambodia, Thailand, Hong Kong, the Philippines and Taiwan had been experiencing extensive outbreaks of a new type of conjunctivitis.

In Japan, a virus was isolated in 1971 (Kono *et al.* 1972) and it is suggested that this agent is an enterovirus of a new serotype. No neutralization was observed with antisera against poliovirus 1–3, echovirus 1–33 (except types 10 and 28), reovirus 1, Coxsackie B1–6, Coxsackie A7, 9 and 16. Electron microscopy showed virions with cubic symmetry, and a diameter of 29 nm.

An outbreak of acute conjunctivitis was observed in Singapore in 1970, but the virus isolated appeared to be antigenically different from Singapore Epidemic Conjunctivitis 1970 virus (Yin-Murphy & Lim, 1972).

In 1971, North Africa experienced an outbreak of the new epidemic conjunctivitis and 10 strains of an enterovirus were isolated in Morocco. The results reported here suggest that these strains are similar to those obtained in Singapore and Japan.

Cross neutralization between the Japanese strain and the antiserum prepared against it in Tokyo and the Moroccan strain R6 and the antiserum prepared against it in Lyon suggests that the Japanese and North African strains are identical.

The monkey antiserum prepared against the Moroccan strain did not neutralize any of the existing prototype enteroviruses and, with the Japanese and Singapore results (Kono *et al.* 1972; Lim & Yin-Murphy, 1971; Yin-Murphy, 1972; Yin-Murphy & Lim, 1972) this reinforces the suggestion that the enterovirus isolated in each place is a new serotype.

REFERENCES

Bourdieu, J. F. (1972). A propos de l'épidémie de conjonctivite observée au Maroc en 1971. Thèse, Faculté de Médicine, Toulouse.

Chatterjee, S., Quarcoopome, C. O. & Apenteng, A. (1970*a*). An epidemic of acute conjunctivitis in Ghana. *Ghana Medical Journal* **9**, 9–11.

Chatterjee, S., Quarcoopome, C. O. & Apenteng, A. (1970*b*). Unusual type of epidemic conjunctivitis in Ghana. *British Journal of Ophthalmology* **54**, 628–30.

Kono, R., Sasagawa, S., Ishii, K., Siginra, S., Ochi, M., Matsumiya, H., Uchida, Y., Kameyama, K., Kaneko, M. & Sakurai, N. (1972). Pandemic of new type of conjunctivitis. *Lancet* i, 1191–4.

Lim, K. H. & Yin-Murphy, M. (1971). An epidemic of conjunctivitis in Singapore in 1970. *Singapore Medical Journal* **12**, 247–9.

Parrott, W. F. (1971). An epidemic called Apollo. An outbreak of conjunctivitis in Nigeria. *Practitioner* **206**, 253–5.

Yin-Murphy, M. (1972). An epidemic of Picornavirus conjunctivitis in Singapore. *Southeast Asian Journal of Tropical Medicine and Public Health* **3**, 303–9.

Yin-Murphy, M. & Lim, K. H. (1972). Picornavirus epidemic conjunctivitis in Singapore. *Lancet* ii, 857–8.

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Recent trends in human salmonellosis in England and Wales: the epidemiology of prevalent serotypes other than *Salmonella typhimurium*

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SUMMARY

In the period 1960–70 meat and poultry products caused over 70% of successfully investigated outbreaks of human salmonellosis. The number of human incidents of salmonellosis declined from 1960 to 1966, but then more than doubled between 1966 and 1971. This increase was mainly due to a threefold increase of incidents of salmonella serotypes other than *Salmonella typhimurium*. The serotypes which increased most and contributed significantly to this trend were *S. enteritidis*, *S. panama*, *S. stanley*, *S. virchow*, *S. agona*, *S.* 4,12:d:- and *S. indiana*.

Strong evidence exists that these serotypes have a path of infection from animal feedingstuffs to the pig and poultry animal reservoirs to pork and poultry foods to man. Cattle appear to be a less important source than pigs and poultry and this may be because the nature and content of their feed is different.

The importance of the control of the pig and poultry reservoirs of salmonella infections is stressed and a significant role of animal feedingstuffs in the maintenance of these reservoirs strongly suggested.

INTRODUCTION

Since 1960 two major trends in the epidemiology of human salmonellosis have become evident. These are an increase in the proportion of outbreaks caused by meat and meat products, and an increase in the number of human incidents caused by salmonella serotypes other than *S. typhimurium*.

From 1949 to 1959 meat and meat products were the commonest vehicle of infection, accounting for 47% of the outbreaks; 27% were attributed to eggs and egg products, 15% to sweetmeats and 11% to other foodstuffs (Galbraith, 1961). In the period 1960–70, however, the proportion of outbreaks associated with meat products increased to 71%, while eggs and egg products were responsible for only 4% (Reports, 1961–4; Vernon, 1965–7; Vernon, 1969–70; Vernon, unpublished). The decline in the importance of egg products was due to the introduction of legislation in January 1964 requiring the compulsory pasteurization of domestic and imported liquid egg used in the manufacture of egg products (Statutory Instrument, 1963). Some producers carried out voluntary pasteurization for a few years before legislation was introduced.

Table 1. *Number of human incidents of* Salmonella *infection reported to the Epidemiological Research Laboratory*, 1960–71

	1960	1961	1962	1963	1964	1965	1966	1967	1968	1969	1970	1971
S. typhimurium	2907	2503	1864	1820	1725	1721	1407	1810	1654	1512	1865	2124
Other salmonellas	1047	1268	982	1149	1368	1224	1089	1449	2142	3305	3360	3540
Total	3954	3771	2846	2969	3093	2945	2496	3259	3796	4817	5225	5664

Table 2. *Number of human incidents of certain prevalent serotypes reported to the Epidemiological Research Laboratory*, 1960–71

	1960	1961	1962	1963	1964	1965	1966	1967	1968	1969	1970	1971
S. panama	9	7	8	12	39	86	95	209	379	307	517	247
S. stanley	68	42	50	65	19	50	85	262	169	195	144	28
S. virchow	7	5	2	25	25	10	2	31	253	326	123	93
S. enteritidis	145	90	93	64	122	180	114	143	271	649	650	511
S. 4,12:d:-	—	—	—	—	—	—	—	—	38	156	50	30
S. agona	—	—	—	—	—	—	—	—	—	2	232	620
S. indiana	—	—	—	1	2	10	11	90	87	164	143	180

Table 1 shows the number of human incidents of salmonella infection reported to the Epidemiological Research Laboratory for the years 1960–71. The number of incidents due to *S. typhimurium* fell from 2907 in 1960 to 1407 in 1966 and fluctuated between that figure and 2124 in 1971. In contrast incidents due to other salmonellas remained fairly constant until 1966 and then increased each year from 1089 in 1966 to 3540 in 1971 – more than a triple increase. Total salmonella infections, therefore, declined from 3954 in 1960 to 2496 in 1966 and then more than doubled to 5664 in 1971.

The second change was thus a very large increase in the number of incidents of salmonella serotypes other than *S. typhimurium* after 1966. The serotypes which increased most in incidence and contributed significantly to this trend were – *S. enteritidis*, *S. panama*, *S. stanley*, *S. virchow*, *S. agona*, *S.* 4,12:d:- and *S. indiana*. This report examines the epidemiological features associated with the prevalence of these serotypes.

SOURCES OF INFORMATION

Reports of isolations have been taken from the annual reports of food poisoning of the Public Health Laboratory Service (Reports, 1950–64; Vernon, 1965–7; Vernon, 1969–70) from Taylor *et al.* (1965) and from information abstracted from the Communicable Disease Reports of the Public Health Laboratory Service for the years 1964–71.

THE SEROTYPES

S. panama

S. panama was not isolated from humans in the United Kingdom before the war. In 1940 Scott examined the mesenteric lymph glands of pigs, but *S. panama* was not one of the serotypes found. During the war dried egg contaminated with salmonellas was imported into the United Kingdom from the U.S.A., Canada and the Argentine and any found unfit for human consumption was distributed to animal food manufacturers. Some of it was fed to pigs, without sterilization, and in 1944 pig mesenteric glands were examined to see whether salmonellas had been introduced into the pig population. *S. panama* was isolated from four such samples in 1944 (Medical Research Council, 1947) and in the same year it was isolated from a human case. From 1944 to 1963, however, *S. panama* caused only 68 human incidents, but after 1963 the number increased considerably (Table 2) reaching a peak in 1970.

From the mid-1960's *S. panama* was isolated on several occasions from animal, including pig, feed ingredients, from pigs, abattoirs and bacon factories; and from pork, sausages and made-up meat.

Investigation of human incidents

In August and September 1967 there were 154 human isolations of *S. panama*, a notable seasonal increase over the three previous years. Cases were widely distributed throughout the country. People of all age groups were affected, several elderly people severely enough to require hospital admission, and two patients

died. Most cases were sporadic in occurrence, but a few family and hospital outbreaks were reported.

In an investigation of sporadic cases in London (unpublished) it was not possible to find a common vehicle and source of infection. In one case, however, cold roast pork appeared to be the vehicle of infection and it was possible to trace its source via a butcher and wholesaler to an abattoir where *S. panama* was isolated from three of several swabs taken from pigs.

In a family outbreak in 1969 firm bacteriological evidence was secured that cooked ham was the vehicle of infection and in 1970 that roast pork was the vehicle in a large general outbreak affecting over 300 persons.

Firm epidemiological evidence was thus obtained that pork products were responsible for transmitting *S. panama* to man. More recently *S. panama* has been found to have contaminated processed poultry. Between 1969 and 1971 a total of 29 isolations from poultry were reported. The chief source of human infection remains, however, pigs.

S. stanley

S. stanley was isolated from humans in the United Kingdom before the last war. It caused very few human cases until 1955 when there were 101 human incidents, most of these being attributable to pork (Report, 1956). There were 56 incidents in 1956, but few in the following years until 1960 when there were 68 incidents. The number of incidents remained fairly steady until 1965. There was an increase in 1966 and a peak was reached in 1967 when *S. stanley* was the commonest human serotype other than *S. typhimurium* (Table 2). After 1967 the number of incidents declined, but *S. stanley* remained among the commonest human serotypes until 1970.

S. stanley was isolated a number of times from coconut in the early 1960's. Thereafter it was isolated most commonly from animal, including pig, feed ingredients, pigs, abattoirs, bacon factories and sausages and made-up meat. There were very few isolations from poultry.

Investigation of outbreaks

During the period 1964 to 1969 there were five outbreaks due to *S. stanley* in which pig products were implicated bacteriologically or epidemiologically. The foods responsible were boiled ham (twice), pork products, pork sausage and pork pies. In a sixth outbreak sausage (unspecified) was implicated.

In June and July 1968 an outbreak affecting 13 persons, with one fatal case, occurred in the West Riding of Yorkshire. The cases were associated with pork products and isolations of *S. stanley* were made from an abattoir supplying the suspected pork.

At the end of August 1968, an outbreak affecting 46 persons occurred in a West Midlands Hospital and a further 49 cases were reported from West Midlands counties. Investigation of 15 cases in two districts strongly suggested that infection was distributed in cooked meat, pork pies and sausages prepared by one local manufacturer. This manufacturer and six of the retail outlets were investigated.

One excreter of *S. stanley* was found at the factory and nine excreters at four of the six retail outlets.

As with *S. panama*, there was strong evidence that *S. stanley* infection originated from pigs and was transmitted via abattoirs and pork products to man.

S. virchow

S. virchow was very rarely isolated in the United Kingdom until 1968. The first single human case occurred in this country in 1942. From 1941 to 1953 there were only four human incidents. Twenty-one incidents occurred in 1955 and then only a few until 1963 when there were 25, the same number being recorded in 1964 (Table 2). From 1965 to 1967 there were 43 incidents. In 1968, 253 incidents occurred and a further increase occurred in 1969, when there were 326 incidents.

Since 1967 chickens have been the main non-human source of *S. virchow*. It has been isolated on numerous occasions from poultry for human consumption and also from poultry processing plants, chickens on farms and feed ingredients, particularly products of poultry origin such as feather meal and poultry offal meal. It has been found on only a few occasions in pigs and other animal sources.

Investigation of outbreaks

Most cases of *S. virchow* in 1968 and 1969 occurred in North West England. Cases first started to occur in this region towards the end of 1967 when there was an outbreak in a county borough and the surrounding districts. The vehicle of infection was chicken and *S. virchow* was isolated from faecal specimens of staff and chicken samples at three chicken retailers, and from staff members and a sewer swab at two processing plants supplying them. Cloacal swabs of birds arriving at one of the plants yielded *S. virchow* on culture.

In the summer of 1968 a second outbreak due to chickens occurred in the North West (Semple, Turner & Lowry, 1968). The source was traced to a packing station where *S. virchow* was isolated from chicken carcasses. The organism was also found in chickens on farms supplying the packing station (Pennington, Brooksbank, Poole & Seymour, 1968).

Undoubtedly, *S. virchow* was introduced into chicken flocks in the North West and gave rise to contaminated processed chicken which resulted in outbreaks and sporadic cases of human infection.

S. enteritidis

S. enteritidis was prevalent in England and Wales when records started in 1923. In the 1940's it caused between 10 and 50 human incidents annually. Between 1950 and 1967 the number of incidents varied between 70 and 200. Table 2 shows that the number of incidents almost doubled in 1968 when 271 were recorded and more than doubled again in 1969 when over 600 incidents occurred, the same number being reported in 1970. The number of incidents declined slightly in 1971.

In the early 1960's *S. enteritidis* was isolated on a few occasions from egg products but since 1967 poultry, particularly chickens, has been the only human food in which it has been found in substantial numbers. The organism has been isolated

from abattoirs and poultry factories, from the main food animal sources, particularly poultry, and from feed ingredients.

Investigation of outbreaks

Thirty-five outbreaks due to *S. enteritidis* were reported from 1966 to 1969. One outbreak occurred amongst persons who attended two wedding receptions in September 1969; of 250 guests, 55 were taken ill. *S. enteritidis* was cultured from the faeces of 45 patients. The incriminated food appeared to be cooked chicken. At the first reception the chicken was cooked, allowed to cool and served cold. At the second reception the chicken had been cooked, cooled and then re-heated. One firm of caterers cooked the chickens for both wedding receptions. *S. enteritidis* was cultured from one of two unconsumed whole cooked chickens from the caterers.

This outbreak was the only one in which bacteriological proof of a food vehicle was obtained. In the other outbreaks no food was available for examination, or no food samples had been taken. But in nine of the outbreaks a food was implicated on epidemiological grounds. Chicken was the vehicle in six; turkey, scotch egg and liver pâté in one each.

It is clear that the cause of the increase in *S. enteritidis* infections was contaminated poultry from infected flocks.

S. 4,12:d:-

S. 4,12:d:-, an un-named monophasic serotype, was isolated for the first time in England and Wales in 1968.

There is circumstantial evidence that this serotype may have been introduced into England and Wales by an imported feed ingredient. *S.* 4,12:d:- was isolated from three sewer swabs at a poultry processing plant in September 1968. Subsequently it was isolated at the plant from chicken carcasses and giblets, factory water and sewer swabs on a number of occasions until the end of January 1969. It was isolated from cloacal swabs taken from chickens which arrived at the processing plant from three chicken broiler farms and subsequently from the faecal droppings on one of these farms, but not from the feed. Cultures made from infertile eggs at the hatchery where the chickens were hatched were negative. Samples of feed and droppings from two breeding farms supplying the eggs to the hatchery were also negative. However, feed supplied to one of the breeding farms contained South African fish pellets from a consignment that arrived in England and Wales in August 1968. *S.* 4,12:d:- was isolated from a sample taken at the manufacturer in January 1969. It was also found in a sample of meat and bone meal from the same feed compounder in December 1969. Thus, a contaminated imported consignment of a feed ingredient appears to have introduced the serotype into a chicken breeding flock, whence it was transmitted via eggs to the chicken broilers and so to processed chickens and equipment in the processing plant.

Table 2 shows the number of human incidents of *S.* 4,12:d:- from 1968 to 1971. In 1969, the year after its introduction, it was amongst the ten commonest serotypes isolated from humans. The number of incidents fell in 1970 and 1971.

Chicken has been the commonest food vehicle of *S.* 4,12:d:- but there have also

been several isolations from pork, sausages and made-up meat. The organism has often been found in chicken factories and abattoirs, in chickens and pigs and in feed ingredients.

S. 4,12:d:- was probably introduced into England and Wales in 1968 by an imported South African feedstuff. It rapidly established itself in the domestic pig and poultry population, contaminated pork and poultry foods and caused human outbreaks and sporadic cases throughout the country.

S. agona

S. agona had only rarely been isolated in England and Wales before 1970. In 1969 two cases were reported in patients who had returned from Spain (Public Health Laboratory Service Report, 1972). In May 1970 there were a number of human incidents in the North of England and Wales (Public Health Laboratory Service Report, 1970). Poultry was implicated in three incidents and pork in one. *S. agona* was isolated from a mincing machine and chopping block in a food shop which supplied one affected family with chickens. It was also isolated from a wooden stacking tray on a farm where the chickens had been killed. In June 1970 *S. agona* was isolated from chicken carcasses from a farm in the West Midlands. Both the farmer and his wife had had gastro-enteritis in May and *S. agona* was isolated from the farmer and his two children, who were symptomless. *S. agona* was also isolated from 12 of 19 sets of broilers' giblets from a batch killed in June 1970 in the North East and from imported fish meal used as raw material for animal food in the same area.

In 1970 and 1971 *S. agona* was frequently isolated from processed poultry, pork, sausages and made-up meat; and from poultry processing plants, abattoirs, chickens, pigs and feed ingredients.

Table 2 shows that in 1970 there were 232 human incidents of *S. agona* whereas in 1971 there were 620 and it was the second commonest serotype isolated from man.

How did *S. agona* first get into England and Wales? The most likely explanation for the appearance of a new serotype seems to be importation in an animal feed ingredient. *S. agona* was isolated from imported fish meal in May 1970 and it is likely that this introduced the organism into domestic livestock in England and Wales. Since then it has been isolated from feed ingredients including meat and bone meal, feather meal and poultry offal meal. All these are treated animal by-products. It is likely that it is now being maintained through re-cycling of these treated animal wastes which are fed back to the animals. It is clear that pork and poultry have become contaminated causing numerous cases of human infection.

S. indiana

The first isolation of *S. indiana* in England and Wales was from a sample of fish meal in 1958. In 1959 and 1960 isolations were made from American meat meal. The first recorded human incident of *S. indiana* occurred in 1963, but there were only a few in the following years until 1967 when there were 90 incidents (Table 2).

The number of incidents increased from 1969 to 1971 during which time *S. indiana* has been among the 10 commonest salmonellas causing human infection.

The increase in human incidents was accompanied by numerous isolations from food sources, particularly chicken, from pigs and poultry and from feed ingredients.

Investigation of outbreaks

In 1969, *S. indiana* was isolated from eight of ten patients with food poisoning in the North of England (Public Health Laboratory Service Report, 1969). The patients had all eaten spit-roasted poultry from a local shop. The shop received eviscerated unfrozen chickens from the company's own processing plant. They were kept in a refrigerator until required and then cooked on a rotary spit for 70 min. at a temperature of 350° F. After cooking, the chickens were removed from the spits and placed whole or quartered in the shop window where, even with a fan operating, the temperature was found to be as high as 84° F. A number of samples of chickens and swabs from various articles of equipment used in the preparation and handling of the chickens were examined for salmonellas with negative results, though subsequently *S. senftenberg* was isolated from two uncooked carcasses. However, at the processing plant, *S. indiana* and other serotypes were isolated from carcasses and rinse water.

Pork has also been shown to be the vehicle of infection in outbreaks due to *S. indiana*.

It is clear that *S. indiana* has gained access to the domestic poultry and pig population, contaminated poultry and pork after slaughtering and processing, and caused cases of human infection.

DISCUSSION

The salmonella serotypes which have been prevalent during recent years are closely associated mainly with pigs and poultry, but cattle do not appear to be an important source. There are probably two main reasons for this. First, there has been a greater increase in the amount of pork and poultry produced and consumed than in that of beef and veal. Between 1964 and 1971 the annual production of beef and veal increased from 862 to 938 thousand tons, an increase of 9%, while consumption did not increase, but remained at about 1170 thousand tons annually. In the same period the annual pig meat production increased from 776 to 938 thousand tons and the number of pig slaughterings increased from 12,804 to 15,957 thousands, increases of 20%. The estimated consumption of poultry meat increased from 15·8 lb. per head of population in 1964 to 23·6 lb. in 1971, an increase of 49% (*Meat and Dairy Produce Bulletins*). During this period imports of beef and veal did not increase, but remained at about 250 thousand tons annually, a quarter of total consumption; imports of pig meat (mostly bacon), remained at about 400 thousand tons annually, about half the quantity home-produced; and imports of poultry meat, only a small fraction of home production, remained at about 10,000 tons annually (*Meat and Dairy Produce Bulletins*).

The second reason concerns feeding practice. There are two important differences between the feeding of cattle on the one hand and pigs and poultry on the other. First, 90–100% of compounded cattle feeds are in the form of pellets and cubes in which the salmonella content is reduced by heating during manufacture. In contrast, only 50–60% of pig feed and 55% of poultry breeder feed is pelleted (Riley, 1967). Secondly, pigs and poultry farmed under intensive conditions are dependent on prepared feeds for all their protein requirements and it is necessary to include in their rations sources of animal proteins such as meat and bone meal and fish meal, known to be frequently contaminated with salmonellas (PHLS Working Group, Skovgaard & Nielsen, 1972). Cattle, on the other hand, graze on pastures and do not require the same proportion of concentrated animal proteins in their compounded feeds which contain mainly vegetable proteins such as groundnut. The fact that the animal by-products known to be contaminated are fed mainly to pigs and poultry and not to cattle, and that it is pigs and poultry that have been implicated in the rise in the number of human infections by serotypes other than *S. typhimurium*, strongly suggests that these animal proteins have contributed significantly to this increase.

There is strong circumstantial evidence that imported feed ingredients introduced three serotypes, *S.* 4,12:d:-, *S. agona* and *S. indiana*, into England and Wales. These types were subsequently isolated from other feed ingredients such as meat and bone meal, feather meal and poultry offal meal, which are treated animal by-products. They continued to be isolated from the animals so that it is likely that they are maintained in their animal hosts by re-cycling through the feed ingredients.

The fluctuation in incidence of some of the serotypes is difficult to explain. It is possible that serotypes whose incidence has not varied so greatly such as *S. typhimurium* and *S. enteritidis* are able to establish a host–parasite relationship that allows for their intestinal carriage and transmission from animal to animal indefinitely. Those serotypes whose prevalence has fluctuated may be more dependent on introduction in feedingstuffs for their maintenance in animals than are *S. typhimurium* and *S. enteriditis*. In a longitudinal study of salmonellas in feed, on farms and in abattoirs (Lee, Ghosh, Mann & Tee, 1972) it was shown that feed introduced a number of serotypes into the pigs, but the longest they were found in pig faeces was one month.

Investigation of incidents and outbreaks showed a path of infection from the animal reservoir to the food vehicle to man. It is difficult to break the chain of infection once carcass contamination has taken place. There may be cross-contamination between raw and cooked meats and, by handling, to other foods and equipment. Salmonellas may survive high cooking temperatures if frozen foods are not adequately thawed throughout their depth. A subinfective dose of salmonellas may multiply to an infective dose if foods are left to stand at room temperatures. The importance of the study and control of the primary animal reservoir is thus obvious.

The role of feedingstuffs in introducing serotypes into these reservoirs and their maintenance in them has recently been investigated (PHLS Working Group *et al.*

1972; Lee *et al.* 1972). In the first of these studies a comparison was made between serotypes occurring in pigs in Denmark where there is legislation requiring the sterilization of feed ingredients of animal origin whether imported or home produced, and in England where there is no such legislation. In the second study infection in pigs at abattoirs was related to contamination of feeds on the farm. It was concluded from these studies that feedingstuffs played a significant role in infecting pigs in England and Wales with serotypes other than *S. typhimurium*. The same conclusion is reached if the serotypes in poultry in Denmark (Hansen & Marthedal, 1970) are compared with those in England and Wales.

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REFERENCES

GALBRAITH, N. S. (1961). Studies of human salmonellosis in relation to infection in animals. *Veterinary Record* **73**, 1296–303.

HANSEN, H. C. & MARTHEDAL, H. E. (1970). *Salmonella typhimurium* in poultry. (Four years of systematic investigations in breeding centres.) *XIV World's Poultry Congress, Madrid*, September 1970.

LEE, J. A., GHOSH, A. C., MANN, P. G. & TEE, G. H. (1972). Salmonellas on pig farms and in abattoirs. *Journal of Hygiene* **70**, 141–50.

Meat and Dairy Produce Bulletin, Commonwealth Secretariat. (1968) **21**, no. 7, 35; no. 11, 68; no. 12, 31.

Meat and Dairy Produce Bulletin, Commonwealth Secretariat. (1969) **22**; (1970) **23**; (1971) **24**; (1972) **25**.

MEDICAL RESEARCH COUNCIL (1947). The bacteriology of spray-dried egg, with particular reference to food poisoning. *Medical Research Council Special Report Series* no. 260.

PENNINGTON, J. H., BROOKSBANK, N. H., POOLE, P. M. & SEYMOUR, F. (1968). *Salmonella virchow* in a chicken-packing station and associated rearing units. *British Medical Journal* iv, 804–6.

PHLS WORKING GROUP, SKOVGAARD, N. & NIELSEN, B. BREST (1972). Salmonellas in pig and animal feedingstuffs in England and Wales and in Denmark. *Journal of Hygiene* **70**, 127–40.

PUBLIC HEALTH LABORATORY SERVICE REPORT. *British Medical Journal* (1969), **4**, 118; (1970), **3**, 353; (1972), **4**, 559.

REPORTS (1950–64). Food poisoning in England and Wales, 1949–1963. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service*, **9**, **10** and **13–23**.

RILEY, P. B. (1967). Salmonella infection: The position of animal food and its manufacturing process. *Royal Society of Health Conference on Salmonella Infection*, October 1967.

SCOTT, W. M. (1940). Salmonellas in healthy pigs at slaughter. *Proceedings of the Royal Society of Medicine* **33**, 366–8.

SEMPLE, A. B., TURNER, G. C. & LOWRY, D. M. O. (1968). Outbreak of food poisoning caused by *Salmonella virchow* in spit-roasted chicken. *British Medical Journal* iv, 801–3.

STATUTORY INSTRUMENT (1963). *The Liquid Egg (Pasteurization) Regulations*, 1963, no. 1503. London: H.M.S.O.

TAYLOR, J., KING, G. J. G., PAYNE, D. J. H., SANDIFORD, B. R. & STEVENSON, J. S. (1965). Sources of Salmonellae, 1951–1963. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* **24**, 164–229, 236–77.

VERNON, E. (1965–7). Food poisoning in England and Wales, 1964–1966. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* **24**, 321–33; **25**, 194–207; **26**, 235–49.

VERNON, E. (1969–1970). Food poisoning and salmonella infections in England and Wales 1967–1968. *Public Health, London* **83**, 205–23; **84**, 239–60.

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Serological studies with purified neuraminidase antigens of influenza B viruses

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SUMMARY

Neuraminidase (N) can be extracted from virus particles of influenza B strains by treatment with trypsin, in a form which is free from the viral HA and has specific immunological activity. The N antigen of B/LEE/40 behaves differently from that of 1965–6 strains in gel diffusion and enzyme inhibition tests with animal antisera raised by infection or by artificial immunization with the homologous or heterologous strains. The frequency and titres of NI antibody detected in human sera by B/LEE antigen are different from those found with antigen from B/Eng/13/65. The latter antibody appears to contribute to the effect of serum HI antibody in protecting volunteers exposed to a deliberate intranasal challenge infection of the B/Eng/13/65 strain.

INTRODUCTION

In the thirty-three years since the first isolation of influenza type B viruses (Francis, 1940) their world-wide distribution has been repeatedly observed, but they have never given rise to pandemics like those seen recurrently with influenza A viruses. The outbreaks tend to be localized and to affect children more commonly than adults in whom individual illnesses may be so mild as to pass unrecognized as anything more than a 'cold' unless serological investigations are performed. This may mean that type B viruses inherently lack virulence or epidemicity, but it is possible that exposure to any one strain may confer broad protection against subsequent strains or reduce the clinical effects of re-infection, as occurs with the parainfluenza and respiratory syncytial myxoviruses. The infectivity of type B virus is significantly reduced in volunteers with high pre-challenge titres of serum HI antibody against the homologous virus haemagglutinin (HA) (Hobson, Curry, Beare & Ward-Gardner, 1972). However, there has been considerable antigenic drift in the HA of strains isolated between 1940 and 1972 (Chakraverty, 1972*a*), and it seems unlikely that HI antibody against the prototype B/LEE strain of 1940 would have any protective value against present-day strains. It thus seems pertinent to investigate whether any one influenza B infection might induce widely reactive antibody against a type-common neuraminidase (N), which could

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contribute to the short-term effect of relatively strain-specific HI antibody and might play a major role in long-term immunity to type B influenza.

The role of N antigens has been less thoroughly investigated for type B than for type A viruses, largely because of technical problems. Segregation of HA and N antigens by genetic recombination is relatively easy for type A, but has not been achieved with type B. The use of suspensions of whole virions, containing both HA and N antigens, to measure NI antibody in postinfective serum tends to show false enzyme inhibition because of steric hindrance by homologous HI antibody also present in the serum. Recently the treatment of various type B strains with trypsin by a modification of the method of Noll, Aoyagi & Orlando (1962) has allowed both HA and N antigens to be separated in high yield (Brown, Hobson & Curry, 1973) and the present paper reports studies with partially purified N to investigate the NI responses of experimental animals and man after infection with various type B viruses and the possible role of NI antibody in conferring resistance to influenza B.

MATERIALS AND METHODS

The derivation and cultivation of all the virus strains used have already been described (Brown *et al.* 1973).

For the preparation of separate HA and N antigens, virus was recovered from infected allantoic fluids by centrifugation, washed in phosphate buffered saline (PBS A) and concentrated to 6000 HA units per ml. Trypsin (Difco 1/250) was added to a final concentration of 10 mg./ml. and after incubation with constant mixing at 37° C. for 45 min. the reaction was stopped by the addition of soybean trypsin inhibitor (BDH) to a final concentration of 10 mg./ml. Centrifugation of the mixture at 40,000 ***g*** for 1 hr. yielded a deposit containing virus particles with HA activity only, whereas the supernatant contained all the enzyme activity of the original virus suspension but was devoid of HA. Before use in the tests described below, N was separated from other viral material, trypsin and soybean extract by passage through a 1·5 × 20 cm. column of Sephadex G75 (Pharmacia). Enzyme activity was recovered quantitatively in the first of four protein peaks, which was shown by polyacrylamide gel electrophoresis to contain a single protein.

The enzyme was assayed by the Warren method with fetuin substrate, and NI titrations of sera were made with freshly prepared batches of separated enzyme; details of the methods are as described by Hobson *et al.* (1972).

Rabbit antisera were obtained by intramuscular inoculation at multiple sites with column-purified N (the amount obtained from 3000 HA units of virus) emulsified in an equal volume of complete Freund adjuvant (Calbiochem); the animals were bled 4 weeks later. Guinea-pig postinfective sera were obtained by cardiac puncture of 8-week-old animals 14 days after the second of two monthly intranasal instillations of 10^4 EID 50 (egg infective doses) of the appropriate virus strain in 0·05 ml. of PBS A; animals were inoculated under light ether anaesthesia. All procedures and housing were in separately ventilated quarantine rooms to avoid cross-infection. No clinical illness was noticed in any animal. No HI or NI activity was found in preinfective sera from these guinea-pigs.

Table 1. *The specific activity of B/LEE virus fractions obtained by treatment with trypsin*

		HA titre per ml.	N* titre/ml.	Serum titres of rabbits twice inoculated i.v. with 1 ml. of the fraction in soluble form: HI	NI†
Whole virus		5120	100	3072	500
Trypsinized virus	Deposit‡	2560	< 2	1536	15
	Supernatant‡	< 2	110	12	300

* Dilution of fraction which gives an optical density of 0·5 in the Warren test after release of NANA from 4·8 mg. fetuin substrate.

† Dilution of serum which gives 50% inhibition of this standard dose of N.

‡ Fractions were reconstituted to the original volume of whole virus suspension.

The volunteer experiments from which human sera were derived, virus challenge procedures and scoring of infection rates were as detailed by Beare, Hobson, Reed & Tyrrell (1969) and Hobson *et al.* (1972).

RESULTS

Separation of HA and N antigens

B/LEE virus was treated by the procedure described above. As shown in Table 1, viral HA apparently was recovered quantitatively with the virus particles after high-speed centrifugation; this deposit fraction had no detectable N enzymic activity. Inoculation into rabbits gave high HI titres but very little NI antibody. Conversely, the supernatant fraction had no detectable HA, but contained all the enzymic activity of the original virus suspension: rabbit antisera showed high NI titres but only low titres of HI antibody. It is probable that the small cross-activity of each fraction represents residual contamination which might be removable by more extensive purification procedures.

Serological responses of infected guinea-pigs

Pairs of animals were infected with B/LEE, B/Tai/4/62 or B/Ann Arbor/1/66 which are known to show large differences in their HA antigens. Convalescent sera were titrated with the separated HA and N antigens of each virus (Table 2). The HA antigens detected an entirely monospecific homologous HI response. B/LEE N demonstrated NI antibody only in homologous serum. The N antigens of B/Tai and B/Ann Arbor were inhibited to some degree by all the sera, but those from the homologously infected animals gave the highest titre; both of these antigens were considerably less effective in detecting NI antibody in B/LEE-infected guinea-pigs than was the homologous B/LEE N.

The cross-reactivity of rabbit antisera

Even after a more artificial and intensive immunization procedure than was used for the guinea-pigs, the N fractions of the three viruses again showed antigenic

Table 2. *The serological response of guinea-pigs after intranasal infection with* 10^4 *EID*50 *of influenza B viruses*

Test	Source of antigen	Serum antibody titres in guinea-pigs infected with		
		LEE	TAI	AA
HI	B/LEE/40	**384**	< 6	< 6
	B/TAI/4/62	< 6	**192**	< 6
	B/Ann Arbor/1/66	< 6	< 6	**1536**
NI*	B/LEE/40	**640**	< 10	< 10
	B/TAI/4/62	160	**750**	400
	B/Ann Arbor/1/66	25	600	**640**

* Titre = the dilution of serum which gives 50% inhibition of that dose of N which produces an optical density of 0·5 at 549 nm. (NANA release) from 4·8 mg. fetuin in the Warren test with normal rabbit serum.

Table 3. *The reaction of purified N from three type B virus strains with the corresponding immune rabbit antisera*

Source of N	NI titre* of antiserum raised against		
	LEE	TAI	13/65
B/LEE/40	**500**	300	20
B/TAI/4/62	120	**2000**	180
B/Eng/13/65	120	1000	**400**

* The measurement of titres is as described in Table 2.

heterogeneity in the NI test with rabbit antisera (Table 3) and in gel diffusion tests with the various antigens (Plate 1) B/LEE N appeared distinct from the N of the two more recent viruses.

The distribution of NI antibody in normal human sera

Sera from 92 unvaccinated adults with no recent history of influenza were titrated with the separated HA and N of B/Eng/13/65 virus, which is closely related antigenically to the B/Ann Arbor strain. NI antibody was detected in 87 (94%) sera (Fig. 1), but HI antibody was present in only 55 (60%). There was little correlation between the two titres, except that in general those with high HI titres also showed high NI titres. However, in those with no HI antibody, NI titres were found over a range of 1/50–1/500.

Sera from 80 unvaccinated normal adults were tested at a single dilution of 1/100 for their enzyme-inhibitory activity against comparable standardized doses of N fractions of B/LEE and B/Eng/13/65. Most sera (Fig. 2) showed NI activity against both antigens, but there was no apparent correlation between the two; the results suggest that each antigen might be measuring a separate and distinct NI antibody.

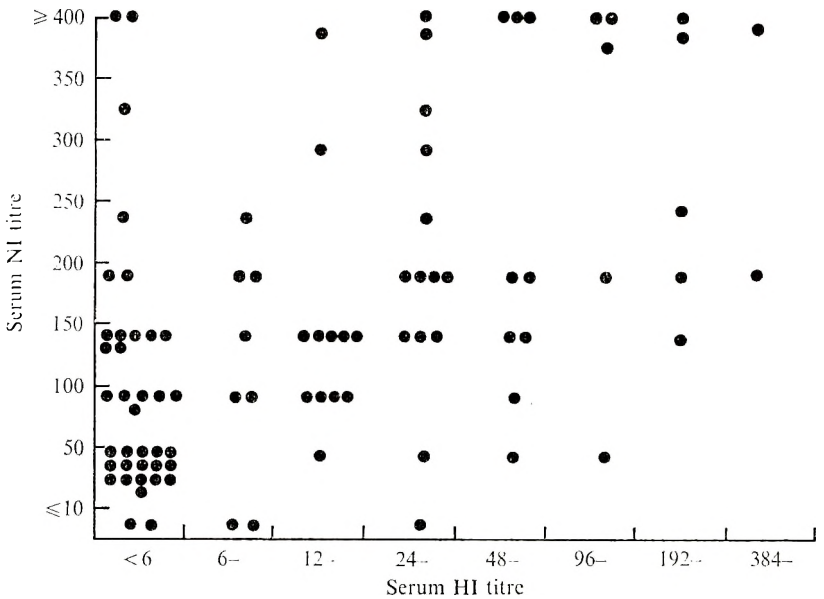

Fig. 1. The distribution of HI and NI serum antibody titres in 92 unvaccinated adults.

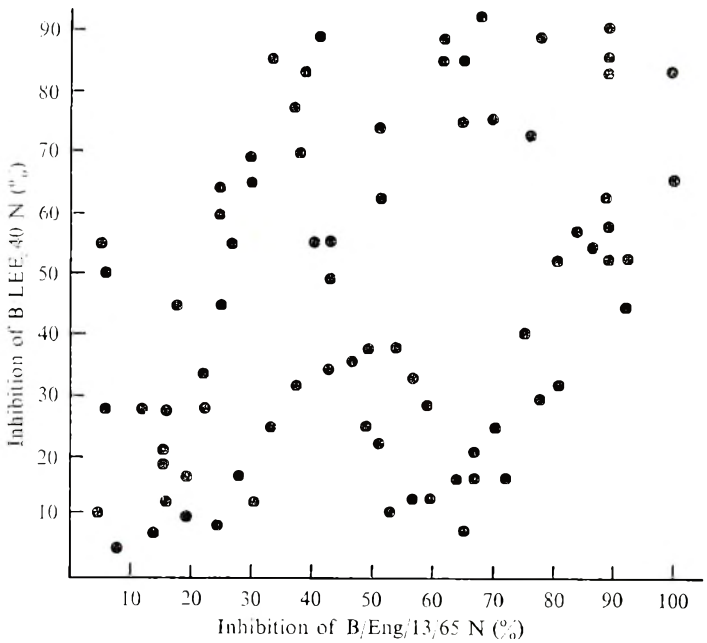

Fig. 2. The serum NI activity of 80 normal adults measured with the N antigen of two different influenza type B viruses. Each serum was used at a single dilution of 1/100 in the NI test (see text).

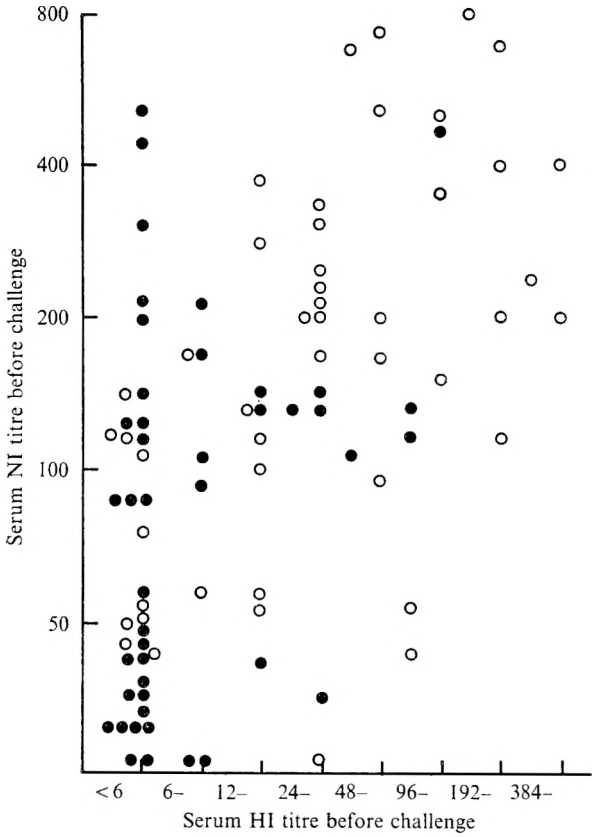

Fig. 3. The relation of serum HI titre and serum NI titre to protection against subsequent challenge infection of 91 volunteers with the homologous B/Eng/13/65 virus. Result of challenge: ● = infected; ○ = not infected.

Table 4. *The effect of pre-existing HI and NI serum antibodies on infection in 91 volunteers challenged with the homologous influenza B virus*

	Pre-challenge immune status of volunteer			
	High HI High NI	High HI Low NI	Low HI High NI	Low HI Low NI
Total no.	15	6	23	47
No. infected	1	2	10	31
Infection rate %	7	33	43	66

High HI ⩾ 48. High NI ⩾ 150.

The relation of NI antibody to protection against influenza

Ninety-one randomly chosen volunteers were given a challenge intranasal spray of B/Eng/13/65 virus immediately after a sample of serum had been obtained for HI and NI titrations against the homologous virus. The distribution of proved infections (Fig. 3) was predominantly in those with low pre-existing serum HI titres, but it also seemed that those with low titres of NI antibody were more susceptible to influenza than those with titres of 1/150 or greater. However, further

analysis of these results (Table 4) suggest that those with high titres of NI antibody alone were less well protected than those with high HI titres alone, whilst those with high titres of both antibodies showed the lowest infection rate after challenge.

DISCUSSION

The influenza B viruses isolated from 1940 onwards do not fall into distinct subtypes of different temporal incidence or in different species of host animal, as do the influenza A strains. Although their HA antigens show considerable diversity, this is generally regarded as antigenic drift within a single type rather than antigenic shift between subtypes, as seen in type A (Pereira, 1969; Chakraverty, 1972*a*). Thus, until recently it was believed that the viral neuraminidase would be likely to have a single type-common antigenic constitution; however, Chakraverty (1972*b*) found that animal antisera raised against a variety of type B strains may show widely differing NI titres according to the virus strain employed in the test. Our present findings provide further evidence of antigenic heterogeneity of type BN; in particular the prototype B/LEE/40 differs sharply from more recently isolated strains in the reactivity of enzyme inhibitory antibody it can induce or detect.

In earlier attempts, such as our own (Hobson *et al.* 1972), to define the role of NI antibody in protection against type B influenza, measurement of preinfective titres with strains other than the homologous challenge virus could lead to unduly pessimistic conclusions. We had previously shown that NI titres against B/LEE did not bear any significant relation to immunity in adult volunteers given a deliberate challenge infection with B/Eng/13/65 virus, whereas it is clear from the present study that pre-challenge NI titres measured by B/Eng/13/65 are of some predictive value in relation to the result of subsequent exposure to the homologous strain, as was suggested for influenza A strains by Slepushkin *et al.* (1971).

Since the N antigens of type B are apparently not homogeneous, it seems unlikely that NI antibody against one strain would confer long lasting protection against the epidemic spread of strains emerging decades later. However in the short term, NI antibody against current strains related to B/Eng/13/65 may add to the protective effect of homologous serum HI antibody in volunteers where both are present, and may be important in protecting those without HI antibody. The greater frequency of NI than HI antibodies in the present panel of volunteers might merely reflect the greater sensitivity of the NI titration, but it is possible that N antigen has changed less frequently than HA over the last 4–8 years; repeated exposure during this time may thus recall or maintain effective NI antibody levels more commonly than HI antibody, and thus offset to some extent the effect of antigenic drift.

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REFERENCES

BEARE, A. S., HOBSON, D., REED, S. E. & TYRRELL, D. A. J. (1969). A comparison of live and killed influenza virus vaccines. *Lancet* ii, 418–20.

BROWN, J. D., HOBSON, D. & CURRY, R. L. (1973). Serological studies with purified neuraminidase antigens of influenza B viruses. In *Influenza Vaccines of Man and Horses*. Symposia Series on Immunobiological Standardization. Basle: Karger. (In the Press.)

CHAKRAVERTY, P. (1972*a*). Antigenic relationships between influenza B viruses. *Bulletin of the World Health Organization* **45**, 755–66.

CHAKRAVERTY, P. (1972*b*). Antigenic relationships between the neuraminidases of influenza B viruses. *Bulletin of the World Health Organization* **46**, 473–6.

FRANCIS, T. (1940). A new type of virus from epidemic influenza. *Science, New York* **91**, 405–8.

HOBSON, D., CURRY, R. L., BEARE, A. S. & WARD-GARDNER, A. (1972). The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *Journal of Hygiene* **70**, 767–77.

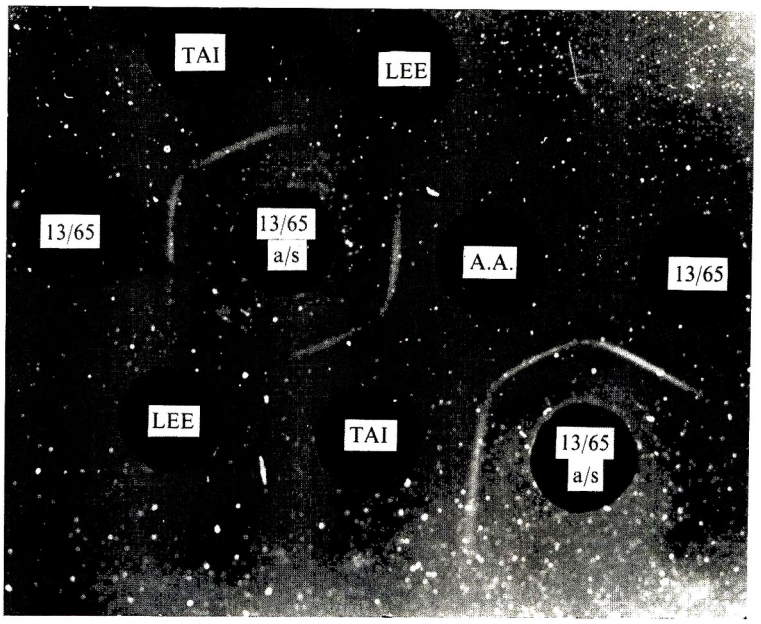
NOLL, H., AOYAGI, T. & ORLANDO, J. (1962). The structural relationship of sialidase to the influenza virus surface. *Virology* **18**, 154–7.

PEREIRA, H. G. (1969). Influenza: antigenic spectrum. *Progress in Medical Virology* **11**, 46–71.

SLEPUSHKIN, A. N., SCHILD, G. C., BEARE, A. S., CHINN, S. & TYRRELL, D. A. J. (1971). Anti-neuraminidase antibody and resistance to vaccination with live A2/Hong Kong influenza virus vaccines. *Journal of Hygiene* **69**, 571–8.

EXPLANATION OF PLATE

Plate 1. The reactivity of rabbit antiserum, raised by the isolated N antigen of B/Eng/13/65 virus, in gel diffusion against isolated N antigens of four type B strains. The four virus N antigens were obtained from: B/LEE/40 (LEE); B/Taiwan/4/62 (TAI); B/Ann Arbor/1/66 (A.A.); B/Eng/13/65 (13/65).


TAI
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Selective elimination of Enterobacteriaceae species from the digestive tract in mice and monkeys

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SUMMARY

In mice and in monkeys, 'selective' elimination of Enterobacteriaceae species from the digestive tract of animals with a sensitive flora was accomplished by oral treatment with nalidixic acid (1 mg./g. body weight in mice and 0·4 mg./g. body weight in monkeys). During treatment, the concentration of enterococci (and also of *Candida albicans* in the monkey) remained unaltered. This indicates that the fraction of the anaerobic microflora which is responsible for the colonization resistance of the digestive tract is not affected by the treatment. An important consequence seems to be, that elimination of yeast and fungi with fungistatic drugs can be started at the same time as elimination of Enterobacteriaceae is attempted.

INTRODUCTION

In patients and animals with impaired immune competence, the digestive tract is often the portal of entry for infections caused by potentially pathogenic (p.p.) micro-organisms such as *Pseudomonas aeruginosa*, *Escherichia coli* and other Enterobacteriaceae species as well as streptococci, staphylococci, yeasts and fungi. Decontamination of the digestive tract by treatment with oral antibiotics is being used increasingly to prevent such infections. However, this approach is of questionable value because decontaminated individuals are easily colonized by bacteria from the environment which are resistant to the antibiotics used for decontamination. Very small numbers of bacteria can colonize a decontaminated individual, because of the previous elimination of certain anaerobic species that are responsible for the colonization resistance (CR) of the digestive tract (van der Waaij, Berghuis-de Vries & Lekkerkerk, 1971). For this reason a different method of decontamination is needed that eliminates only the p.p. micro-organisms while leaving the anaerobes unaffected. The present experiments were conducted to determine whether the use of nalidixic acid is an effective alternative method to antibiotic decontamination for selective elimination of Enterobacteriaceae species from the digestive tract.

Table 1. *Results of* in vitro *test of sensitivity of Entobacteriaceae species present in ten faecal samples from monkeys*

	mg. drug/ml.			
Drug tested	0·027	0·29	2·1	23
Furoxone	6*	2	0	0
Nalidixic acid	2	0	0	0

* Number of positive cultures per 10 samples.

MATERIALS AND METHODS

Antimicrobial agents

Initially two chemotherapeutic drugs, nalidixic acid and furoxone, gave promising results in *in vitro* sensitivity tests (Table 1). The use of furoxone was discontinued because, when supplied in effective doses, it induced vomiting in several monkeys and caused anorexia in mice. Accordingly, only results with nalidixic acid (N.A.) are reported in this study.

Sensitivity testing

The sensitivity of the Enterobacteriaceae species in the faeces of animals to be treated was determined by suspending 1 part of fresh faeces from each animal in 9 parts of saline. The suspensions were subsequently streaked on Endo agar; disks consisting of 10, 25, 50 and 100 μg. nalidixic acid were then placed on the plates. After 24 hr. of incubation at 37° C. the plates were read.

Animals

Thirty female ND2 mice, 12 weeks old (30–35 g.) and eight young rhesus monkeys (*M. mulatta*, 2·2–3·5 kg.) were used in this study.

Eight monkeys were found to have a sensitive Enterobacteriaceae flora. Thus any organisms isolated from the faeces of these eight monkeys during treatment would have had to be due to a colonization from an exogenous source.

Housing

All animals were caged individually in clean disinfected cages with wire mesh grids. The mice were given a standard pelleted ration (Hope Farms). The monkeys were maintained on fresh fruit, vegetables, and a standard pelleted primate ration (Hope Farms). Drinking water was supplied *ad libitum*. Food was purposely not sterilized in order to prevent a reduced intake due to a change in palatability. A reduction in food intake during treatment could thus be ascribed to nausea due to nalidixic acid.

Oral treatment

N.A. was supplied to the mice in the drinking water. Commercially available 500 mg. tablets were dissolved in sterilized water (pH = 9) to achieve a concentration of 10 mg./ml. The average dose of 1 mg. N.A./g. body weight was effective

in eliminating Enterobacteriaceae species. To each monkey, 500 mg. N.A. was administered orally twice daily in 5 ml. of water (pH = 9) to obtain an average dose of 0·3–0·5 g./kg. body weight per day. The mice were treated in this way for 3 weeks, and monkeys for 16 days.

Determination of effect of treatment on CR and disappearance of Enterobacteriaceae species

CR and effect of the N.A. treatment on the Enterobacteriaceae population were determined by daily quantitative and qualitative studies in the faeces. A sample of 0·1 g. faeces was suspended in 0·4 ml. of Brain Heart Infusion broth (BHI broth, DIFCO); 0·05 ml. of this suspension was transferred to cup 1 of a plastic tray containing 0·5 ml. of broth per cup. The suspension was then serially diluted through eight dilution steps of 1/11 by transferring 0·05 ml. with diluting loops (Flow laboratories). After incubation of the tray, subcultures from each cup were made on Endo agar for the Enterobacteriaceae species and on Aesculin azide agar (Sneath, 1956) for enterococci. The presence of *Candida* species in the cultures from the monkey samples was determined by observing growth on Endo plates after 3 days incubation. In the mouse experiments, subcultures of each dilution were also made. However, the mouse cultures were subcultured on Sabauroud agar, since the yeast species that had been isolated from the mice rarely grew on Endo agar. The concentration both of the enterococci and the yeasts in the faeces was determined because they are not sensitive to nalidixic acid and an increase in number of either would be one indication of a decrease in the CR of the digestive tract.

Oral sampling

In the mice the oral cavity was swabbed daily with cotton swabs moistened with broth. The swabs were cultured in BHI broth after which subcultures were made on selective plates as described above. The oral cavity of the monkeys was not swabbed.

RESULTS

In mice there was a transitory reduction in the water intake during the first 2 days of treatment after which normal intake was restored. Food consumption was normal in both animal species and no weight loss occurred during treatment. The consistency of the faeces remained normal both in mice and monkeys, while all Enterobacteriaceae species were eliminated within 9 days (Figs. 1, 2). The oral swabs in the mice also were negative after 9 days. Thereafter, a positive swab was found occasionally. This is attributed to the fact that the food and water were not sterilized. In no case, however, was a yeast or a fungus species isolated from the oral cavity during treatment. The concentration of enterococci remained constant in the faeces of both mice and monkeys, as did that of *Candida albicans* (Fig. 3), which was present in the faeces of three monkeys at the start of the study. Five other monkeys were negative for candida when they entered the study and

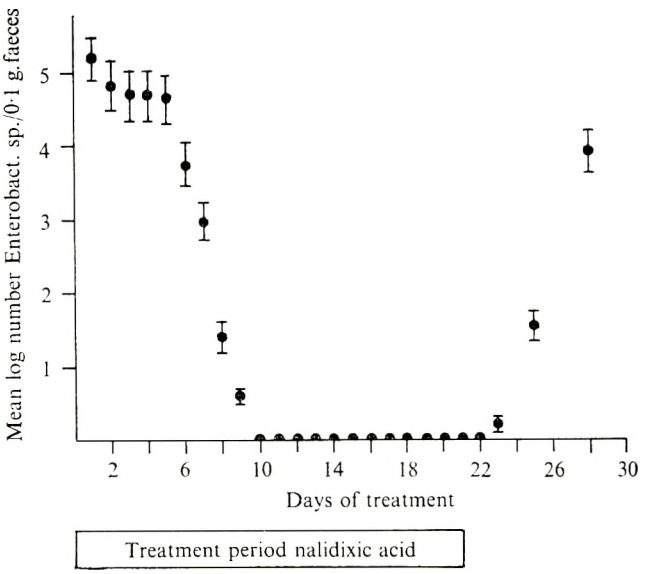

Fig. 1. Mean and standard deviation of the Enterobacteriaceae concentration in the faeces of mice during and after N.A. treatment.

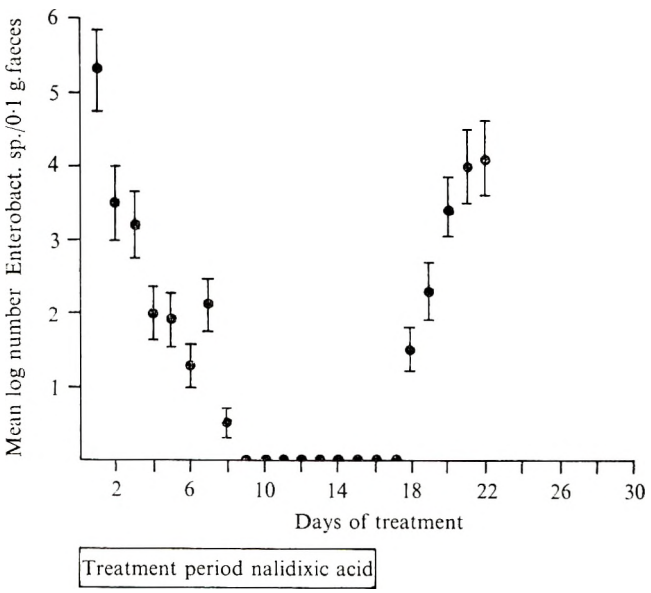

Fig. 2. Mean and standard deviation of the Enterobacteriaceae concentration in the faeces of monkeys during and after N.A. treatment.

remained negative during treatment. No yeasts or fungi were isolated from oral swabs and faeces of the mice.

DISCUSSION

This study has shown that Enterobacteriaceae can be successfully eliminated from the faecal flora both of mice and monkeys in about 1 week by oral adminis-

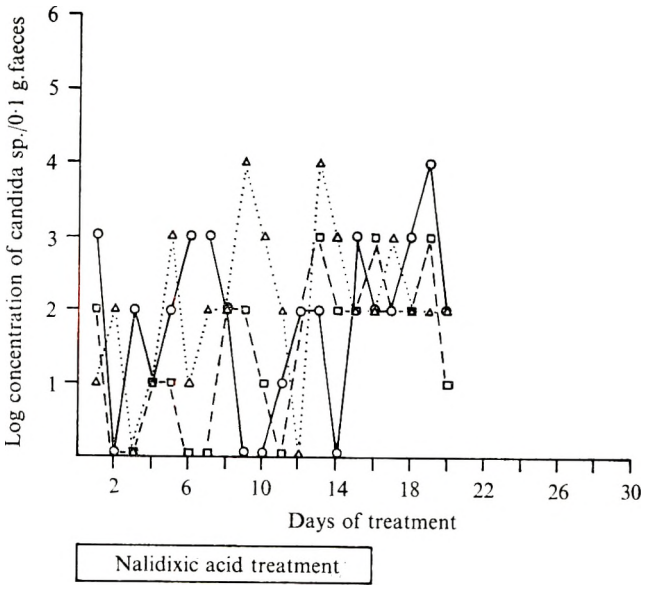

Fig. 3. The concentration of *Candida albicans* in the faeces of three monkeys during and after N.A. treatment.

tration of N.A. Inasmuch as the concentration of enterococci remained unchanged during the treatment, it appears that the CR was unaffected. The fact that the *C. albicans* concentration remained low in the monkeys during treatment further supports the interpretation that N.A. does not affect CR.

The CR of the digestive tract is the resistance to growth and multiplication encountered by micro-organisms after ingestion. The CR for several p.p. bacterial species has been determined both in mice and monkeys, and has been expressed as the log of the oral dose of bacteria of a certain species resulting in a colonization for 2 weeks or longer in 50% of a group of animals (van der Waaij *et al.* 1971). The CR apparently is caused by anaerobic bacterial species (Wensinck & Ruseler-van Embden, 1971) which act largely through the host by stimulating g.i.-peristalsis (Abrams & Bishop, 1967).

In previous experiments it was determined that Enterobacteriaceae species can be eliminated effectively from the digestive tract of mice by systemic treatment with kanamycin (van der Waaij, 1968). It has also been shown that systemic antibiotic treatment reduces the CR to some extent (van der Waaij, Berghuis & Lekkerkerk, 1972). N.A. is only available for oral administration. However, it was used in this study because about 95% is readily absorbed from the g.i. tract, and because it has a limited range which affects Enterobacteriaceae species while leaving enterococci and other bacterial species unaffected. Thus, the enterococci could be used as an indicator of the CR because, if CR decreased as a result of N.A. treatment, the concentration of enterococci in the faeces would increase (van der Waaij *et al.* 1972).

The mechanism by which N.A. eliminates Enterobacteriaceae from the intestinal lumen is not known. However, it is unlikely that it acts directly on the

intestinal contents. A rough estimate of the concentration of N.A. in the faeces of the monkeys was made with a 'sensitive' *Esch. coli* strain. The results indicated that if free N.A. were present in the faeces of these animals, its concentration would not have been more than 7 μg./g. This concentration is not sufficiently high to eliminate all Enterobacteriaceae species. On the other hand, the absorbed part of the drug may well have reached a sufficiently high concentration in the intestinal mucosa to prevent adherence of Enterobacteriaceae species to the mucosa. Recent observations (Williams & Gibbons, 1972) indicate that immunoglobin A (IgA) may act in about the same way and prevent bacterial colonization by preventing adherence to the mucosa. If prevention of adherence is a factor in the mechanism of N.A. action, this could explain why a week elapsed before the animals were free while, in animals decontaminated with antibiotics, Enterobacteriaceae species disappear in 2 days (van der Waaij, de Vries & Lekkerkerk, 1970).

Clinical observations in patients by Dankert (1973) also indicate that selective decontamination may be feasible. Kidney transplantation patients undergoing moderate immuno-suppression were treated with the combination trimethoprim and sulphamethoxazole. These two drugs were also administered orally. They are also readily absorbed; however, they have a much wider range than N.A. During treatment the concentration of Enterobacteriaceae species in the faeces decreased to zero in about 1 week. As in our experiments, the concentration of enterococci, yeasts and fungi was determined at constant intervals. During treatment no change in *C. albicans* concentration was reported. After treatment, however, a slight increase occurred. The enterococci behaved differently. They rose in concentration from 10^6/g to 10^8/g of faeces. This may indicate that the drugs used decrease the CR for enterococci to some extent.

The elimination of yeasts and fungi from the digestive tract by nystatin or amphotericin B is more often successful in the presence of a microflora that causes a good CR than in its absence, such as during antibiotic decontamination. Thus, it is technically preferable to eliminate yeasts before antibiotic decontamination is started. A 4-day treatment period with an adequate antimycotic drug is usually sufficient provided that oral administration of antimycotics is then continued during the antibiotic decontamination period. When N.A. or a similar drug is used, yeasts, fungi and Enterobacteriaceae species can be eliminated simultaneously because the anaerobes responsible for CR remain unaffected.

The results of this study suggest that patients with a N.A. sensitive Enterobacteriaceae flora can now be considered for selective decontamination. It should be stressed, however, in this context, that *Pseudomonas aeruginosa* is not sensitive to N.A. Although N.A. apparently does not affect the CR, so that low dose contaminations with Enterobacteriaceae species will not take, pasteurization of food and oral fluids seems advisable. As in mice, low dose contamination may occur in the oral cavity and thus create a potential hazard.

REFERENCES

ABRAMS, G. D. & BISHOP, J. E. (1967). Effect of the normal microbial flora on gastro-intestinal motility. *Proceedings of the Society for Experimental Biology and Medicine* **126**, 301.

DANKERT, J. (1973). Biotyping of Enterobacteriaceae: a method to determine the efficacy of the barrier function of isolation units. Proc. IVth Int. Symp. on Germfree Research, New Orleans, 1972. In *Germfree Research* (ed. J. B. Heneghan), p. 59.

SNEATH, P. H. A. (1956). Cultural and biochemical characteristics of the genus *Chromobacterium*. *Journal of General Microbiology* **15**, 70.

WAAIJ, D. VAN DER (1968). The persistent absence of Enterobacteriaceae from the intestinal flora of mice following antibiotic treatment. *Journal of Infectious Diseases* **118**, 32.

WAAIJ, D. VAN DER, DE VRIES, J. M. & LEKKERKERK, J. E. C. (1970). Eliminating bacteria from monkeys with antibiotics. In *Infections and Immunosuppression in Subhuman Primates* (ed. H. Balner and W. I. B. Beveridge), Copenhagen: Munksgaard, p. 21.

WAAIJ, D. VAN DER, BERGHUIS-DE VRIES, J. M. & LEKKERKERK-VAN DER WEES, J. E. C. (1971). Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. *Journal of Hygiene* **69**, 405.

WAAIJ, D. VAN DER, BERGHUIS, J. M. & LEKKERKERK, J. E. C. (1972). Colonization resistance of the digestive tract of mice during systemic antibiotic treatment. *Journal of Hygiene* **70**, 605.

WENSINCK, F. & RUSELER-VAN EMBDEN, J. G. H. (1971). The intestinal flora of colonization-resistant mice. *Journal of Hygiene* **69**, 413.

WILLIAMS, R. C. & GIBBONS, R. J. (1972). Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. *Science, New York* **177**, 697.

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Experimental pertussis infection in the marmoset: type specificity of active immunity

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SUMMARY

Although we have failed to produce either paroxysmal cough or vomiting in rhesus monkeys, cynomolgus monkeys and marmosets, we have found in marmosets several features of pertussis infection similar to those seen in children with whooping cough: catarrh, persistence of colonization of the naso-pharynx with *Bordetella pertussis* for 4–11 weeks, change of serotype during colonization and inability of type 1 organisms to establish themselves as the predominant serotype.

As in children, we have found that intramuscular vaccine of type 1,2,3 was more effective than type 1,2 in preventing persistent infection with the currently prevalent serotypes 1,2,3 and 1,3. A mixed vaccine (1,2,3 and 1,3) seemed to produce agglutinin 3 in the serum more consistently than a pure type 1,2,3 vaccine. The duration of colonization, after naso-pharyngeal challenge, was greatly reduced in animals with agglutinin 3.

Local immunity, resulting from previous infection, was even more effective than a good vaccine in preventing subsequent persistent colonization. Marmosets may be useful in studying the possible development of aerosol pertussis vaccine for human use.

INTRODUCTION

There is much evidence (see Preston & Stanbridge, 1972) to indicate that the type-specific pertussis agglutinogens (2 and 3) play an important role in immunity to whooping cough in the child. However, in the absence of a field-trial to compare the efficacies of vaccines made from different serotypes of *Bordetella pertussis*, the importance of these antigens is still denied by some workers at international level (Pittman, 1970; Cohen, Hannik & Nagel, 1971).

The experimental animal most widely used in the study of pertussis immunity is the mouse, which can be given a lethal infection by intracerebral injection. Passive type-specific immunity against such infection has been demonstrated by Preston & Evans (1963), but attempts to show active type-specific immunity have failed (Andersen & Bentzon, 1958; Preston & Te Punga, 1959). The most likely explanation (Preston, 1966) is that, in the mouse, the species-specific agglutinogen (factor 1) is more important than the type-specific factors (2 and 3), whereas in man the converse may apply – hence our desire to study type-specific immunity in animals more closely related to man.

Attempts to produce experimental pertussis infection in the respiratory tract of primates range from failure (e.g. Mallory, Horner & Henderson, 1913) to claims that monkeys coughed paroxysmally and sometimes vomited (e.g. Klimenko, 1909; Sauer & Hambrecht, 1929; Lin, 1958; Huang *et al.* 1962). Immunity to re-infection was reported and also successful immunization with pertussis vaccine, but the serotypes of the vaccine strains and challenge strains have not been recorded.

MATERIALS AND METHODS

Strains of Bordetella pertussis

Strain 41633 was isolated in Coventry during the recent whooping-cough survey of the Public Health Laboratory Service (1969). By single colony subculture we isolated, from this one source, pure cultures of the three common serotypes (1,2,3; 1,2; 1,3). All three were lyophilized and they were subsequently used both for attempts to infect marmosets and also for vaccines with which marmosets were immunized.

For intracerebral challenge of vaccinated mice (Kendrick, Eldering, Dixon & Misner, 1947) we used the standard challenge strain, W.18–323, which in our laboratory is a type 1 strain (Preston, 1966).

Serotyping of pertussis strains and vaccines, and estimation of pertussis agglutinins in sera. Full details of these techniques have been described previously (Preston, 1970*b*).

Pertussis vaccines

Growth from charcoal-blood-agar (Oxoid, 1965), incubated at 35–36° C., was harvested in phosphate-buffered saline (pH 7·4) and killed with 0·1% formaldehyde. The bacterial content of each suspension was estimated by comparison with the U.S. National Institutes of Health opacity standard. Marmosets were vaccinated intramuscularly, in the thigh, with four injections over a period of 2 weeks, the total dose for each animal being *ca.* 6×10^{10} bacteria. The hyper-immunized animals (Table 7) received further injections, bringing the total dose to *ca.* 15×10^{10} bacteria.

Marmosets were challenged about 2 weeks after the last injection of vaccine.

Marmosets

The early experiments (Tables 1 and 3) were performed on a number of common marmosets that had been either imported into this country or bred in our Department, but subsequently we used only animals bred in our Department. Their weights ranged from 150 to 350 g., and their ages from 4 months to about 3 years; but we attempted, in each experiment, to ensure that pairs or larger groups were similar in weight-range and sex.

For inoculation in the naso-pharynx, lyophilized strains of *Bord. pertussis* were subcultured three times within a week on charcoal-blood-agar, the third culture was suspended in the basal medium of Cohen & Wheeler (1946), and its bacterial content was estimated by opacity (see above). An inoculum of 0·1 ml., containing

ca. 50×10^9 bacteria, was instilled slowly from a syringe, through a blunted needle, along the floor of the marmoset's nose.

Tiny pernasal swabs, made from brass-wire (*ca.* 0·35 mm. diameter), were used for recovery of *Bordetella* from the naso-pharynx. These were inoculated immediately onto charcoal-blood-agar medium containing penicillin (0·25 units per ml.). For details of culture and identification, see Preston (1970*b*).

Samples of blood (3–4 ml.) were obtained from the femoral vein.

Sedation of primates. Rhesus and cynomolgus monkeys and, for intratracheal inoculation, marmosets were sedated with 'Sernylan' (Parke, Davis and Co., Hounslow, London, England) which was given intramuscularly (1 mg./kg. of body weight) $\frac{1}{2}$–1 hr. before inoculation. Marmosets were inoculated in the naso-pharynx, swabbed and bled without sedation.

RESULTS

Preliminary attempts to infect primates with Bordetella pertussis

Our initial attempts to establish pertussis infection in primates made use of six common marmosets that had been housed in our Department for about 2 years. These were inoculated with two different serotypes of strain 41633 in various doses and by different routes (Table 1), a separate room being used for each serotype. The highest dose, 180×10^9 bacteria, resulted in death 2 days after inoculation. With the other five animals, nothing more than a nasal discharge or catarrh was observed: they did not whoop or vomit. Bouts of coughing did occasionally occur, but only during feeding; and these were observed in normal and inoculated marmosets alike.

Because experimental pertussis infection of rhesus monkeys has been reported previously (Sauer & Hambrecht, 1929) we acquired a young pair, male and female. Both had coughs on arrival, but we did not isolate *Bord. pertussis*, *Bord. parapertussis* or *Bord. bronchiseptica* from either of them, nor were *Bordetella* antibodies detectable in their sera. When they had recovered from their coughs, each was inoculated into the trachea, through the mouth and larynx, with 100×10^9 organisms of strain 41633 (type 1,3). Neither animal showed any ill effect, nor was *Bord. pertussis* recovered with pernasal swabs on any occasion over the next 8 weeks. However, antibodies to *Bord. bronchiseptica* factor *c* (Andersen, 1953) developed in the sera of both animals during this period.

Our failure to infect rhesus monkeys may have been due partly to interference-immunity (Evans & Perkins, 1954) or local antibody resulting from concurrent mild infection with *Bord. bronchiseptica*, but we wondered whether attenuation of the infecting strain of *Bord. pertussis* by lyophilization may have been a factor, and also innate immunity of that type of monkey. We obtained two cynomolgus monkeys, male and female, from the same dealer, and inoculated each of them, partly intratracheally and partly intranasally, with 100×10^9 organisms of a type 1,2,3 strain of *Bord. pertussis* freshly isolated in Manchester. Again there were no signs of illness, and pernasal swabbing failed to recover *Bord. pertussis*. But these

Table 1. *Six non-vaccinated marmosets inoculated in the upper respiratory tract with* Bordetella pertussis, *type* 1,2 *or type* 1,3

Marmoset ...		M3	M5	M1	M2	M4	M6
Inoculum	Route ...	Pernasal	Intratracheal	Intratracheal	Intratracheal	Pernasal	Intratracheal
	Dose ($\times 10^9$ orgs.) ...	30	125	110	55	45	180
	Strain ...	Type 1,2		Type 1,3			
* Serotype(s) isolated with pernasal swabs during *N*th week after inoculation	Week (*N*) 1	1,2	1,2	1,3	1,3	1,3	1,3†
	2	1,2	1,2	1,3	1,3	1,3	—
	3	1,2; 1	1,2	1,3; 1	1,3	1,3	—
	4	1,3; 1	1,2; 1	1,3; 1	1,3; 1	1,3	—
	5	1,3	1,2	1,2; 1,3	1,3; 1	None	—
	6	1,3	1,2; 1	None	None	1,3	—
	7	1,3	None	None	None	None	—
	8–26	None	None	None	None	None	—
Duration of detectable pertussis colonization (days) ...		46	42	30	32	39	2

* This table records only predominant serotypes, and ignores those constituting up to 20% of the colonies tested from the primary plate cultures (see Table 2).

† Died 2 days after inoculation.

Table 2. *Detailed serotyping of* Bordetella pertussis *recovered with pernasal swabs from four marmosets, two inoculated with type* 1, 2 *and two with type* 1, 3

Marmoset ...		M3				M5				M1				M2			
Inoculum ...		Type 1, 2								Type 1, 3							
Serotypes of colonies tested ...		1, 2, 3	1, 2	1, 3	1	1, 2, 3	1, 2	1, 3	1	1, 2, 3	1, 2	1, 3	1	1, 2, 3	1, 2	1, 3	1
	Week (*N*)																
* No. of colonies of each serotype during *N*th week after inoculation	1	0	18	0	0	0	18	0	0	0	0	12	0	0	0	12	0
	2	0	18	0	0	0	12	0	0	0	0	12	0	0	0	6	0
	3	0	6	0	7	0	12	0	0	1	1	4	2	1	0	11	0
	4	0	0	1	2	2	6	2	4	0	2	11	5	0	0	14	4
	5	0	0	5	0	0	11	1	1	0	1	1	0	0	0	7	3
	6	0	0	18	0	1	5	1	4	0	0	0	0	0	0	0	0
	7	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0

* Each marmoset was swabbed two or three times per week, and about 6 colonies were tested from each primary culture.

animals both had *Bordetella* antibodies in their sera on arrival, and we isolated *Bord. bronchiseptica* type *c* from them on two occasions during the third week after inoculation.

With failure to infect rhesus and cynomolgus monkeys, we reverted to the use of marmosets. Although we had produced no signs of illness except catarrh in the marmoset, we had noted (Tables 1 and 2) several features of pertussis infection that are found in the child (see Preston & Stanbridge, 1972): colonization of the naso-pharynx with *Bord. pertussis* persisted for 4–7 weeks, and change of serotype occurred during colonization; also, type 1 organisms were apparently unable to establish themselves as the predominant serotype, even though they appeared during the course of infection. In order to give ourselves the best chance of detecting signs of pertussis infection, such as coughing, whooping or vomiting, we decided to continue with a large dose (50×10^9 bacteria) which, however, our preliminary study (Table 1) suggested would be sublethal. Also, as there appeared to be no advantage in using intratracheal inoculation, rather than the simpler pernasal procedure, we adopted the latter technique for subsequent experiments.

Relative efficacies of different vaccines against challenge with Bordetella pertussis *of serotypes* 1, 2, 3 *and* 1, 3 (*the types most commonly recovered from children in recent years*)

Against subsequent type 1, 3 challenge (Table 3), vaccines containing antigen 3 had a considerable effect on the duration of colonization. Compared with 36 days for non-vaccinated controls (M13, M31), animals M10, M17 and M11 were colonized for only 8–10 days. Vaccine that did not include antigen 3 appeared to be less effective in eliminating the infection: colonization persisted in M7 and M14 for 20–22 days. (Colonization in M12 lasted as long as in the controls, but this animal had produced no detectable antibody to the factor 3 component of the type 1, 3 vaccine that it received, Table 8.)

The serotypes recovered from animals in which infection persisted for more than 14 days were influenced by the vaccine given previously: infection persisted with a predominance of type 1, 3 in M7 and M14 which had been immunized against antigen 2 (type 1, 2 vaccine); but, in M12, M13 and M31, mutants containing antigen 2 (type 1, 2, 3 and type 1, 2) emerged.

Against subsequent type 1, 2, 3 challenge (Table 4), the influence of the factor 3 content of the vaccine was again apparent. The duration of colonization in M39, M44, M37 and M42 (type 1, 2, 3 or type 1, 3 vaccine) was appreciably less (8–16 days) than in the non-vaccinated controls, M35 and M40 (34–72 days). In all of these animals except M42, the challenge strain mutated from type 1, 2, 3 to type 1, 3 during the course of the infection.

In animals M32 and M43, immunized with type 1, 2 vaccine, these mutants appeared to influence the duration of colonization: in M43 the type 1, 3 mutants did not establish themselves and the infection was eliminated within 14 days, but in M32 a predominance of type 1, 3 developed and the infection persisted for 34 days – as long as in one of the non-vaccinated controls.

These alternative responses to a type 1, 2, 3 challenge after type 1, 2 vaccine were seen again in five more marmosets (Table 5). Rapid elimination occurred, within

Table 3. *Four pairs of marmosets challenged with type* 1,3 Bordetella pertussis *in the naso-pharynx after intramuscular immunization with heterologous and homologous vaccine*

Vaccine ...		Type 1,2,3		Type 1,2		Type 1,3		None	
Marmoset ...		M10	M17	M7	M14	M11	M12	M13	M31
Challenge-strain ...		Type 1,3							
	Week (*N*)								
* Serotype(s) isolated with pernasal swabs during *N*th week after challenge	1	1,3	1,3	1,3	1,3	1,3	1,3	1,3	1,3
	2	1,3	1,3	1,3	1,3	1,3	1,3	1,2,3; 1,3	1,3
	3	None	None	1,3	1,3	None	1,2,3; 1,3	1,2,3; 1,2; 1,3	1,2,3; 1,3
	4	None	None	None	1,3	None	1,3	1,2; 1,3	1,2,3; 1,2; 1,3
	5	None	None	None	None	None	1,2; 1,3	1,2,3; 1,2	1,2
	6	None	None	None	None	None	1,3	1,2	1,2; 1
	7–9	None	None	None	None	None	None	None	None
Duration of detectable pertussis colonization (days) ...		8	10	20	22	10	36	36	36

* See first footnote to Table 1.

Table 4. *Four pairs of marmosets challenged with type* 1,2,3 Bordetella pertussis *in the naso-pharynx after intramuscular immunization with homologous and heterologous vaccine*

Vaccine ...		Type 1,2,3		Type 1,2		Type 1,3		None	
Marmoset ...		M39	M44	M32	M43	M37	M42	M35	M40
Challenge-strain ...		Type 1,2,3							
	Week (*N*)								
* Serotype(s) isolated with pernasal swabs during *N*th week after challenge	1	1,2,3; 1,3	1,2,3; 1,3	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3
	2	1,3	1,3	1,3	1,2,3; 1,3	None	1,2,3	1,2,3; 1,3	1,2,3
	3	1,3	None†	1,3	None	1,3	None	1,3	1,2,3; 1,3
	4	None	—	1,3	None	None	None	1,3	1,3
	5	None	—	1,3	None	None	None	1,3	1,3
	6	None	—	None	None	None	None	None	1,3
	7	None	—	None	None	None	None	None	1,2,3; 1,3
	8–10	None	—	None	None	None	None	None	1,3
	11	NT	—	NT	NT	NT	NT	NT	1,3
	12–15	NT	—	NT	NT	NT	NT	NT	None
Duration of detectable pertussis colonization (days) ...		16	13	34	13	16	8	34	72

NT = not tested.

* See first footnote to Table 1.

† This animal had apparently eliminated the pertussis infection by the end of the 2nd week; it died during the 3rd week but *Bord. pertussis* was not recovered *post mortem*.

Table 5. *Five more marmosets challenged with type* 1, 2, 3 Bordetella pertussis *in the naso-pharynx after intramuscular immunization with type* 1, 2 *vaccine*

Vaccine ...		Type 1, 2				
Marmoset ...		M33	M48	M52	M41	M45
Challenge-strain ...		Type 1, 2, 3				
	Week (*N*)					
* Serotype(s) isolated with pernasal swabs during *N*th week after challenge	1	1, 2, 3	1, 2, 3	1, 2, 3; 1, 3	1, 2, 3	1, 2, 3; 1, 3
	2	1, 2, 3; 1, 3	1, 3	1, 3	1, 2, 3	1, 3
	3	1, 3	1, 3	1, 3	None	None
	4	1, 3	None	1, 3	None	None
	5	1, 3	None	1, 3	None	None
	6	1, 3	None	None	None	None
	7	1, 3	1, 3	None	None	None
	8	1, 3	1, 3	None	None	None
	9	1, 3	None	None	None	None
	10–12	None	None	None	None	None
Duration of detectable pertussis colonization (days) ...		57	50	32	11	14

* See first footnote to Table 1.

Table 6. *Assessment by the intracerebral mouse-protection test of the relative potencies of the type* 1, 2, 3 *and type* 1, 2 *vaccines used in the immunization of marmosets*

Vaccine (intraperitoneal)		Mortality of mice. No. dead/no. challenged*			PD 50 ($\times 10^8$ orgs.)†
Serotype	Dose ($\times 10^8$ orgs.)	Expt. 1	Expt. 2	Total	
1, 2, 3	100	1/10	0/10	1/20	14·3
	20	4/10	6/11	10/21	
	4	7/10	9/11	16/21	
1, 2	100	0/10	2/11	2/21	9·6
	20	3/8	2/11	5/19	
	4	6/10	9/11	15/21	

* Challenged intracerebrally with *ca.* 150 LD 50 of strain W. 18–323 (type 1).
† Estimated by the method of Reed & Muench (1938).

14 days, in M41 and M45: in M41, type 1, 3 mutants did not replace the parent challenge-strain, and in M45 the type 1, 3 mutants did not become well established but were recovered from the naso-pharynx in only small numbers during the second week. Conversely, in M33, M48 and M52, type 1, 3 mutants did establish themselves and gave rise to a much more persistent infection which lasted for 32–57 days. (The two types of response were *not* related to the sex, age or weight of the animals.)

From Tables 3 and 4 it can be seen that the four animals that were immunized with type 1, 2, 3 vaccine eliminated the subsequent challenge (type 1, 3 or type

1,2,3) in 8–16 days (average, 12 days), whereas the four immunized with type 1,2 vaccine harboured the challenge for 13–34 days (average, 22 days). The greater efficacy of the type 1,2,3 vaccine could be explained, as indicated above, on the basis of type-specific immunity, with emphasis on the importance of antigen 3 in these particular experiments. It was also possible, however, that the two vaccines differed in potency in some manner independent of their agglutinogen content, although care had been taken to standardize them to the same opacity. They were therefore compared by the intracerebral mouse-protection test of Kendrick *et al.* (1947) and Table 6 shows that it was the type 1,2 vaccine which appeared to be slightly more potent by this test, although the difference was not significant.

Efficacies of pertussis vaccines: difficulty in achieving adequate antibody-3 response

The above experiments indicated that the immune response to the factor 3 component of a vaccine may be vitally important in protection against challenge with organisms possessing antigen 3 (type 1,2,3 or type 1,3). Moreover, we noted that one animal (M12) had responded poorly to factor 3 in the type 1,3 organisms with which it was vaccinated.

We therefore decided to immunize a further series of animals with a higher dose of vaccine (see Materials and Methods) and to compare the responses of groups of marmosets to vaccines of type 1,2,3, of type 1,2 and of type 1,3 and also to a vaccine consisting of equal parts of type 1,2,3 and type 1,3 organisms.

Table 7 shows an obvious correlation between the titre of agglutinin 3, at the time of challenge, and the duration of colonization with the type 1,3 challenge strain. Animals in which agglutinin 3 was detectable, even at a low titre (M59, M61, M62, M58, M63, M36, M38) eliminated the challenge-strain in 7–13 days (average, 10 days), whereas animals in which agglutinin 3 was not detectable (M46, M60, M50) harboured the challenge-strain for 18–29 days (average, 23 days). High titres of agglutinins 1 and 2, in these three animals, did not compensate for lack of agglutinin 3.

The absence of agglutinin 3 in M50 was expected, as this animal had received type 1,2 vaccine. But in M46 and M60 we had further examples of a failure to respond to the factor 3 component of a vaccine, even after a higher dose. We note also that, although the number of animals was too small to draw convincing conclusions, there tended to be a better response to factor 3 in the animals that were vaccinated with a mixture of type 1,2,3 and type 1,3 organisms than in those that received type 1,2,3 alone. In both groups, the response to factors 1 and 2 appeared to be more than adequate.

Local immunity from previous infection: better protection than by parenteral vaccine

Table 8 summarizes the results of a first challenge with type 1,3 or type 1,2,3 organisms in thirteen marmosets, some of which had been previously vaccinated. With the exception of animal M43, which has been mentioned previously, there was a correlation between the duration of colonization and the level of serum-agglutinin

Table 7. *Ten marmosets challenged with type* 1,3 Bordetella pertussis *in the naso-pharynx after intramuscular hyperimmunization with various vaccines*

Vaccine ...		Types 1,2,3/1,3			Type 1,2,3				Type 1,2	Type 1,3	
Marmoset ...		M59	M61	M62	M46	M58	M60	M63	M50	M36	M38
Serum-agglutinin titres when challenged	Factor 1	160	320	320	80	1280	1280	320	160	80	80
	Factor 2	640	1280	> 1280	1280	> 1280	1280	1280	> 2560	< 5	< 5
	Factor 3	40	5	40	< 5	10	< 5	5	< 5	320	320
Challenge-strain ...		Type 1,3									
* Serotype(s) isolated with pernasal swabs during *N*th week after challenge	Week (*N*) 1	1,3	1,3	1,3	1,3	1,3	1,3	1,3	1,3	1,3	1,3
	2	1,3	1,3	1,3	1,3	1,3	1,3	None	1,3	1,3	1,3
	3	None	None	None	1,3	None	1,3	None	1,3	None	None
	4	None	None	None	1,3	None	1,3	None	None	None	None
	5	None	None	None	1,3	None	None	None	None	None	None
	6–8	None	None	None	None	None	None	None	None	None	None
Duration of detectable pertussis colonization (days) ...		10	10	8	29	13	23	7	18	10	13

* See first footnote to Table 1.

Table 8. *Thirteen marmosets re-challenged with* Bordetella pertussis *in the naso-pharynx after elimination of previous challenge*

Vaccine ...		Type 1,2,3		Type 1,2		Type 1,3		None		Type 1,2		Type 1,3	None	
Marmoset ...		M10	M17	M7	M14	M11	M12	M13	M31	M32	M43	M42	M35	M40
Serum-agglutinin titres at first challenge	Factor 1	160	40	20	20	40	40	<10	<5	10	20	20	<5	<5
	Factor 2	20	160	<10	<10	<10	<10	<10	<5	20	5	<5	<5	<5
	Factor 3	20	10	<10	<10	10	<10	<10	<5	<5	<5	5	<5	<5
First challenge ...		Type 1,3								Type 1,2,3				
Duration of colonization (days) ...		8	10	20	22	10	36	36	36	34	13	8	34	72
Antigens detected from pernasal swabs ...		1:3		1:2:3		1:3	1:2:3			1:2:3				
Serum-agglutinin titres at second challenge	Factor 1	80	160	40	160	320	80	20	<5	40	40	40	<2	<2
	Factor 2	40	320	40	40	<10	<5	<5	<5	160	40	<5	<2	<2
	Factor 3	80	40	<5	<5	40	40	<5	20	<5	<5	80	<2	<2
Second challenge ...		Type 1,2,3								Type 1,3				
*Duration of colonization (days) ...		1	1	3	2	1	2	1	10	4	4	3	4	4
Antigens detected from pernasal swabs ...		1:2:3								(All 3 previously detected)				
Serum-agglutinin titres at third challenge	Factor 1						80	80	<5					
	Factor 2						<5	<5	<5					
	Factor 3	Not tested					80	<5	40	Not tested				
Third challenge ...							Type 1,3							
*Duration of colonization (days) ...							7	2	4					

* After second and third challenge, pernasal swabbing was continued for three weeks (and longer with some animals); the serotype that was recovered, during the few days that colonization persisted, remained the same as that of the challenge-strain.

Table 9. *Comparative efficacy of different vaccines in enabling marmosets to eliminate* Bordetella pertussis *from the naso-pharynx after challenge with organisms possessing antigen* 3 (*type* 1,2,3 *or type* 1,3)*

Vaccine ...	Types 1,2,3/1,3	Type 1,2,3	Type 1,2	Type 1,3	None
No. of marmosets challenged ...	3	8	10	6	4
No. in which colonization persisted less than 14 days ...	3	5	2	4	0
Percentage ...	100	63	20	67	0

* Data from Tables 3, 4, 5 and 7.

for factor 3: those animals with detectable agglutinin 3 eliminated the challenge within 14 days, whereas it persisted much longer in the others.

During the period of colonization, organisms possessing antigens 1 and 3 were recovered from all 13 animals and antigen 2 was detected (either from the original challenge strain or by mutation from a type 1,3 challenge) in organisms recovered from all but three of the animals (M10, M17, M11). But detectable serum-agglutinins did not always develop in the absence of previous vaccination. In particular, animals M35 and M40 produced no detectable serum-agglutinin for any of the three pertussis factors. Nevertheless, on second challenge, even with a strain heterologous to that used for the first challenge, there was very rapid elimination in 1–10 days (average, 3 days). This suggests that the local immunity resulting from previous infection may be much more effective than even a good vaccine in preventing subsequent persistent colonization.

DISCUSSION

The efficacy of intramuscular pertussis vaccine, in hastening the elimination of *Bord. pertussis* subsequently instilled into the nasopharynx of marmosets, has been shown to depend on the level of serum-agglutinins produced in response to the vaccine (Tables 7 and 8). The challenge-strains used in these experiments were of type 1,2,3 or type 1,3 – the types most commonly recovered from children with whooping cough in many countries over the last 10 years (see Preston, 1970*a*; Preston & Stanbridge, 1972; Shmilovitz, Preston, Zaltser & Cahana, 1972; Public Health Laboratory Service, 1973). Since the challenge always included antigen 3, it is not surprising that the level of agglutinin 3 was found to be of great importance, and to this extent the active immunity after vaccination was found to be type-specific – high levels of agglutinins 1 and 2 did not compensate for lack of agglutinin 3. Thus Table 5 shows the limited efficacy of type 1,2 vaccine in protecting against type 1,2,3 infection: as expected, none of these five marmosets had detectable agglutinin 3 when challenged, and prolonged colonization depended on chance mutation of the challenge strain to type 1,3 and early proliferation of this mutant before the original type 1,2,3 challenge could be eliminated with the aid of agglutinins 1 and 2 alone.

Type 1,3 strains possess predominantly antigen 3, with little antigen 1 (Preston, 1971), whereas type 1,2 and type 1,2,3 strains have a high content of antigen 1 and of antigen 2. Immunity to all three serotypes may therefore be achieved by agglutinin 3 together with either agglutinin 1 or agglutinin 2. This would explain why Pillemer's SPA vaccine (Pillemer, Blum & Lepow, 1954), which produced a good antibody-response to factors 1 and 3 (Preston & Te Punga, 1959), gave good protection against whooping cough (Medical Research Council, 1959) at a time when the predominant serotypes in the population were 1,2,3 and 1,2 (Preston, 1963).

In many animals, mutants of the challenge strain proliferated during the course of infection – for example in M1 (Table 1) type 1,3 mutated via type 1 to type 1,2; in M3 (Table 1) type 1,2 mutated via type 1 to type 1,3; in M12, M13 and M31 (Table 3) type 1,3 mutated via type 1,2,3 to type 1,2; in M5 (Table 2) type 1,2 probably mutated directly to type 1,2,3 and also mutated, via either type 1,2,3 or type 1, to type 1,3; and in many animals (Tables 4 and 5) type 1,2,3 mutated to type 1,3. In many of these cases, the selection (or lack of selection) of the mutant was clearly determined by the vaccination history of the animal. This would adequately explain the otherwise rather puzzling finding of the Public Health Laboratory Service (1973) that in 22 of 158 households the serotypes isolated from different children in one house were not the same.

The superiority of type 1,2,3 vaccine over type 1,2 vaccine (Tables 3 and 4) could be explained by the improved ability of these vaccinated animals to eliminate a type 1,3 challenge or type 1,3 mutants from a type 1,2,3 challenge. For the best immunity, vaccine should stimulate the production of all three agglutinins. As in children (Abbott, Preston & Mackay, 1971) it seems that the response to factor 3 is the most difficult to achieve; and Table 7 shows that, when the factor 2 component was reduced by mixing type 1,2,3 organisms with type 1,3 in equal parts, the response to factor 3 was better than with type 1,2,3 organisms alone. Table 9 shows the relative efficacies of vaccines with various combinations of the three pertussis agglutinogens, and suggests that the mixed 1,2,3/1,3 vaccine may well give the best immunity. The criterion, of colonization persisting for less than 14 days from challenge, has been chosen on the grounds that, if a child were colonized for less than 14 days, it would have reached only the catarrhal phase of pertussis infection before the organisms were eliminated, and this would probably result in only mild illness, not recognizable clinically as whooping cough.

Lastly, it is clear from Table 8 that the local immunity resulting from previous infection of marmosets was usually more effective than the best serum-agglutinin immunity achieved by intramuscular vaccine, and this confirms the findings of Huang *et al.* (1962) who showed that immunity to re-infection did not depend on detectable serum-agglutinin. Holt (1972) has also suggested the importance of local immunity and the possibility of introducing aerosol vaccination for children. We would note that nothing is yet known about the type-specificity of such local immunity. After colonization with *any* live serotype, local IgA is likely to include antibody to all three pertussis factors, because of mutations. However, with mutation occurring so readily *in vivo*, it is unlikely that a safe, live, attenuated

vaccine could be developed in the case of *Bord. pertussis*. Before an aerosol vaccine could be introduced, it would be necessary to study the type-specificity and the likely duration of such immunity resulting from locally administered *killed* vaccine, and it appears that the marmoset would be an excellent experimental animal for such studies.

Meanwhile we stress once again (see Preston & Stanbridge, 1972) the importance of an adequate response to the agglutinogen 3 component of parenteral vaccine.

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REFERENCES

ABBOTT, J. D., PRESTON, N. W. & MACKAY, R. I. (1971). Agglutinin response to pertussis vaccination in the child. *British Medical Journal* i, 86.

ANDERSEN, E. K. (1953). Serological studies on *H. pertussis*, *H. parapertussis* and *H. bronchisepticus*. *Acta pathologica et microbiologica scandinavica* **33**, 202.

ANDERSEN, E. K. & BENTZON, M. W. (1958). The failure to show correlation between type-specificity and protection in experimental pertussis in mice. *Acta pathologica et microbiologica scandinavica* **43**, 106.

COHEN, H. H., HANNIK, C. A. & NAGEL, J. (1971). Success and limitations of vaccination against pertussis. *Scientific Publication* no. 226, p. 323. Washington, D.C.: Pan American Health Organisation.

COHEN, S. M. & WHEELER, M. W. (1946). Pertussis vaccine prepared with phase I cultures grown in fluid medium. *American Journal of Public Health* **36**, 371.

EVANS, D. G. & PERKINS, F. T. (1954). Interference immunity produced by pertussis vaccine to pertussis infection in mice. *British Journal of Experimental Pathology* **35**, 603.

HOLT, L. B. (1972). The pathology and immunology of *Bordetella pertussis* infection. *Journal of Medical Microbiology* **5**, 407.

HUANG, C. C., CHEN, P. M., KUO, J. K., CHIU, W. H., LIN, S. T., LIN, H. S. & LIN, Y. C. (1962). Experimental whooping cough. *New England Journal of Medicine* **266**, 105.

KENDRICK, P. L., ELDERING, G., DIXON, M. K. & MISNER, J. (1947). Mouse protection tests in the study of pertussis vaccine. A comparative series using the intracerebral route for challenge. *American Journal of Public Health* **37**, 803.

KLIMENKO, W. N. (1909). Die Aetiologie des Keuchhustens. Experimenteller Keuchhusten. *Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten* Abt. I (Orig.) **48**, 64.

LIN, T. M. (1958). Experimental whooping cough in monkey. *Journal of the Formosan Medical Association* **57**, 505.

MALLORY, F. B., HORNER, A. A. & HENDERSON, F. F. (1913). The relation of the Bordet-Gengou bacillus to the lesion of pertussis. *Journal of Medical Research* **27**, 391.

MEDICAL RESEARCH COUNCIL (1959). Vaccination against whooping-cough. *British Medical Journal* i, 994.

OXOID MANUAL (1965). 3rd ed., p. 90. London: Oxoid Ltd.

PILLEMER, L., BLUM, L. & LEPOW, I. H. (1954). Protective antigen of *Haemophilus pertussis*. *Lancet* i, 1257.

PITTMAN, M. (1970). *Bordetella pertussis* – bacterial and host factors in the pathogenesis and prevention of whooping cough. In *Infectious Agents and Host Reactions* (ed. S. Mudd), p. 249. Philadelphia: Saunders.

PRESTON, N. W. (1963). Type-specific immunity against whooping-cough. *British Medical Journal* ii, 724.

PRESTON, N. W. (1966). Potency tests for pertussis vaccines: doubtful value of intracerebral challenge test in mice. *Journal of Pathology and Bacteriology* **91**, 173.

PRESTON, N. W. (1970*a*). Pertussis: the epidemiological situation in various countries – serotypes. In *International Symposium on Pertussis*, Bilthoven 1969. *Symposia Series in Immunobiological Standardization* vol. 13, p. 18. Basel: Karger.

PRESTON, N. W. (1970*b*). Technical problems in the laboratory diagnosis and prevention of whooping-cough. *Laboratory Practice* **19**, 482.

PRESTON, N. W. (1971). The importance of the different serotypes of *Bordetella pertussis* in the effectiveness of pertussis vaccines. *Scientific Publication* no. 226, p. 371. Washington, D.C.: Pan American Health Organisation.

PRESTON, N. W. & EVANS, P. (1963). Type-specific immunity against intra-cerebral pertussis infection in mice. *Nature, London* **197**, 508.

PRESTON, N. W. & STANBRIDGE, T. N. (1972). Efficacy of pertussis vaccines: a brighter horizon. *British Medical Journal* iii, 448.

PRESTON, N. W. & TE PUNGA, W. A. (1959). The relation between agglutinin production by pertussis vaccines and their immunising potency in mice. *Journal of Pathology and Bacteriology* **78**, 209.

PUBLIC HEALTH LABORATORY SERVICE (1969). Efficacy of whooping-cough vaccines used in the United Kingdom before 1968. A preliminary report. *British Medical Journal* iv, 329.

PUBLIC HEALTH LABORATORY SERVICE (1973). Efficacy of whooping-cough vaccines used in the United Kingdom before 1968. Final report. *British Medical Journal* i, 259.

REED, L. J. & MUENCH, H. (1938). A simple method of estimating fifty per cent endpoints. *American Journal of Hygiene* **27**, 493.

SAUER, L. W. & HAMBRECHT, L. (1929). Experimental whooping-cough. *American Journal of Diseases of Children* **37**, 732.

SHMILOVITZ, M., PRESTON, N. W., ZALTSER, H. & CAHANA, A. (1972). Whooping cough in northern Israel. *Israel Journal of Medical Sciences* **8**, 1936.

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The bacteriological examination of urine: a computer-aided study

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SUMMARY

For 6 months details of every patient who had his or her urine sent to a laboratory for bacteriological examination and the result of such examination were entered on a computer-card. A total of 15,606 cards were completed with information in code recording the sex and age of a patient, the origin of the request, the presence or absence in the urine of an excess of protein or cells, the culture result and the name of any significant organism isolated together with its sensitivity to various antimicrobial drugs. This information was interrelated in a computer, and in some cases the resulting numerical details were expressed as rates so as to eliminate the effect of uneven sex and age distribution. In this way the occurrence of urinary tract infection and the type of infecting organism in persons of either sex at various ages was examined according to whether the patient was in hospital or general practice. The sensitivity pattern of each type of significant organism isolated was established according to its source. The association between patients of either sex and various ages who had, or did not have, bacteriologically evident infections and the presence in their urine of an inflammatory exudate was investigated. Finally, the capacity of each type of infecting organism to produce such an exudate was estimated.

It was shown that hospital and general practice experience of urinary tract infections differed widely, with regard both to the age and sex distribution of those suffering from it, and to the causative organisms concerned and their sensitivities to antimicrobial drugs. It is suggested that these differences were so great that conclusions drawn from any study of this subject conducted in one of the two areas cannot be applied to the other, and that those derived from a mixture of the two will vary according to the relative sizes of each of the components.

INTRODUCTION

A computer-card was punched with coded details of each urine specimen sent to the Hospital Microbiology and Public Health Laboratory, Plymouth, during the 6 months from October 1972 to March 1973. No publicity was given to the study so that there would be no change in the way specimens were submitted. They were examined by simple methods appropriate in a busy laboratory, and the cards

which resulted were put into an IBM 1130 computer, which sorted the information and extracted certain relations.

The laboratory serves 400,000 people, more than half of whom live in the City of Plymouth. Specimens are accepted from hospitals and from general practice, and as the nearest similar laboratory is 40 miles away it may be assumed that all bacteriology for this population is done in one place. It was therefore possible to apply population statistics for the area to the information collected and so allow comparisons to be made of the incidence of infection and distribution of infecting organisms in patients of either sex at various ages.

METHODS

Clean-catch or mid-stream urine specimens were passed, or were transferred immediately after passing, into sterile plastic screw-capped 1 fl-oz. (28 ml.) bottles, each containing 0·5 g. of boric acid powder (Porter & Brodie, 1969). They were brought to the laboratory by hand, or sent there by post or by public or other transport, depending on the distance and the communications available. In many cases specimens were examined within 4 hr. of being passed, but some which came through the post were as much as 36 hr. old by the time they were dealt with.

Examination began by centrifuging 7 ml. of urine at 500 *g* in a 12 × 100 mm. glass tube for 7 min. The supernatant resulting was decanted, and the deposit resuspended in the amount of urine adhering to the glass by flicking the bottom of the tube. A drop of this was examined microscopically under a coverslip, at first at low power. A representative area was chosen and pus cells were counted in not less than five fields each of 0·31 mm. diameter, at a magnification of 280. The average number of cells per field was graded into one of four classes for reporting, though for the computer four or less cells were recorded as negative, and five or more as positive. Each specimen was tested for the presence of protein by adding 3 vol. of 3% sulphosalicylic acid to 1 vol. of urine; any turbidity which developed was compared with proteinometer standards (Gallenkamp Ltd). Four degrees of positivity were reported, but for the computer less than 10 mg. of protein per 100 ml. was recorded as negative, and more than this as positive. When indicated a direct sensitivity test was done by spreading about 0·03 ml. (three calibrated loopfuls) of urine on 'Wellcotest' sensitivity-test agar (Wellcome Reagents Ltd) in a Petri dish and adding one of the 'Multodisks' described below. Whether a direct sensitivity was done or not, a half Petri dish of cystine–lactose–electrolyte-deficient (CLED) medium (Mackey & Sandys, 1966) was spread with 0·01 ml. urine, using a fused calibrated loop. After overnight incubation the dishes were examined and the results on CLED medium were reported and coded according to the following conventions:

Culture negative	No growth	No colonies in the spread area
	No significant growth	Less than 100 colonies in the spread area
	Culture of doubtful significance	More than 100 colonies made up of two or more different organisms

Culture positive	Single pathogen	More than 100 colonies* in pure or near pure culture
	Mixed growth of two or three pathogens	More than 100 colonies each of two or rarely three organisms
	Mixed growth with one predominant pathogen	More than 100 colonies of the predominant organism, together with a mixed background growth

* Urines aspirated suprapubically were reported as positive if they gave any growth. Specimens collected via an indwelling catheter were regarded as positive if they gave more than 10 colonies when cultured as described.

Organisms leading to positive culture reports were divided into 12 named types which were separately coded for entry on computer-cards, according to the following criteria:

Gram-negative bacilli	
1. *Escherichia coli*	Characteristic colonial morphology, lactose positive, indole positive
2. *Klebsiella* spp.	Characteristic colonial morphology, lactose positive, indole negative
3. *Proteus* spp.	Characteristic colonial morphology, lactose negative, strong urease production
4. Paracolons	Variable colonial morphology, lactose negative, negative or weak urease production
5. Coliforms	Variable colonial morphology, lactose positive
6. *Pseudomonas* spp.	Characteristic colonial morphology and pigment (oxidase positive)
Gram-positive cocci	
7. Faecal streptococci	Characteristic colonial morphology (catalase negative)
8. *Staphylococcus albus*	Characteristic colonial morphology, coagulase negative, novobiocin sensitive (catalase positive)
9. *Micrococcus* spp.	Characteristic colonial morphology, coagulase negative, novobiocin resistant (catalase positive)
10. *Staphylococcus aureus*	Characteristic colonial morphology, coagulase positive (catalase positive)
Others	
11. *Candida* spp.	Characteristic morphology
12. Other organisms	

(Criteria in parentheses were only used in cases of doubt.)

Where a direct sensitivity test had been done a report was issued after 24 hr. but to code the organism for the computer it was subjected to any additional tests needed so as to allocate it to one of the types listed.

Strains other than *Candida* spp. from positive cultures were tested for their sensitivity to a selected group of antimicrobial drugs. Where a direct sensitivity had not been done, representative colonies of the organism were suspended in peptone-water to an optical density judged by eye as being sufficient to produce a semi-confluent growth on sensitivity-test agar inoculated with about 0·03 ml. of the suspension, spread with a bent glass rod. A 'Multodisk' (Oxoid Ltd) was added, bearing the antimicrobial drugs listed below, at the concentrations noted. A disk with the first eight drugs was used for the Gram-negative types, excluding

Pseudomonas spp., and for faecal streptococci. A second disk from which the next two antibiotics on the list were reported routinely was used for the remaining Gram-positive organisms. The last three antibiotics on the list were used for strains of *Pseudomonas* spp.:

Cotrimoxazole	25 μg.	Tetracycline	50 μg.
Kanamycin	30 μg.	Penicillin	1·5 units
Cephalexin	30 μg.	Erythromycin	10 μg.
Nitrofurantoin	200 μg.	Gentamicin	10 μg.
Nalidixic acid	30 μg.	Colistin	200 μg.
Ampicillin	25 μg.	Carbenicillin	100 μg.
Sulphonamide	500 μg.		

After about 18 hr. incubation, sensitivity results were read according to the sizes of the zones of inhibition of bacterial growth produced by the drugs. These were measured by eye where the results were clear-cut or by using a ruler when the size of any zone was close to the measurement separating sensitive from resistant strains. The reading for each drug was reported and coded as sensitive or resistant according to whether the zone was larger or smaller than the critical size decided for the organism concerned. This had been determined for each drug by testing a large number of isolates together with a known sensitive reference strain of each type of organism under the same conditions as used in the test. The zone sizes measured in this way usually had a bimodal distribution corresponding to sensitive and resistant organisms, and the critical size dividing sensitive from resistant strains was chosen at a point between the two peaks. When a drug failed to produce a biphasic curve when tested in this way its minimum inhibitory concentration against a number of the organisms concerned was measured, and the critical zone size chosen by reference to the results. The 'Multodisk' used for testing Gram-positive species included novobiocin (5 μg.). This antibiotic was used to divide coagulase-negative staphylococci into *Staphylococcus albus* (sensitive) and *Micrococcus* spp. (resistant) (Mitchell & Baird-Parker, 1967) for this study.

In addition to the laboratory results other information was entered on the computer-cards. This included the patient's sex and age, the latter coded in one of the eight groups shown in the 'Results' section, or where necessary as 'not given'. The origin of the specimen, from a medical in-patient (MIP), surgical in-patient (SIP), hospital out-patient (OP), or from the patient of a general practitioner (GP), was added.

It did not prove possible to identify second or third specimens coming from the same patient during the trial, so duplicate or multiple entries were not excluded from the computer except in the case of those sent for examination only for *Mycobacterium tuberculosis*. Some clinicians regularly re-examined patients' urine after treatment, and a few patients had repeated tests carried out on their urine during the 6 months of the survey.

Before the beginning of the study proper, the whole system was put into operation for a month to practise methods and to prove the computer programme. Thereafter the cards produced were summarized monthly in the computer, and the

Table 1. *Details of urines examined, according to the sex of the patients and the sources of the specimens*

Source		Males	Females	Totals	
Hospital	MIP*	1,504	2,667	4,171	10,044 (64·4)
	SIP*	1,935	2,320	4,255	
	OP*	458	1,160	1,618	
GP*		1,208	4,354	5,562	(35·6)
Totals		5,105 (32·7)	10,501 (67·3)	15,606	

* (Percentages are given in parentheses. * MIP, Medical in-patient; SIP, surgical in-patient; OP, out-patient; GP, patient of a general practitioner.)

Table 2. *The distribution, percentage, of the six varieties of culture result which led to positive or negative reports*

Result reported	Percentage			
	Males	Females	Both	
Culture negative				
No growth	67·8	51·6	57·0	80·2
No significant growth	16·4	26·1	22·9	
Culture of doubtful significance	0·3	0·3	0·3	
Culture positive				
Single pathogen	13·2	19·2	17·2	19·8
Mixed growth with two or three pathogens	1·4	1·3	1·3	
Mixed growth with one predominant pathogen	0·9	1·5	1·3	

results used to check the internal reproducibility of the study, which proved to be high. Full interrelationships were established after the third and the sixth months.

RESULTS

A total of 15,606 specimens of urine were examined and were recorded on computer-cards during the study. Two-thirds were from female patients and one-third were sent by general practitioners; Table 1 gives their sources more completely. The distribution of the six defined types of culture result is given in Table 2. In all, one specimen in five was culture-positive. This ratio held almost exactly when three of the four sources were considered individually, but specimens from hospital out-patients produced only one positive in eight examined. Urines from females were positive more often than those from males, and they had a higher incidence of results indicating contamination.

Fig. 1 shows the rate at which examinations of urine were carried out and positive results found in various groups of patients defined by sex and age. The actual numbers observed have been converted into rates per 10,000, using the Registrar General's population figures (*The Registrar General's Statistical Review of England and Wales for the Year 1970*) for the area as a basis. In Table 3 the number of organisms of each type which led to a positive culture report is given, first as a simple total and then as a rate of such isolations per 1000 patients having their

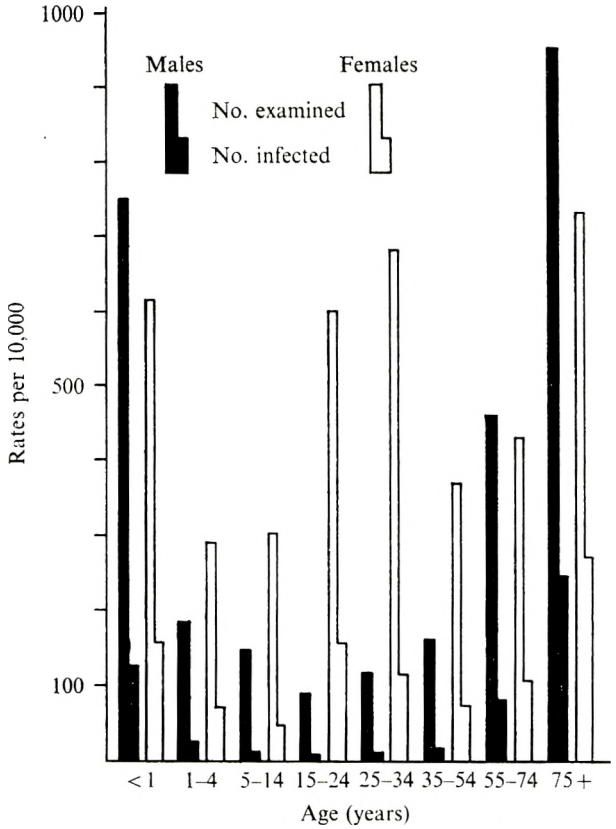

Fig. 1. The number of bacteriological examinations of samples of urine from a population divided into 16 groups by sex and age, expressed as a rate per 10,000 patients in each group (whole column) together with the incidence of bacteriologically proved infections, similarly divided and expressed (broad lower part of each column).

urines examined from each of the sources, divided according to the sex of the patients. This table does not include four isolations which did not fit into any of the 11 named groups. They were classified for the computer as 'other organisms'; all were non-faecal streptococci. Table 4 shows the rate of isolation of each type of organism per 10,000 patients in each of several groups defined by sex and age. At the bottom of this table is given the rate per 10,000 at which patients in each of the sex and age groups had their urine examined, divided to show whether they were being seen in hospital or in general practice.

When the results of the examinations of urine for protein and cells were looked at in detail, it became clear that no purpose would be served by dealing with the four possible types of finding separately. The summary given in Table 5 keeps some separation of protein and cell results but otherwise any specimen with an excess of protein or cells or both was regarded as containing an inflammatory exudate and was called 'exudate positive'. The proportion of patients who were exudate positive in each of the groups defined by sex and age is shown in Fig. 2, where those with a positive culture result have been separated from those found to be negative. Table 6 displays the association between each type of pathogen and the presence or absence of an exudate.

Table 3. *The number of each type of organism isolated expressed as a total and as a rate per 1000 patients from each source who were examined for the presence of a urinary infection*

No. of isolations	Types of organism	Isolations/1000 examinations from each source							
		MIP*		SIP*		OP*		GP*	
		M	F	M	F	M	F	M	F
1509	*Escherichia coli*	46	130	41	107	35	79	60	135
472	*Proteus* spp.	37	47	27	36	15	8	29	24
290	Faecal streptococci	22	22	23	22	17	11	17	14
203	Paracolons	9	13	11	12	11	8	12	18
155	*Staphylococcus albus*	16	7	29	10	4	6	8	3
144	Coliforms	12	15	8	13	2	4	7	6
133	*Klebsiella* spp.	11	15	10	8	2	7	3	6
116	*Micrococcus* spp.	1	3	6	3	0	5	3	18
108	*Candida* spp.	7	10	11	18	0	2	2	1
94	*Pseudomonas* spp.	12	8	9	7	4	4	7	1
47	*Staphylococcus aureus*	7	1	8	3	9	0	2	0

* See key at foot of Table 1.

Table 7 gives the results of sensitivity tests done between the antimicrobial drugs listed and the organisms isolated. These have been divided according to whether the specimen yielding each organism came from general practice, or from one of the hospital (MIP, SIP, OP) sources.

Finally, 20 organisms identified as paracolons, together with 20 coliforms and 25 *Proteus* spp. randomly isolated and initially identified according to the criteria given, were examined in rather greater detail so as to discover the major species making up these types. Fifteen of the paracolons (75%) proved to be lactose-negative *Escherichia coli*; the remainder were *Enterobacter* spp. or lactose-negative *Klebsiella* spp. Twelve (60%) of the coliforms were *Esch. coli* of atypical morphology on CLED medium, and the remainder were atypical *Klebsiella* spp., half of them being indole positive. Of the *Proteus* spp., 23 (94%) were *Pr. mirabilis*, and there was one *Pr. vulgaris* and one *Pr. morgani*.

DISCUSSION

The 1-to-4 positive-to-negative ratio of culture results observed here is artificially small because the study includes patients whose urine was examined more than once, often as a test of cure. Others (Gallagher, Montgomerie & North, 1965; Mond, Percival, Williams & Brumfitt, 1965; Steensberg *et al.* 1969; Brooks & Maudar, 1972; Dove *et al.* 1972) have noted ratios of about 1-to-1 in smaller series where repeat examinations were excluded and in which there was likely to have been critical patient selection. There are a number of studies (Fry, Dillane, Joiner & Williams, 1962; Loudon & Greenhalgh, 1962; Steensberg *et al.* 1969) from general practice in which urinary tract infection is seen predominantly as a disorder of young and middle-aged women. When the general practice component in the

Table 4. *The number of isolations of the types of organism named per* 10,000 *males or females in the age-groups shown, together with a comparison of the rates of presentation of patients from these sex and age groups in hospital and general practice*

Types of organism or source	Numbers per 10,000 in each sex and age group															
	<1		1–4		5–14		15–24		25–34		35–54		55–74		75+	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Escherichia coli	60	106	8	44	2	32	1	54	4	64	6	45	23	56	67	125
Proteus spp.	10	11	6	8	5	3	1	10	0	12	1	11	13	19	65	49
Faecal streptococci	7	14	5	5	1	2	1	9	2	11	2	5	12	7	31	28
Paracolons	7	0	0	3	2	4	0	9	1	7	2	5	5	7	14	15
Staphylococcus albus	23	4	2	2	0	1	0	7	1	4	2	1	9	2	28	5
Coliforms	3	18	1	4	0	1	0	1	1	4	0	2	5	6	20	18
Klebsiella spp.	0	7	0	0	0	1	0	2	0	4	1	1	6	5	8	24
Micrococcus spp.	3	0	2	0	0	1	0	20	0	10	0	1	2	1	3	2
Candida spp.	0	0	1	2	0	1	0	2	0	1	0	2	4	6	15	14
Pseudomonas spp.	13	4	0	6	0	1	0	0	0	1	2	1	4	2	11	11
Staphylococcus aureus	7	0	2	0	0	1	0	0	1	0	0	0	4	1	6	5
MIP* SIP* OP*	706	567	111	122	77	134	63	305	71	333	101	176	386	317	875	614
GP*	44	46	71	167	69	168	25	290	45	341	58	187	65	108	76	108

* See key at foot of Table 1.

Table 5. *Results, percentage, of the examination of male and female urines for protein and for excess cells, related to culture findings*

		Percentage		
		Culture positive	Culture negative	Totals
Males	Protein positive	10	24	100
	Protein negative	5	61	
	Protein and/or cells positive	13	27	100
	Protein and cells negative	3	57	
Females	Protein positive	12	15	100
	Protein negative	10	63	
	Protein and/or cells positive	16	20	100
	Protein and cells negative	6	58	

Table 6. *The percentages of patients with infections due to the organisms named who also had either protein or an excess of cells or both (inflammatory exudate) in their urine*

Types of organism	Inflammatory exudate %	Types of organism	Inflammatory exudate %
Micrococcus spp.	92	*Klebsiella* spp.	76
Staphylococcus aureus	87	Coliforms	76
Candida spp.	86	*Pseudomonas* spp.	75
Paracolons	84	*Escherichia coli*	72
Proteus spp.	81	*Staphylococcus albus*	70
Faecal streptococci	76		

present study is examined on its own by referring to the last two rows of Table 4 and to Fig. 1 good agreement with these studies is seen even though the effect of using rates for greater comparability alters the shape of the curve. However, when hospital and general practice experience is combined and in particular when rates are used to even out the disparity in the sizes of the various age groups, a different picture emerges. Examination for urinary tract infection and its bacteriological diagnosis is seen in Fig. 1 to have affected both sexes almost equally at the extremes of age. Table 4 shows that most of these young and old patients were in hospital. Fig. 1 also confirms the higher incidence of examination for and diagnosis of urinary infection in females in the early years of sexual activity not seen in males. It must be added that because bacteriological help in the diagnosis and treatment of urinary tract infections is more likely to be sought in hospital than in general practice, the relative heights of the columns in Fig. 1 must not be taken as absolute indices of these infections in a community. The preponderance of females in middle life seen in general practice must outweigh hospital experience of involvement of both sexes at the extremes of age, but the absolute incidence of such cases has not been established in this study. However, this does not invalidate a comparison between the occurrence of urinary symptoms leading to a bacteriological examination, and the diagnosis of an infection in males and females of different ages illus-

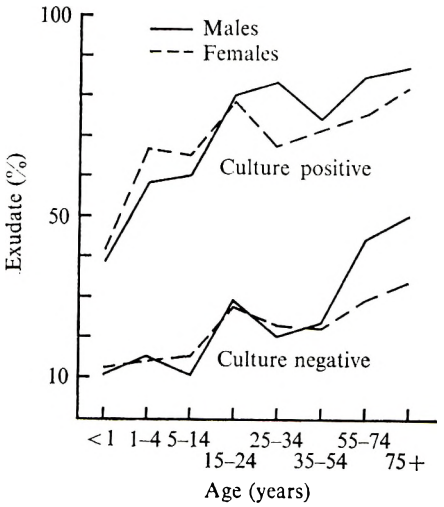

Fig. 2. The incidence of an inflammatory exudate (excess protein or cells or both) in urine, by sex and age, divided according to the presence or absence of significant bacteriuria.

trated in Fig. 1. Table 2 shows that the suspicion of urinary tract infection proved to be bacteriologically correct more often in women (22·0%) than in men (15·5%), and this table also gives an indication of the degree of contamination in specimens from females, whose urines gave rise to a greater incidence of reports of 'no significant growth' and 'mixed growth with one predominant pathogen' than did specimens from males, though the difference between the sexes was less than might have been expected. This, together with the generally low incidence of doubtful results, is thought to have been due to the use of boric acid, which had the effect of turning urine into its own bacteriostatic transport medium.

Tables 3 and 4 taken together show the distribution of the various urinary pathogens in a population. A study of these tables and of Fig. 1 shows how widely different conclusions might be reached about the incidence of urinary tract infection and the distribution of its causative organisms by being selective in the choice of the patients surveyed. However, it is clear that *Escherichia coli* was the dominant pathogen in the urine of those of both sexes of all ages from all sources, with the single exception of males in the 5–14 age group, among whom *Proteus* spp. was more common. The dominance of *Esch. coli* is even more striking when the 75% of paracolons which proved to be lactose-negative strains of this organism and the 60% of coliforms which were also *Esch. coli*, though colonially atypical, are added. The number of lactose-negative *Esch. coli* that were found is nearly 10% of all the strains of this organism isolated, a proportion which agrees with the findings of another group (McAllister *et al.* 1971) and with an estimate of the incidence of such strains among *Esch. coli* in general (Edwards & Ewing, 1964). *Esch. coli* was quite evenly spread among patients from the different sources given in Table 3, and it produced almost the lowest incidence of associated inflammatory exudate (Table 6). The sensitivity to antimicrobial drugs of strains of this organism isolated

Table 7. *The percentage of strains which were resistant to various antimicrobial drugs among the organisms isolated, divided according to their origin in hospital or general practice*

Types of organism	Source	Resistant strains (%)												
		G	PN	SXT	TE	F	K	NA	CL	P (PN)	E	CN	CS	PY
Escherichia coli	Hospital	33	28	9	23	4	6	3	9	—	—	—	—	—
	GP	20	17	2	14	2	1	1	5	—	—	—	—	—
Proteus spp.	Hospital	12	10	7	98	99	2	6	15	—	—	—	—	—
	GP	10	6	8	99	99	2	3	10	—	—	—	—	—
Faecal streptococci	Hospital	98	3	9	50	1	92	98	95	—	—	—	—	—
	GP	96	1	2	43	4	95	99	96	—	—	—	—	—
Paracolons	Hospital	30	38	14	18	21	6	12	30	—	—	—	—	—
	GP	20	23	5	18	11	3	2	16	—	—	—	—	—
Coliforms	Hospital	49	67	28	43	17	14	15	26	—	—	—	—	—
	GP	41	47	6	35	26	6	9	24	—	—	—	—	—
Klebsiella spp.	Hospital	52	83	28	34	19	6	15	17	—	—	—	—	—
	GP	26	74	15	22	22	11	11	27	—	—	—	—	—
Staphylococcus albus	Hospital	20	—	21	66	1	26	99	—	72	27	—	—	—
	GP	14	—	19	43	0	19	95	—	62	5	—	—	—
Micrococcus spp.	Hospital	18	—	21	45	0	15	100	—	70	13	—	—	—
	GP	1	—	1	10	0	0	100	—	65	2	—	—	—
Staphylococcus aureus	Hospital	20	—	18	45	0	15	100	—	77	33	—	—	—
	GP	38	—	14	38	0	0	100	—	88	38	—	—	—
Pseudomonas spp.	Hospital	—	—	—	—	—	—	—	—	—	—	3	5	24
	GP	—	—	—	—	—	—	—	—	—	—	7	0	43

Key: G, sulphonamide; PN, ampicillin; SXT, cotrimoxazole; TE, tetracycline; F, nitrofurantoin; K, kanamycin; NA, nalidixic acid; CL, cephalexin; P, benzyl penicillin; E, erythromycin; CN, gentamicin; CS, polymyxin, colomycin; PY, carbenicillin.

from patients in general practice was quite different from that of those found in hospital (Table 7), the latter being noticeably more resistant.

Proteus spp., almost exclusively *Pr. mirabilis*, were distributed somewhat like *Esch. coli*, though with a slight predilection for males, particularly the old and the very young, and a tendency to be found in patients in medical wards in hospital more often than elsewhere. The organisms were more frequently associated with an inflammatory exudate than *Esch. coli*, and although they showed a smaller variation in sensitivity pattern between isolates from hospital and general practice, those from general practice were on the whole more sensitive. The position of *Proteus* spp. as the second most common organism isolated in urinary tract infections is usual (Loudon & Greenhalgh, 1962; Gallagher *et al.* 1965; Mond *et al.* 1965; McAllister *et al.* 1971) except in the series where selection of patients by age and sex or by source has been such as to impose a different pattern (Steensberg *et al.* 1969; Dove *et al.* 1972).

The next most common organism at about 9% of all was the faecal streptococcus. The age, sex and source distribution of this organism follows that of *Proteus* spp. closely. As with other Gram-positive organisms, the reported incidence of infections with this agent in a number of series has been variable (Garrod, Shooter & Curwen, 1954; Loudon & Greenhalgh, 1962; McAllister *et al.* 1971) and although this may be due to patient selection, other possibilities exist which will be mentioned later.

The distribution of, and sensitivity test results on, the paracolons and coliforms followed those of the species into which the more detailed survey divided them. Thus the paracolons, most of which were lactose-negative *Esch. coli*, had a similar distribution to that organism, whereas the coliforms which included significant numbers of *Klebsiella* spp. as well as *Esch. coli* occupied an intermediate position between the two. *Klebsiella* spp. were found more commonly in hospital practice, were more resistant to antibacterial drugs, and compared with *Esch. coli* were more often found in the very old. *Pseudomonas* spp. were hospital strains with a predilection for the old and the very young, with a slight male preponderance.

The Gram-positive bacteria isolated from cases of urinary tract infection made an interesting study. The faecal streptococcus has been widely accepted as a pathogen and it figures in most published series, though with variations in its proportional incidence, as has been noted. This variation is much greater with the other Gram-positive species. For instance, one group found that 4·06% of 1556 causal organisms isolated from cases of urinary tract infection were Gram-positive (Arneil, McAllister & Kay, 1973); in making this statement the authors appear to be referring to a contribution to a collaborative study (McAllister *et al.* 1971) in which these Gram-positive organisms are given as *Streptococcus faecalis* 4·0% and *Staphylococcus aureus* 0·06%, with no isolations of coagulase-negative staphylococci. At the other end of the scale another group report that 24% of 55 organisms isolated from urine obtained by suprapubic aspiration from women aged between 16 and 60 were coagulase-negative staphylococci (Dove *et al.* 1972). This figure is notable, because it is almost identical with that which can be derived from Table 4 when infections due to *Staph. albus* and *Micrococcus* spp. for the age-

and sex-groups covered by the investigation quoted are combined. The pathogenic status of these organisms has been accepted by many for some time (Garrod *et al.* 1954; Gallagher *et al.* 1965; Mond *et al.* 1965; Steensberg *et al.* 1969), and they have been the subject of specific study (Pereira, 1962; Mitchell, 1965). In the series reported here 8·3% of all significant organisms were coagulase-negative staphylococci, divided by their sensitivity to novobiocin into *Staph. albus* (4·7%) and *Micrococcus* spp. (3·6%). Separated in this way these organisms had very different distributions, the *Micrococcus* spp. being the second most common pathogen in women aged between 15 and 34, while *Staph. albus* was found chiefly among male patients in surgical wards at the extremes of age. Of all organisms micrococci were the most productive of inflammatory exudate at 92%, whereas *Staph. albus* stood at the other end of the scale with only 70% associated exudate (Table 6). It may be that the latter organism frequently contaminated urine samples collected in infancy. Its remarkably high incidence in males under 1 year old might have been because it colonized the preputial sac and thus contaminated specimens collected in adhesive bags with significant multiplication before the urine reached boric acid in the final container. The remaining Gram-positive bacterial pathogen, *Staph. aureus*, follows *Staph. albus* in being found chiefly in old or very young males in hospital.

There are probably several reasons for the wide variation in the reported incidence of Gram-positive organisms in urinary tract infections. One is that a few bacteriologists would not accept *Staphylococcus albus* or *Micrococcus* spp. as pathogens, so that any growth of these was ignored. Another may result from the use of MacConkey's medium for urinary bacteriology. This medium was not designed for work with Gram-positive organisms, and its ability to support their growth is variable. The introduction of CLED medium, based on the electrolyte-deficient medium of Sandys (1960), provides a cheap non-inhibitory alternative which gives good colonial differentiation while suppressing the swarming of *Proteus* spp. A third possibility is that the *Micrococcus* spp. may be pathogenic in the so-called urethral syndrome, but that it is ignored if the figure of 100,000 organisms per ml. for an infection is rigidly applied and particularly if the small colonies which this organism produces are wrongly interpreted numerically when using the dip-inoculum technique. The efficient bacteriostatic action of boric acid has allowed the lower standard of 10,000 organisms per ml. to be adopted here with a confidence strengthened by the agreement just noted between the rates of isolation of significant coagulase-negative staphylococci in this study and those reported by Dove *et al.* (1972) who used suprapubic aspiration for the collection of their specimens.

Candida spp. are absent from several lists of pathogens of the urinary tract, appearing only in larger or more specialized works (Seneca, Longo & Peer, 1968; Rocha, 1972). In the present series they were reported as significantly present more often than *Pseudomonas* spp. and *Staph. aureus*, with an incidence of 3·3% among all pathogens. The paucity of previous records may reflect the fact that many reports in this field have come from general practice, whereas significant isolations of *Candida* spp. are seen in Table 3 to have been hospital strains. The suggestion

that their presence in urine may follow contamination from the female genital tract is countered by the observation that they were isolated from either sex with equal frequency, and that as generators of inflammatory exudate *Candida* spp. were near the top of the list at 86%. The status of this organism as a potential pathogen in the urinary tract perhaps deserves more attention.

The distribution of inflammatory exudate in urine revealed by this survey shows once more how unreliable is the practice of using a test for an excess of either protein or cells as the sole criterion in diagnosing a urinary tract infection. Table 5 shows that on the basis of the presence of protein alone one-third of male and nearly half of female infections would have been missed, and that one-third of the males and a quarter of the females presenting with urinary symptoms would have been treated for bacteriologically non-existent infections. Table 5 also shows that when protein and cells were considered together the rate of error was diminished but that it remained unacceptably high. This fact has been publicized (Loudon & Greenhalgh, 1962; Mond *et al.* 1965; Brooks & Maudar, 1972), but the practice is still so widespread as to require the point to be made again. In Fig. 2 the incidence of inflammatory exudate by sex and age and the presence or absence of a positive culture result has been plotted. Those with symptoms referable to the urinary tract, but without bacteriologically demonstrable infection at the time of examination, had an incidence of inflammatory exudate which increased almost linearly with age. Males and females moved very similarly in this respect until the onset of prostatic enlargement in the male began to have an effect. In the presence of bacteriologically proved infection the incidence of exudate was increased by about 50% at all ages in both sexes, so that the 'infected' curves for both sexes remained almost parallel to the non-infected ones at all ages except in those under 1 year where there was a lower incidence of exudate in the presence of positive culture results. This may have been due to bacteriological mis-diagnosis in this age group, owing to the difficulty of collecting specimens aseptically. Those who have compared the results of the examination of urine samples gathered from infants by the usual methods with those of specimens withdrawn by suprapubic aspiration might agree. Clarification of this point is clearly important so long as urinary infection and chronic pyelonephritis are linked in that order as cause and effect.

It has been suggested that the choice of antimicrobial drug for use in clinically diagnosed acute urinary tract infection before a bacteriological diagnosis is available or where no laboratory examination is to be made, should be from a list of drugs arranged in order of preference as the result of studies of the sensitivity of a large number of urinary pathogens brought up-to-date from time to time (McAllister *et al.* 1971). The results presented here suggest that a single list should not be used for this purpose. The sensitivity of strains from general practice and hospital differed sufficiently widely for any single set of figures to depend on the relative sizes of the contributions from the two sources. Also, the distribution of the groups of pathogen involved in urinary tract infection changed according to the age, sex and source of the patients yielding them, so that by varying the composition of a trial group the pathogens concerned and hence the sensitivity results for all patients taken together alter. Finally, if one or other potential

pathogen is under-represented for any reason, remarkably misleading suggestions may result. It seems possible that the best advice can only be given if the sort of information in Tables 3 and 4 is available, so that the most likely pathogens can be identified according to the circumstances, and the 'best' antimicrobial drug chosen. For instance, the most likely cause of a urinary tract infection in a woman of 24 in general practice was either *Esch. coli* or *Micrococcus* spp. Turning to the antibiotic sensitivity pattern of these organisms in general practice (Table 7), it appears that cotrimoxazole represented the best choice of drug for this patient. It also seems that ampicillin might have failed in a significant number of cases, and that nalidixic acid was a poor choice because of the complete resistance of the *Micrococcus* spp. to this drug.

The Head of the Department of Mathematics, Plymouth Polytechnic, kindly allowed access to his Department's computer. Members of his staff gave invaluable assistance in programming and operating it.

REFERENCES

ARNEIL, G. C., MCALLISTER, T. A. & KAY, P. (1973). Measurement of bacteriuria by plane dipslide culture. *Lancet* i, 94–5.

BROOKS, D. & MAUDAR, A. (1972). Pathogenesis of the urethral syndrome in women and its diagnosis in general practice. *Lancet* ii, 893–8.

DOVE, G. A., BAILEY, A. J., GOWER, P. E., ROBERTS, A. P. & DE WARDENER, H. E. (1972). Diagnosis of urinary-tract infection in general practice. *Lancet* ii, 1281–3.

EDWARDS, P. R. & EWING, W. H. (1964). In *Identification of Enterobacteriaceae*, p. 62. Minneapolis: Burgess Publishing Company.

FRY, J., DILLANE, J. B., JOINER, C. L. & WILLIAMS, J. D. (1962). Acute urinary infections. Their course and outcome in general practice with special reference to chronic pyelonephritis. *Lancet* i, 1318–21.

GALLAGHER, D. J. A., MONTGOMERIE, J. Z. & NORTH, J. D. K. (1965). Acute infections of the urinary tract and the urethral syndrome in general practice. *British Medical Journal* i, 622–6.

GARROD, L. P., SHOOTER, R. A. & CURWEN, M. P. (1954). The results of chemotherapy in urinary infections. *British Medical Journal* ii, 1003–8.

LOUDON, I. S. L. & GREENHALGH, G. P. (1962). Urinary tract infections in general practice. *Lancet* ii, 1246–8.

MCALLISTER, T. A., PERCIVAL, A., ALEXANDER, J. G., BOYCE, J. M. H., DULAKE, C. & WORMALD, P. J. (1971). Multicentric study of sensitivities of urinary tract pathogens. *Postgraduate Medical Journal* **47** (suppl.), 7–18.

MACKEY, J. P. & SANDYS, G. H. (1966). Diagnosis of urinary infections. *British Medical Journal* i, 1173.

MITCHELL, R. G. (1965). Staphylococci and urinary infection. *British Medical Journal* i, 1127.

MITCHELL, R. G. & BAIRD-PARKER, A. C. (1967). Novobiocin resistance and the classification of staphylococci and micrococci. *Journal of Applied Bacteriology* **30**, 251–4.

MOND, N. C., PERCIVAL, A., WILLIAMS, J. D. & BRUMFITT, W. (1965). Presentation, diagnosis, and treatment of urinary-tract infections in general practice. *Lancet* i, 514–6.

PEREIRA, A. T. (1962). Coagulase-negative strains of staphylococcus possessing antigen 51 as agents of urinary infection. *Journal of Clinical Pathology* **15**, 252–3.

PORTER, I. A. & BRODIE, J. (1969). Boric acid preservation of urine samples. *British Medical Journal* ii, 353–5.

The Registrar General's Statistical Review of England and Wales for the Year 1970. Part II. London: H.M. Stationery Office (1972).

ROCHA, H. (1972). In *Urinary Tract Infection and its Management* (ed. D. Kaye), p. 11. Saint Louis: The C.V. Mosby Company.

SANDYS, G. H. (1960). A new method of preventing swarming of *Proteus* spp. with a description of a new medium suitable for use in routine laboratory practice. *Journal of Medical Laboratory Technology* **17**, 224–33.

SENECA, H., LONGO, F. & PEER, P. (1968). Candida pyelonephritis and candiduria: the clinical significance of Candida albicans in urine cultures. *Journal of Urology* **100**, 266–9.

STEENSBERG, J., BARTELS, E. D., BAY-NIELSEN, H., FANØE, E. & HEDE, T. (1969). Epidemiology of urinary tract diseases in general practice. *British Medical Journal* iv, 390–4.

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Serological evidence of infection with Tana and Yaba pox viruses among several species of monkey

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SUMMARY

Sera from cynomolgus monkeys from Malaysia, from Indian rhesus monkeys, from various species of monkeys from Africa and from South America have been examined for neutralizing antibody to Tanapox and Yaba viruses. No antibody was found to either virus in the sera of rhesus monkeys or South American monkeys. A certain proportion of sera from cynomolgus monkeys and various species of African monkey showed antibody to one or other of the viruses, but few of the positive sera showed antibody to both. The results would seem to suggest that infection with the two viruses is endemic in African and Malaysian monkeys but does not occur or is very rare in Indian rhesus and New World monkeys.

INTRODUCTION

Yabapox and Tanapox viruses readily infect macaque monkeys, although Tanapox virus was first isolated from the lesions of a patient who acquired infection during an outbreak in 1963 among Africans living in the Tana River valley in Kenya (Downie *et al.* 1971). Yaba virus has also been shown to be pathogenic for man on experimental infection and one accidental infection in a laboratory technician has been reported (Grace & Mirand, 1963). On epidemiological grounds it has been suggested that African monkeys are the natural hosts of both viruses (Niven *et al.* 1961; Schmidt, 1970; Downie *et al.* 1971). The two viruses, although similar in morphology, produce lesions in monkeys which are clinically and histologically different, the lesions of Yaba viruses being large tumour-like masses involving mainly mesodermal tissues while Tanapox virus produces superficial hyperplastic nodules affecting almost entirely epithelial cells. Although there is some serological overlap (España, 1971; Nicholas, 1970) the viruses are immunologically distinct (Kupper, Casey & Johnson, 1970; Downie & España, 1973).

Tsuchiya & Tagaya (1971) have recently reported on the presence of antibodies to Yaba virus and '1211 agent' in the sera of two species of Asian monkeys (*Macaca iris, M. radiata*) and of African green monkeys (*Cercopithecus aethiops*). This '1211 agent' isolated from an outbreak of disease in macaque monkeys in the Oregon primate centre (Hall & McNulty, 1967; Nicholas & McNulty, 1968) is the same virus as the 'Yaba-like' virus (YLD) isolated by España (1966) from a similar outbreak in the California primate centre and was later shown to be identical with Tanapox virus (Downie & España, 1972). Tsuchiya & Tagaya found

antibodies to Yaba virus in 19·9% of cynomolgus (*M. iris*) sera, 8·4% of bonnet (*M. radiata*) sera but none in rhesus (*M. mulatta*) sera, while in the sera of African green monkeys (*C. aethiops*) the incidence of Yaba antibodies was as high as 76·4%. Antibodies to Tanapox virus (1211 agent) were not found in the sera of any Asian monkeys and only in 5·5% of African green monkeys. A similar survey of antibodies to Yaba and Tanapox virus in monkey sera has now been made in Liverpool and is reported in this paper. Sera from cynomolgus and rhesus monkeys have been tested as well as sera from several species of African and South American monkeys. As shown below the results have differed in certain respects from those obtained by Tsuchiya and Tagaya (1971).

MATERIALS AND METHODS

Cell cultures

All neutralization tests were made in cultures of BSC 1, a continuous line of grivet monkey kidney cells, grown in Leighton tubes. The culture medium was Eagle's medium with 10·0% foetal calf serum added; but the concentration of foetal calf serum was reduced to 1·0% when the tubes were inoculated with serum-virus mixtures.

Viruses

The Yaba virus stock used was prepared from sonically-disrupted BSC 1 cells which had been infected with the 5th subculture of Yaba virus in BSC 1 cells 12 days previously. The original virus had been supplied by Dr Allison in the form of Yaba tumour tissue from an experimentally infected rhesus monkey. The Tanapox virus stock was similarly prepared from virus grown in bottle cultures of BSC 1 cells as described by Downie & España (1972).

Monkey sera

The sera from cynomolgus, rhesus and patas monkeys were kindly supplied by Dr Frank Perkins of the Division of Immunological Products Control of the Medical Research Council. The animals had been bled 5 or 6 weeks after their arrival in this country. The cynomolgus monkeys came from Malaysia, the rhesus monkeys from India and the patas monkeys from Kenya. The sera from most of the African green monkeys were sent by Dr P. B. Stones and Dr McHugh of Pfizer Laboratories. The animals had been collected in the Lake Awasa region of Ethiopia and had been bled 1 week or 5 weeks after they arrived in Sandwich, Kent. A few sera from African green monkeys were from animals caught in Kenya. Seventeen sera from baboons caught in Kenya were generously given by Professor Nelson of the London School of Hygiene and eight sera from baboons caught in Senegal were supplied by Mr Hackett of Shamrock Farms, Essex. The seventeen sera from cercopithecus monkeys in Cameroon were sent by Dr Jan of Institut Pasteur, Yaounde (5 *C. cephus*, 8 *C. nictitans* and 4 *C. pogonias*). Sera from other West African primates were kindly supplied by Dr Nakano of the Communicable Diseases Centre, Atlanta, Georgia. These were 26 chimpanzee sera from the Ivory Coast and 32 cercopithecus and colobus monkeys from Liberia (14 *C. campbelli*,

6 *C. petaurista*, 3 *C. diana*, 1 *C. atys*, 2 *C. nictitans* and 6 *Colobus badius*). Most of the South American sera were sent by Dr Melendez of the Massachusetts primate centre but 13, given by Dr Marguerite Pereira, came from animals caught and bled in the Amazon valley. The 104 South American sera came from 5 species of monkey, namely, spider monkeys (*Atelas geofroyii*) 8, squirrel monkeys (*Saimiri sciureus*) 29, owl monkeys (*Aotus trivirgatus*) 32, marmosets (*saquinus oedipus*) 22 and capuchin monkeys (*cebus albifrons*) 13.

Antisera for the two viruses were prepared in rhesus or vervet monkeys as described by Downie & España (1972) and were used as positive controls along with known negative control monkey sera in each batch of tests.

Neutralization tests

These were carried out as previously described (Downie & España, 1972). All sera were inactivated at 58° C. for 15 min. before test. The test viruses were used in a dilution calculated to give 50–100 lesions in each control culture tube with negative sera. All sera were first screened at a dilution of 1/10. These diluted sera were mixed with virus suspension and the tubes containing the mixtures were rocked in a water bath at 37° C. for 2 hr. before the mixtures were tested. Of each of these, 0·2 ml. was inoculated into each of three or four Leighton culture tubes and 1·2 ml. of culture medium added to each tube before incubation. Tanapox virus lesions were counted after incubation of the tubes for 6–10 days. Yaba lesions, which developed more slowly, were counted after 10–12 days. At first lesion counts were made on the unstained sheets with a binocular microscope at a magnification of × 10. Latterly it was found more convenient to stain the cell sheets in the tubes with 1% Crystal violet and then to count the lesions with a hand lens. Both methods gave essentially the same counts. All sera which showed definite or doubtful neutralization in a dilution of 1 in 10 were retested in three-fold dilutions from 1/10 upwards.

RESULTS

The results of tests for neutralizing antibody to Tanapox virus in the various monkey sera are shown in Table 1 and for antibody to Yaba virus in Table 2. The numbers of sera tested against the two viruses are different because a few sera which were examined for antibody to Tanapox virus were not available when the technique for estimating antibody to Yaba virus was developed. However, all the sera tested for Yaba antibodies were also tested for antibody to Tanapox virus. It will be seen from the results in Tables 1 and 2 that antibodies to both viruses were present in a proportion of the sera from various species of monkeys in East and West Africa and in the sera from cynomolgus monkeys from Malaysia. Neutralizing antibody was not found in the sera of rhesus monkeys from India nor in the sera of various species of monkey from South America. Although the percentage of positive results from the two viruses is roughly the same for the various species of monkeys tested, few individual sera were positive for both viruses (Table 3). Only nine sera showed antibody to both viruses; two were from African green monkeys, three were from cynomolgus monkeys, one from a chimpanzee

Table 1. *Tanapox-neutralizing antibody in monkey sera (screening test at 1/10 serum dilution)*

Monkey species	Place of origin	No. tested	No. positive	% positive
Cercopithecus aethiops	Ethiopia and Kenya	78	13	16·6
Erythrocebus patas	Kenya	50	1	2·0
Papio anubis	Kenya	29	5	17·2
P. anubis	Senegal	8	1	12·5
Cercopithecus (see text)	Fr. Cameroon	17	2	11·8
Chimpanzees	Ivory Coast	26	2	7·7
Cercopithecus and *Colobus* (see text)	Liberia	32	8	25·0
Macaca mulatta	India	61	0	0
M. iris	Malaysia	51	10	19·6
Various (see text)	S. America	104	0	0

Table 2. *Yaba neutralizing antibody in monkey sera (screening test at 1/10 serum dilution)*

Monkey species	Place of origin	No. tested	No. positive	% positive
Cercopithecus aethiops	Ethiopia and Kenya	63	10	15·9
Erythrocebus patas	Kenya	49	6	12·2
Papio anubis	Senegal	8	1	12·5
Cercopithecus (see text)	Fr. Cameroon	17	2	11·8
Chimpanzees	Ivory Coast	26	4	15·4
Cercopithecus and Colobus (see text)	Liberia	32	4	12·5
Macaca mulatta	India	50	0	0
M. iris	Malaysia	51	10	19·6
Various (see text)	S. America	100	0	0

Table 3. *Analysis of results of antibody to Tanapox and Yaba viruses in those monkey species showing antibody*

		Antibody to Yaba virus		
		+	−	Total
Antibody to Tana virus	+	9	24	33
	−	27	184	211
Totals		36	208	244

and three from Campbell's monkeys. The analysis of the results shown in Table 3 indicates that antibody to either virus may be induced independently of the other. It may be of interest to note that of 40 sera from natives in the Tana River area, 20 of which showed antibody to Tanapox virus, none had neutralizing antibody to Yaba virus (Manson-Bahr & Downie, 1973). As mentioned above, all sera showing definite or doubtful neutralization of virus at a serum dilution of 1/10 were retested in dilutions from 1/10 upwards. The titres from the positive samples varied from 1/10 to 1/100, this latter titre being attained by a few sera. The titres found in the present study were similar to those seen in monkeys and humans suffering from clinical infection with Tanapox or Yaba viruses (España, 1971; Downie & España, 1973).

Twenty monkey sera that showed neutralizing antibody to Tanapox virus were tested for antibody by the complement-fixation technique. None was positive in a dilution of 1/5 or higher. The sera were not examined by the complement-fixation inhibition test which Hall, Olsen, Pakes & Yohn (1973) have recently found to give positive results with the sera of monkeys recovered from Yaba and Tanapox infection after the ordinary complement-fixation test has become negative.

DISCUSSION

The results of the serological survey reported here differ in some particulars from those recorded by Tsuchiya & Tagaya (1971). The percentage of cynomolgus monkeys from Malaysia showing neutralizing antibody in their sera to Yaba was similar in both series; but antibody to Tanapox was not found in the Japanese survey, whereas in our survey 20% of the sera showed antibody. The absence of antibody to both viruses from 14 rhesus sera examined by Tsuchiya & Tagaya was confirmed in our experience. However, the high proportion of sera from green monkeys in Uganda showing Yaba antibodies (76%) was not observed in our tests on green monkeys from Ethiopia and Kenya. It may be that the 1/4 serum dilution used in the screening test of the Japanese workers was responsible for their greater proportion of positive results, although there was not a corresponding difference in the results with cynomolgus sera. Furthermore, the very high titres of Yaba antibody found by the Japanese workers in some African cercopithecus sera were not obtained in our tests on similar sera. Another difference in the results concerns the antibody to Tanapox virus in the sera of African monkeys. Whereas only three of 55 cercopithecus sera were found to contain antibody in the Japanese tests, the proportion of positive sera found by us was considerably higher (Table 1).

Clinical infection with Yaba virus was first observed in rhesus monkeys housed in captivity in West Africa, whereas clinical infection with Tanapox was first reported in natives living in Eastern Kenya. Nevertheless, the results of our tests on monkey sera suggest that infection of monkeys with the two viruses is equally prevalent in both East and West Africa.

Our results suggest that various species of monkey in Africa and cynomolgus monkeys in Malaysia suffer from infection, probably latent, with Tanapox or Yaba viruses. Indian rhesus monkeys, which appear to be highly susceptible to clinical infection with both viruses, apparently rarely if ever acquire infection in the wild state. Our tests with sera of several species of South American monkeys failed to provide evidence of infection with either virus in the New World.

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REFERENCES

DOWNIE, A. W., TAYLOR-ROBINSON, C. H., CAUNT, ANNE E., NELSON, G. S., MANSON-BAHR, P. E. C. & MATHEWS, T. C. H. (1971). *British Medical Journal* i, 363–8.

DOWNIE, A. W. & ESPAÑA, C. (1972). Comparison of Tanapox virus and Yaba-like viruses causing epidemic disease in monkeys. *Journal of Hygiene* **70**, 23–32.

DOWNIE, A. W. & ESPAÑA, C. (1973). A comparative study of Yaba and Tanapox viruses. *Journal of General Virology* **19**, 37–49.

ESPAÑA, C. (1966). A Yaba-like disease in Primates. 17th Annual Meeting Animal Care Panel, Chicago. Cited *Laboratory Primate Newsletter* **5**, ii.

ESPAÑA, C. (1971). A pox disease of monkeys transmissible to man. *Medical Primatology. Proceedings of the 2nd Conference of Experimental Medicine and Surgery in Primates* (ed. E. I. Goldsmith and J. Moor-Jankowski), pp. 694–708. Basel: S. Karger.

GRACE, J. T. JR. & MIRAND, B. A. (1963). Human susceptibility to a simian tumour virus. *Annals of the New York Academy of Sciences* **108**, 1123–8.

HALL, A. S. & MCNULTY, W. P. JR. (1967). A contagious pox disease in monkeys. *Journal of the American Veterinary Medical Association* **151**, 833–8.

HALL, R. D., OLSEN, R. G., PAKES, S. P. & YOHN, D. S. (1973). Differences in clinical and convalescent-phase antibodies of rhesus monkeys infected with monkey pox, Tanapox and Yabapox viruses. *Infection and Immunity* **7**, 539–46.

KUPPER, J. L., CASEY, H. W. & JOHNSON, D. K. (1970). Experimental Yaba and benign epidermal monkeypox in rhesus monkeys. *Laboratory Animal Care* **20**, 979–88.

MANSON-BAHR, P. E. C. & DOWNIE, A. W. (1973). The persistence of Tanapox in the Tana River Valley. *British Medical Journal* ii, 151–3.

NICHOLAS, ANN H. (1970). A pox virus of primates. II. Immunology. *Journal of the National Cancer Institute* **45**, 907–14.

NICHOLAS, ANN H. & MCNULTY, W. P. (1968). *In vitro* characteristics of a poxvirus isolated from rhesus monkeys. *Nature, London* **217**, 745–6.

NIVEN, JANET S. F., ARMSTRONG, J. A., ANDREWES, C. H., PEREIRA, H. G. & VALENTINE, R. C. (1961). Subcutaneous 'growths' in monkeys produced by a poxvirus. *Journal of Pathology and Bacteriology* **81**, 1–14.

SCHMIDT, R. H. (1970). *Infections and Immunosuppression in Subhuman Primates* (ed. H. Balner and W. I. B. Beveridge), p. 87. Copenhagen: Munksgaard.

TSUCHIYA, Y. & TAGAYA, I. (1971). Sero-epidemiological survey on Yaba and 1211 virus infections among several species of monkeys. *Journal of Hygiene* **69**, 445–51.

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Differentiation of smallpox and camelpox viruses in cultures of human and monkey cells

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SUMMARY

The cytopathic effect of smallpox and camelpox viruses has been compared in cell cultures derived from human and monkey tissues. The two viruses could easily be distinguished in HeLa, GMK–AH1, BSC–1 and WISH cells, camelpox producing multinucleate giant cells and smallpox producing rounding up of individual cells. In other cells (LLC–MK2, HEK and 2RhK) the differences were not so marked, and in some (VERO, HEL, K and HuTh) no differences were seen.

INTRODUCTION

Recent work in this laboratory has shown that the techniques commonly used in smallpox diagnostic laboratories fail to distinguish between strains of camelpox virus isolated in Iran, and strains of smallpox virus isolated in Tanzania (Baxby, 1972). However, subsequent preliminary experiments (Bedson, 1972) suggest that camelpox and smallpox viruses produce different types of cytopathic effect (CPE) in HeLa cells. Previous studies had failed to differentiate the viruses in camel (Mirchamsy & Ahourai, 1971), rabbit and chick cells (Baxby, 1972). The present paper extends these observations by comparing the CPE produced by camelpox and smallpox viruses in a number of cell lines and cell strains of human and monkey origin.

MATERIALS AND METHODS

Virus strains

Three strains of camelpox virus, CM–G1, CM–G2 and CM–S, whose origins have been described elsewhere were used (Baxby, 1972). Also used were the International Reference Strain of smallpox virus (Harvey), two strains of smallpox virus, EA8 and EA17, isolated in Tanzania (Bedson, Dumbell & Thomas, 1963), and strain 64–7275, a so-called 'white' poxvirus isolated from the tissues of a healthy monkey and so far indistinguishable from smallpox virus (Gispen & Brand-Saathof, 1972; Marennikova, Selukhina, Maltseva & Ladnyi, 1972).

Cell cultures

Cell cultures were grown in $6 \times \frac{1}{2}$ in. tubes in one of four media: Eagle's MEM with (i) adult or (ii) foetal calf serum, or Parker's 199 with (iii) adult or (iv) foetal calf serum.

The monkey cells used (with medium shown in parentheses) were as follows: secondary rhesus kidney, 2RhK (iii), three lines of transformed green monkey kidney cells, BSC–1 (ii), GMK–AH1 (ii), VERO (iv) and one line of transformed rhesus monkey kidney cells LLC–MK2 (iii).

The human cells used were two lines of transformed amnion cells, K (iii) and WISH (iv), HeLa cells (i), cell strains derived from human embryo lung, HEL (ii), and kidney, HEK (ii), and secondary human thyroid cells HuTh (iii).

Confluent cell sheets were infected with various doses of virus calculated to give all stages of CPE from very fast total destruction of cell sheets, to the much slower development of isolated foci of infection. Cell sheets were fixed in 5% formal-saline and were photographed unstained using phase-contrast optics (McCarthy, 1960).

RESULTS

The results obtained are summarized in Table 1. The CPE produced by the strains of smallpox virus and the 'white' poxvirus were the same. No differences were found in the type of CPE produced by the three strains of camelpox virus.

The results could be divided into three categories. Cells in which clear-cut differences were found between the behaviour of camelpox and smallpox viruses, those in which differences were not so clear-cut and those in which no differences were found. Any differences found were consistent whatever the dose of virus used. With high doses (10^5 to 10^6 pock-forming units/ml.) CPE was detectable within 24 hr. and soon spread to involve the whole cell sheet. With lower doses (10^2 to 10^3 pfu/ml.) CPE was not detected until the second or third day and resulted in localized plaque production.

Clear differences were found between smallpox and camelpox viruses in HeLa (Plate 1, figs. 1, 2), GMK–AH1 (figs. 3, 4), BSC–1 and WISH cells. In all these cells camelpox virus produced multinucleate giant cells (GC CPE), whereas the CPE of smallpox virus was characterized by the rounding up of infected cells (round cell (RC) CPE), with the occasional production of cells with long cytoplasmic strands (strand cell (SC) CPE). With low doses smallpox virus produced characteristic 'hyperplastic foci' – small aggregations of heaped-up cells (Pirsch & Purlson, 1962). Camelpox virus produced small holes in the cell sheet caused by the detachment of multinucleate cells.

In LLC–MK2 (figs. 5, 6), HEK and 2RhK cells the differences were not so marked. In LLC–MK2 cells smallpox virus tended to produce round cell CPE with an occasional strand cell (fig. 5), whereas camelpox virus produced a mixed strand cell/round cell CPE (fig. 6). In 2RhK cells smallpox virus produced a mixed round cell/giant cell CPE and camelpox virus a mixed strand cell/giant cell CPE. In HEK cells smallpox virus produced strand cell CPE whereas camelpox virus in addition produced occasional giant cells.

Table 1. *Cytopathic effects of smallpox and camelpox viruses in human and monkey cells*

Cell type	Designation	Viruses	
		Smallpox	Camelpox
Transformed green monkey kidney	VERO	SC–RC	SC–RC
Transformed green monkey kidney	BSC–1	RC–SC	GC
Transformed green monkey kidney	GMK–AH1	RC(SC)	GC
Transformed rhesus monkey kidney	LLC–MK2	RC(SC)	SC–RC
Secondary rhesus monkey kidney	2RhK	RC–GC	SC–GC
Human cervical carcinoma	HeLa	RC	GC
Transformed human amnion	K	RC	RC
Transformed human amnion	WISH	RC	GC
Diploid human embryo lung	HEL	SC(GC)	SC(GC)
Diploid human embryo kidney	HEK	SC	SC(GC)
Secondary human thyroid	HuTh	SC	SC

SC = strand cell CPE, GC = giant cell CPE, RC = round cell CPE. Bracketed entries refer to occasional or very minor type of CPE. For example, SC–RC = predominantly SC with some RC. RC–SC = predominantly RC with some SC. SC(GC) = predominantly SC with occasional or very minor GC.

In HEL (figs. 7, 8), VERO, K and HuTh cells no differences were detected in the type of CPE produced by the viruses.

DISCUSSION

The results described here, although failing to distinguish between smallpox and the 'white' poxviruses, confirm and extend those of Bedson (1972) in indicating that the viruses of smallpox and camelpox are not identical. At the same time the results provide easy means of distinguishing them. It should be remembered that consistent results have not always been obtained with smallpox virus in cell cultures (Mika & Pirsch, 1960; Bedson & Dumbell, 1964; Netter & Piat, 1969; Gispen & Brand-Saathof, 1972) and that a giant cell producing a variant of smallpox virus has recently been selected by repeated passage through monkey kidney cells (Tsuchiya & Tagaya, 1972). Nevertheless, consistent results have been obtained in the author's laboratory with both smallpox and camelpox material passaged through various animals (mouse, monkey, chick embryo) or adapted to various cell cultures (VERO, HeLa, BSC–1).

The results of these and earlier studies (Baxby, 1972) suggest that the ease with which smallpox and camelpox viruses can be distinguished upon isolation depends essentially on the choice of cell used to detect CPE. Hence it is hoped that the information presented here, that the viruses can be differentiated in a number of cell cultures, may be of value to those interested in the comparisons between smallpox and related viruses.

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REFERENCES

BAXBY, D. (1972). Smallpox-like viruses from camels in Iran. *Lancet* ii, 1063.

BEDSON, H. S. (1972). Camelpox and smallpox. *Lancet* ii, 1253.

BEDSON, H. S. & DUMBELL, K. R. (1964). Hybrids derived from the viruses of variola major and cowpox. *Journal of Hygiene* **62**, 147.

BEDSON, H. S., DUMBELL, K. R. & THOMAS, W. R. G. (1963). Variola in Tanganyika. *Lancet* ii, 1085.

GISPEN, R. & BRAND-SAATHOF, B. (1972). 'White' poxviruses from monkeys. *Bulletin of the World Health Organization* **46**, 585.

MCCARTHY, K. (1960). An adaptor for examining tissue cultures in test tubes by phase contrast microscopy. *Virology* **12**, 313.

MARENNIKOVA, S. S., SELUKHINA, E. E., MALTSEVA, N. N. & LADNYI, I. D. (1972). Poxviruses from clinically ill and asymptomatically infected monkeys and a chimpanzee. *Bulletin of the World Health Organization* **46**, 613.

MIKA, L. & PIRSCH, J. (1960). Differentiation of variola virus from other members of the poxvirus group by the plaque technique. *Journal of Bacteriology* **80**, 861.

MIRCHAMSY, H. & AHOURAI, P. (1971). Comparative adaptation of some poxviruses in two cell systems. *Archives of the Institute Razi, Teheran* **23**, 93.

NETTER, R. & PIAT, A. (1969). Essais de culture de quatre souches de virus varioliques sur cellules RK13 et BHK21. *Annales de l'Institut Pasteur* **116**, 820.

PIRSCH, J. & PURLSON, E. H. (1962). A tissue culture assay for variola virus based on the enumeration of hyperplastic foci. *Journal of Immunology* **83**, 632.

TSUCHIYA, Y. & TAGAYA, I. (1972). Isolation of a multinucleated giant-cell-forming and haemagglutinin negative variant of variola virus. *Archiv für die gesamte Virusforschung* **39**, 292.

EXPLANATION OF PLATE

Cytopathic effects produced by smallpox and camelpox viruses. Unstained, phase contrast, × 325.

Fig. 1. Smallpox virus in HeLa cells.

Fig. 2. Camelpox virus in HeLa cells.

Fig. 3. Smallpox virus in GMK–AH1 cells.

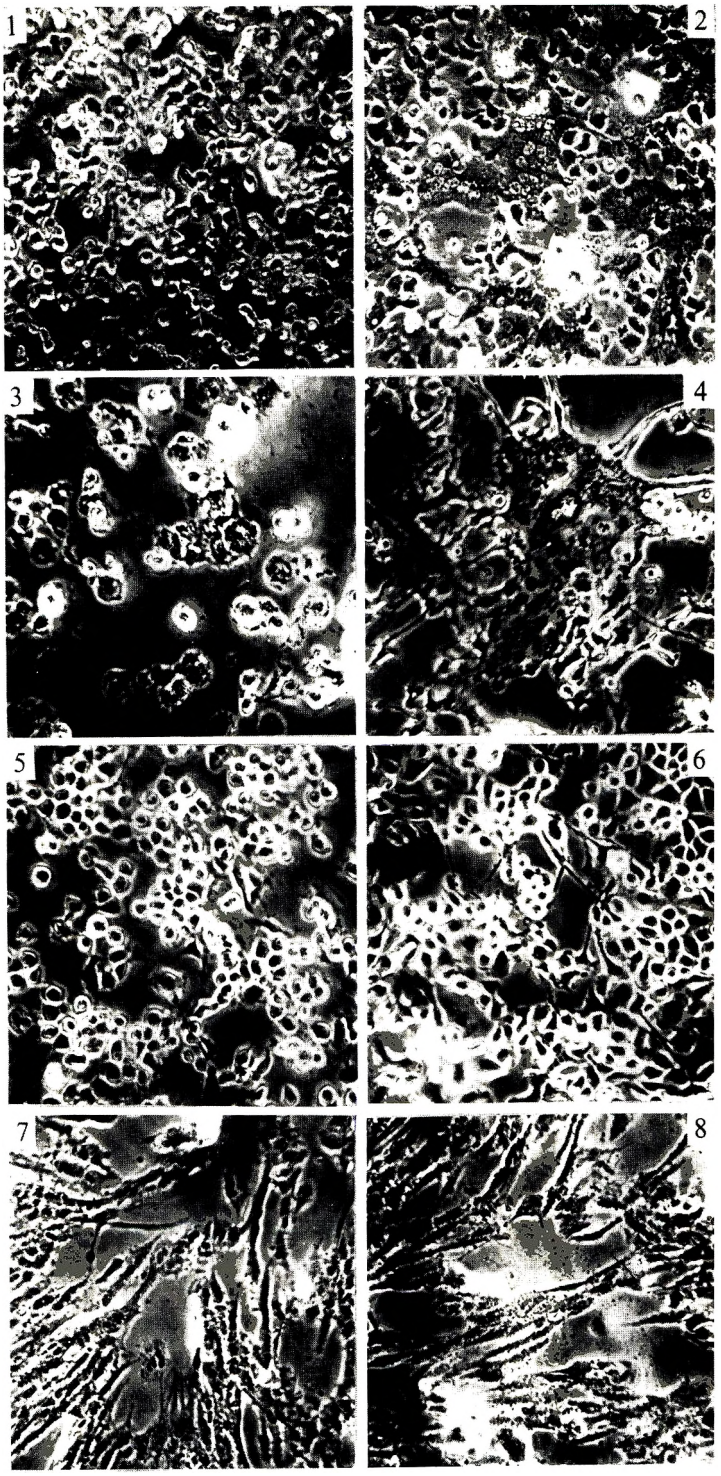
Fig. 4. Camelpox virus in GMK–AH1 cells.

Fig. 5. Smallpox virus in LLC–MK2 cells.

Fig. 6. Camelpox virus in LLC–MK2 cells.

Fig. 7. Smallpox virus in HEL cells.

Fig. 8. Camelpox virus in HEL cells.



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Virus isolations from patients in general practice, 1961–71

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SUMMARY

During the period 1961–71 of 1785 viruses isolated from patients in the general population 503 (28%) were rhinoviruses, 465 (26%) influenza viruses, 248 (14%) enteroviruses, 234 (13%) herpes simplex virus, 132 (7%) parainfluenza viruses, 129 (7%) adenoviruses and 49 (3%) respiratory syncytial virus. Also isolated were 18 strains of mumps virus, 7 coronaviruses and 295 streptococci of groups A, C or G.

Fluctuations were observed in the frequency with which respiratory syncytial virus, parainfluenza virus type 2, and the adenoviruses were isolated over the 10-year period.

Influenza viruses types A and B, parainfluenza viruses types 1 and 2, respiratory syncytial virus, adenoviruses types 3, 4, 6, 7 and 21, and many enteroviruses were all associated with outbreaks.

Infections with influenza viruses A and B and parainfluenza viruses types 1 and 2 came during the winter, whereas those with parainfluenza virus type 3, enteroviruses, and rhinoviruses were more frequently seen in the summer and early autumn.

INTRODUCTION

Between 1954 and 1960 several new viruses were described in association with acute respiratory infections. In order to determine the significance of these agents in the general pattern of acute infections a number of surveys were undertaken (e.g. Medical Research Council, 1965; Fox *et al.* 1966), but few were continued for more than 2 or 3 years. By examining specimens submitted by local practitioners to the virus laboratory at Cirencester over a 10-year period, 1961–71, and the Public Health Laboratory at Gloucester from October 1970 to September 1971 it was possible to determine the relative frequency with which certain viruses or groups of viruses could be isolated from patients within a local population and also the variation in these frequencies with time and season.

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MATERIALS AND METHODS

Population sampled

Between October 1961 and September 1971 specimens were received from patients thought to be suffering from acute virus infections. Most of the patients lived in or around Cirencester, but during the last year patients from a wider area of Gloucestershire were included. Many of the specimens were collected during the course of other investigations (Medical Research Council, 1965; Hope-Simpson & Higgins, 1969; Medical Research Council/Public Health Laboratory Service, 1973) when an attempt was made to secure an unselected sample; at other times specimens were examined without a precise knowledge of the relation of the illness sampled to the total amount or type of sickness present in the community at that time.

Laboratory techniques

Specimens consisted mainly of nose and throat swabs in transport medium, but faeces and material from local lesions were also received. Most specimens were delivered to the laboratory on melting ice within a few hours of collection.

The extent to which nose and throat swabs were examined increased with time. Initially only cultures of human embryo kidney and monkey kidney were used, but within a few months the inoculation of cultures of the Bristol line of HeLa cells and a blood plate were introduced. Cultures of diploid fibroblasts (WI 26 or WI 38) were employed when they became available in October 1962 and specimens were examined in suckling mice after the end of 1962.

From September 1965 onwards a limited number of specimens (approximately 700), generally those negative on examination by these methods, were inoculated into organ cultures of human embryonic ciliated respiratory epithelium. For limited periods specimens were examined in fertile hen eggs, the L132 line of human embryo lung (Davis & Bolin, 1960), or on solid media for the isolation of mycoplasmas.

The methods employed have been described in detail elsewhere (Higgins, Ellis & Boston, 1963; Higgins, Boston & Ellis, 1964; Higgins, 1966).

RESULTS

Frequency of isolation of viruses and variation with time

The number of specimens examined and the distribution of the agents isolated are shown, at monthly intervals, in Fig. 1.

Sample

The number of patients sampled each year ranged between 307 in the first year and 599 in 1968–9 with a larger number of specimens being received in the winter than in the summer months of each year. The disproportionately large number of specimens examined in the colder months from 1968 onwards was the result of the particular interest of one practitioner in influenza (Hope-Simpson, 1970). In all, specimens from well over 5000 patients were examined during the 10-year period.

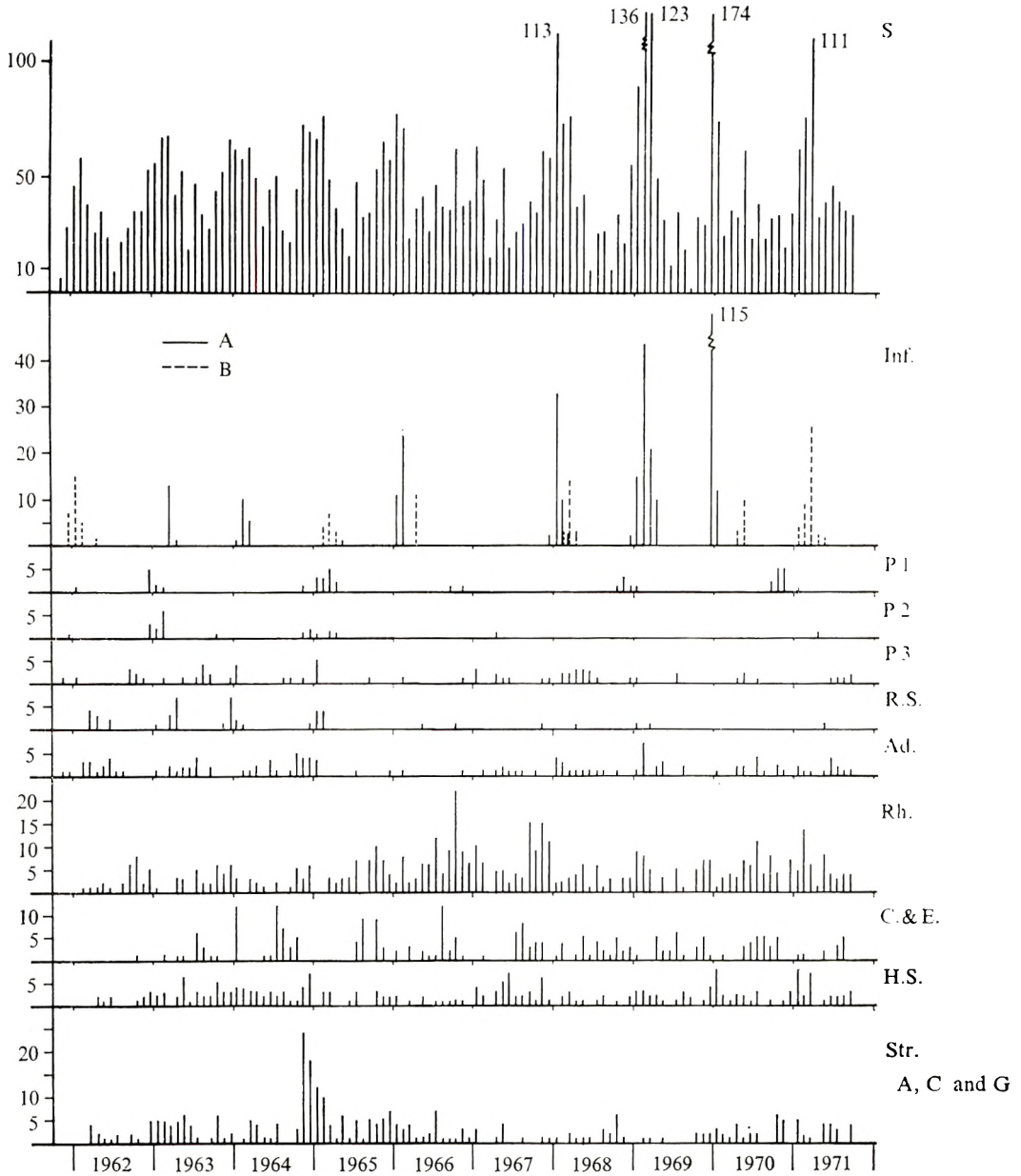

Fig. 1. The number of specimens examined and agents isolated between October 1961 and September 1971. *Key.* S = total samples; Inf. = influenza viruses; P1, P2, P3 = para-influenza 1, 2 and 3 viruses; R.S. = respiratory syncytial virus; Ad. = adenoviruses; Rh. = rhinoviruses; C & E = Coxsackie and echoviruses; H.S. = Herpes simplex virus; Str. = Streptococci of groups A, C or G.

Influenza virus infections

A total of 465 infections with influenza viruses were diagnosed, 333 were with type A virus and 132 with type B virus. There were six outbreaks of infections with influenza virus A and six with influenza virus B during the 10 years and on three occasions infections with influenza A virus preceded those with influenza B virus in the same winter. Strains of one or both types of virus were isolated between January and December of every year, although there was a period in excess of 12 months in 1966 and 1967 when no influenza virus was detected.

Parainfluenza virus infections

Of the 132 parainfluenza viruses isolated, type 3 (68 strains) was the commonest. Infections with type 3 virus showed clustering in time but not in the pronounced fashion that was observed with types 1 and 2. Forty-three strains of type 1 virus were isolated from outbreaks which happened every second year so that five distinct periods were established when parainfluenza virus type 1 was circulating in the community. Parainfluenza virus type 2 behaved in a similar manner for the first 4 years when 19 strains were isolated, but only two further strains were detected in the 6 years beginning in the autumn of 1965. Type 4 virus was not isolated.

Respiratory syncytial virus infections

During the first 4 years well-defined outbreaks of infections with respiratory syncytial virus were observed and 39 strains of the virus were isolated over this period. However, only seven further strains were detected during the next 6 years.

Adenovirus infections

Adenoviruses were most often encountered during the early part of the study. After 2 years when infections with these viruses were rarely seen, adenoviruses were again commonly isolated in the latter half of the period of observation.

A total of 119 strains were isolated, 74 (62%) of which belonged to type 1, 2, or 3. Infections with types 1 and 2 were diagnosed in all but one of the 10 years; 22 of the 26 strains of type 3 virus were isolated during three outbreaks, one in each of 1964, 1968 and 1971. Similarly, 10 of 13 strains of type 4 virus were isolated during two outbreaks, one in 1970 and another in 1971 and six of nine infections with type 6 were between April 1964 and January 1965. Ten strains each of types 7 and 21 were isolated; each virus appeared only twice, type 7 in 1962 and 1969 and type 21 in 1962 and 1963.

Rhinovirus infections

The most commonly isolated agent was a rhinovirus. Five hundred and three strains were detected, of which only 96 (19%) could be grown in monkey kidney tissue culture (M strains). Rhinovirus infections were less frequently diagnosed in the early part of the study when fewer methods for their detection were employed; even so there was not a time when there was a complete failure to isolate these viruses.

Enterovirus infections

A total of 248 enterovirus infections were diagnosed.

(i) *Poliovirus infections.* Twenty-one strains of poliovirus were isolated and in most instances a history of recent vaccination with live attenuated virus or close contact with such a person could be established. None of the patients from whom poliovirus was isolated had evidence of involvement of the central nervous system.

(ii) *Coxsackievirus infections.* Almost half, 114, of the enteroviruses isolated were shown to be Coxsackie A viruses and nine serotypes were represented. Type 16 was the commonest type and 36 of the 38 strains isolated were detected in three periods, 1963–4, 1967, 1969–70. Similarly, 13 of 15 strains of type A9 were isolated during the 12 months from August 1966 to July 1967 and five of six strains of type A6 between June and August 1964. Infections with type A10 were diagnosed mainly (12 of 15 strains) during two outbreaks, one in 1966–7, the other in 1969, whereas 15 out of 18 strains of type A4 were detected in 1965–6 and 1968. Type A2 (10 strains), type A3 (2 strains), type A5 (9 strains) and type A8 (2 strains) were isolated too infrequently or were too widely dispersed in time to provide evidence of outbreaks of infections with these viruses in the community.

All types but type 6 were represented among the 57 Coxsackie B viruses isolated. Twenty-three strains of type B5 were detected – 19 of these infections in two outbreaks, one in 1965 and the other in 1971. Type B2 was the next commonest type; 10 of the 13 strains were isolated during the summers of 1964 and 1967. Type B1 (7 strains), type B3 (9 strains) and type B4 (5 strains) made up the rest of this group.

(iii) *Echovirus infections.* Fifty-six echoviruses of 16 different serotypes were isolated but only types 6 and 9 were detected on more than five occasions. Ten of the 15 infections with type 6 virus were diagnosed between July 1968 and July 1969, and 9 of the 13 strains of type 9 virus between July and November 1969. The remaining serotypes isolated were types 3, 4, 7, 11, 13, 15, 17, 19, 21, 22, 23, 24, 25, and 30.

Herpes simplex virus infections

Isolations of herpes simplex virus were almost as common as those of enteroviruses (234 against 248) and isolations were made with approximately the same frequency during each year of the study.

Streptococcal infections

Of the β-haemolytic streptococci isolated only those belonging to groups A, C or G were recorded; 295 such infections were diagnosed. Most of the streptococci were members of group A; few belonged to group C (23 strains) or group G (17 strains). Isolations were made more frequently during the first half of the study and included a large outbreak of infections in the winter of 1964–5.

Infections with other agents

Mumps virus was isolated on 18 occasions, although infection with this virus was seldom suspected on clinical grounds at the time the patients were swabbed.

No isolations of *Mycoplasma pneumoniae* were made during a search for this agent between September 1963 and October 1966.

Only seven coronavirus infections were proved, but their distribution in time suggests that there were outbreaks of infection with these viruses. One strain of 229E virus was isolated in April 1968 and three further infections with this agent were diagnosed as illnesses in the spring of 1971. Another probable outbreak, in the spring of 1970, was suggested by the isolation of three organ-culture strains from illnesses in March and April of that year. A coronavirus was not isolated from any of the remaining 144 suitably examined specimens collected between 1966 and 1971.

Variation in the frequency of isolation of viruses with season

The frequency with which the various agents or groups of agents were isolated during each of the months of the year is shown in Fig. 2*a–g*. This frequency was calculated by determining the proportion of specimens collected in any one month (e.g. January) for all of the 10 years which yielded a particular virus.

Influenza viruses

The isolation of these viruses was restricted to the winter months and no strain of either influenza A or B virus was isolated between June and November of any year. During the 10 years studied influenza A virus was isolated from a greater proportion of specimens in December, January and February than other months when it was present, whereas influenza B virus showed a tendency to appear later and to be relatively more frequently isolated in March and April. These figures may be biased by the timing of influenza epidemics in the later years as these were more extensively sampled than earlier outbreaks.

Parainfluenza viruses

Although these viruses were never shown to be a dominant cause of illness there was a decided difference in the frequency with which each of the three types was isolated in relation to the time of year. Types 1 and 2 were more frequently isolated during the winter months and no isolations of either type were made from May to August in any year. The reverse was true of type-3 virus which was shown to be a more important cause of illness from April to September than during the remainder of the year.

Respiratory syncytial virus

Respiratory syncytial virus was another virus which was commoner in the cooler months of the year (December–April) with, in this study, the greatest incidence in the spring (April).

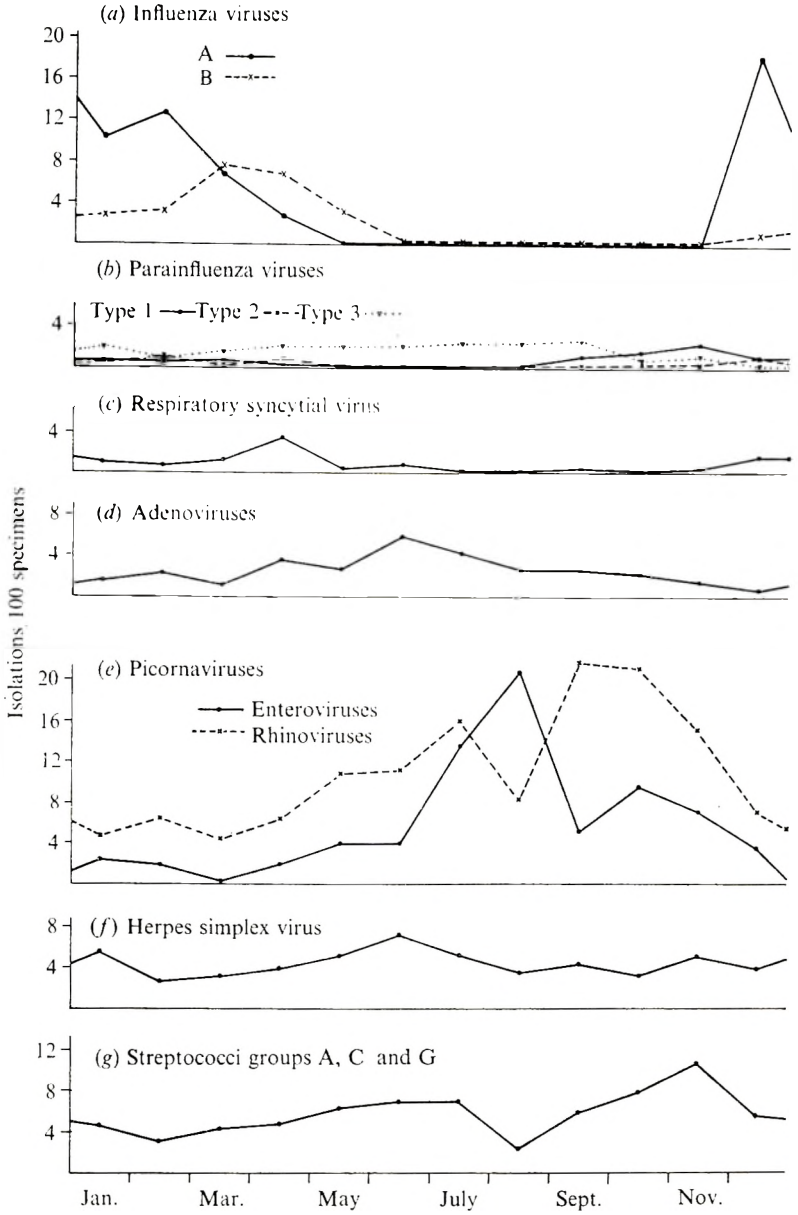

Fig. 2. The monthly variation in the proportion of specimens shown to contain the various agents isolated between October 1961 and September 1971.

Adenoviruses

Although there were a number of periods of a month or more when these viruses were not isolated, these occasions were at different seasons in different years. The isolation rate for adenoviruses was slightly higher in April to July than during other months.

Picornaviruses

For much of the year the isolation rates for enteroviruses and rhinoviruses ran parallel, with rhinoviruses the more common in every month except August. For the first 4 months of the year the isolation rates were low but rose during the summer to reach a peak in July and August for enteroviruses and in September and October for rhinoviruses.

Herpes simplex virus

Herpes simplex virus was demonstrated on average in 2–8% of the specimens examined each month with no special change in frequency at any season.

Streptococci

β-Haemolytic streptococci belonging to groups A, C or G were isolated on average from between 3 and 7% of specimens during most months. However, isolation rates of little over 2% (August) and as high as 10% (November) were also noted.

DISCUSSION

The epidemiology of the viruses encountered will vary according to the type of population studied and the results described here may be applicable only to small urban and semi-rural communities similar to those sampled in this survey.

Unfortunately the nature of the sample examined does not allow an accurate interpretation of the results in terms of infections in the local community as a whole. However, apart from the intensive sampling of respiratory illnesses during outbreaks of influenza in the last four winters there is no evidence that the sample was biased and it is not unreasonable to accept these findings as, at least, indicative of the prevalence of the various agents in the general population.

A more likely source of error is the known variation in efficiency with which the methods employed were able to isolate the different agents sought. For example, influenza viruses were probably isolated from a high proportion of those who were infected and sampled (Higgins & Ellis, 1972) but, despite the use of more than the average number of techniques, the efficiency of isolation of the rhinoviruses was probably less than half that of the influenza viruses. The incidence of respiratory syncytial virus and the parainfluenza viruses was also probably underestimated.

Although coronaviruses were isolated they were sought in less than 5% of the specimens and their importance in the total illness suffered by the community remains to be determined.

These findings show that certain viruses such as influenza virus types A and B, parainfluenza viruses types 1 and 2, respiratory syncytial virus, many of the enteroviruses and some adenoviruses are responsible for outbreaks of infections within a community. Infections with other agents, rhinoviruses as a group (isolates were not typed), other adenovirus serotypes (e.g. types 1 and 2), herpes simplex virus and β-haemolytic streptococci of groups A, C or G are either constantly present in the population or are associated with sporadic cases only.

It is apparent from this study that some viruses are of major importance in causing illness only at certain times of the year. The myxoviruses, with the exception of parainfluenza virus type 3, strongly influence morbidity during the winter months, whereas parainfluenza virus type 3 and the enteroviruses are responsible for a greater proportion of sickness during the summer months. A significant proportion of illness throughout the year is caused by rhinoviruses but these viruses have their greatest impact on the health of the general population in the autumn.

The value of this type of long-term study is the ability to observe the changing pattern of infections in a community. Because all isolations were made in one laboratory any change in the observed frequency with which a particular agent was detected probably reflected the true situation in the community. Where a number of different laboratories take part the question of their relative efficiency in isolating the various agents must arise and lead to doubts as to the significance of the findings. Similar doubts may result from the pooling of the findings in different populations. In this study the virtual disappearance of infections with respiratory syncytial and parainfluenza type-2 viruses after outbreaks in the first 4 years would have been more difficult to establish had the timing of the survey been different or the specimens divided among a number of laboratories. Similarly, the variation in the frequency with which adenoviruses, particularly type 21, and streptococcal infections were encountered and the seasonal fluctuations in the relative importance of rhinovirus infections could only be determined with certainty by localizing the community studied and limiting the number of investigating units.

Long-term studies are also of value in focusing attention on the epidemiological problems which still exist concerning many common viral infections. However, until it is possible to diagnose in the laboratory many of the illnesses which at present remain unconfirmed, it will be difficult to define these problems with accuracy and progress towards solving them will be correspondingly slow.

REFERENCES

Davis, E. V. & Bolin, V. S. (1960). Continuous cultivation of isogenous cell lines from the human embryo. *Federation Proceedings* **19**, 386.

Fox, J. P., Elveback, L. R., Spigland, I., Frothingham, T. E., Stevens, D. A. & Huger, M. (1966). The virus watch program: a continuing surveillance of viral infections in metropolitan New York families. I. Overall plan, methods of collecting and handling information and a summary report of specimens collected and illnesses observed. *American Journal of Epidemiology* **83**, 389–412.

Higgins, P. G. (1966). The isolation of viruses from acute respiratory infections. V. The use of organ cultures of human embryonic nasal and tracheal ciliated epithelium. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* **25**, 283–8.

Higgins, P. G., Boston, D. G. & Ellis, E. M. (1964). The isolation of viruses from acute respiratory infections. II. A study of the isolations made from cases occurring in 1963. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* **23**, 93–105.

Higgins, P. G. & Ellis, M. (1972). The isolation of influenza viruses. *Journal of Clinical Pathology* **25**, 521–4.

HIGGINS, P. G., ELLIS, E. M. & BOSTON, D. G. (1963). The isolation of viruses from acute respiratory infections. A study of the isolations made from cases occurring in a general practice in 1962. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* **22**, 71–84.

HOPE-SIMPSON, R. E. (1970). First outbreak of Hong Kong influenza in a general practice population in Great Britain. A field and laboratory study. *British Medical Journal* iii, 74–7.

HOPE-SIMPSON, R. E. & HIGGINS, P. G. (1969). A respiratory virus study in Great Britain: Review and evaluation. *Progress in Medical Virology* **11**, 354–407.

MEDICAL RESEARCH COUNCIL (1965). A Report of the working party on acute respiratory virus infections. A collaborative study of the aetiology of acute respiratory infections in Britain 1961–4. *British Medical Journal* ii, 319–26.

MEDICAL RESEARCH COUNCIL/PUBLIC HEALTH LABORATORY SERVICE (1973). Survey of acute respiratory infections in patients in general practice and children admitted to hospital. *Postgraduate Medical Journal* **49**, 748–821.

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The distribution in Kenya of bluetongue virus and antibody, and the *Culicoides** vector

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SUMMARY

Sera from domestic cattle, sheep and goats and 11 wild bovid species taken at 41 locations throughout Kenya were screened for antibody to bluetongue virus using the indirect fluorescent antibody method. Positive sera were found at 39 locations and in all the species tested. The distribution of clinical disease was mapped and found to be more restricted than the distribution of antibody. *Culicoides* were collected in light traps at 55 locations throughout Kenya. The presumptive vector of bluetongue in Kenya, *C. pallidipennis*, was the most widespread species, found in 38 locations. Both the distribution of antibody and *Culicoides* have been tabulated by ecological zones to demonstrate that bluetongue virus in its natural state in cattle and wild bovids is far more widespread than clinical bluetongue disease seen in exotic sheep of restricted distribution; and that *C. pallidipennis* has great ecological range.

INTRODUCTION

Bluetongue in Kenya is thought to exist largely in its natural biological state. The virus is transmitted by *Culicoides* midge vectors between wild bovids, indigenous cattle and sheep without apparent clinical disease. The disease hosts are generally exotic wool sheep, which are protected in Kenya by a multi-strain egg-attenuated vaccine. The reservoir and amplifying mammalian hosts, both wild and domestic, are widely distributed throughout Kenya, but little is known of the distribution of bluetongue virus and its vectors.

Walker & Davies (1971) gave results from a small number of locations and postulated an apparent enzootic area which has now been shown to be too restricted. They listed domestic cattle and sheep and eight wild bovid species as containing antibody to bluetongue virus; and *Culicoides milnei*, *C. pallidipennis* and *C. cornutus* as probable vectors. Bluetongue virus was isolated from the first two species and *C. tororoensis* (previously *C. 23*), and *C. pallidipennis* is known as a vector of bluetongue in South Africa (Du Toit, 1944; Nevill, 1971). Khamala (1971) made an analysis of collections of East African *Culicoides* which were obtained for a taxonomic survey (Khamala & Kettle, 1971), by distribution in ecological zones. This indicated that the probable vector species were more widespread than

* Diptera, Ceratopogonidae.

expected, but the range of collection locations was limited by lack of battery-powered traps.

This work has been carried out as an extension of the earlier surveys, with the intention of defining the ecological zones in which the virus, its vectors and mammalian hosts can be found. The work has been hampered to some extent by the dry conditions prevailing since the heavy rainfall year of 1967–8 which has kept the populations of *Culicoides* at a lower level than in 1968 when large numbers of domestic and wild bovids were infected. This has been shown by regular monitoring since 1968 of a sentinel cattle herd (F. G. Davies, to be published).

METHODS

Serology

The indirect fluorescent antibody method described by Pini, Ohder, Whiteland & Lund (1968) has been used to screen most sera for bluetongue antibody. This is a group-specific test. There were some modifications in the preparation of anti-ovine and anti-bovine gamma globulins. The gamma globulins were precipitated with 2·25 M sodium sulphate from ovine or bovine sera. The precipitate formed was resuspended in phosphate buffered saline (PBS) and precipitation repeated twice more. The precipitate was finally resuspended in PBS and dialysed against PBS for 24 to 48 hr., at 4° C. The protein concentration was adjusted to 1% for conjugation.

Conjugation with fluorescein isothiocyanate was at a ratio of 1 mg. of dye to 50 mg. of protein. The dialysis conjugation method of Clark & Shepard (1963) has been found to be better than the earlier conjugation method at 4° C. overnight. Conjugates were further dialysed against PBS and filtered down a column of Sephadex G 25.

Gel precipitation tests with the wild bovid sera and ovine and bovine sera showed lines of identity against the anti-bovine gamma globulin. All sera were stored at −20° C. and were routinely inactivated at 56° C. for 30 min. The wild bovid sera were kindly supplied by various farmers and the Food and Agriculture Organisation Wildlife team at the Veterinary Research Laboratory, Kabete. Sera from domestic animals were collected by members of the Kenya Veterinary Department. Collections of sheep and goat sera were generally made from mixed flocks, described in Kenya as 'shoats'. There was no difference in the results obtained from these when tested separately as goat or sheep sera, and for the purposes of this paper they are presented together. The tissue culture and staining procedures were largely as described by Pini *et al.* (1968).

Entomology

Culicoides were caught in battery-powered light and suction traps similar in construction to that described by Service (1970). Typically, collections were made in sites considered to be favourable to *Culicoides* such as stock pens or waterholes, using from two to six traps with a variety of lights (6 W ultra-violet or white light tubes or 18 W incandescent bulbs) and augmented with carbon dioxide from dry

ice in plastic bags on the traps. Most locations were visited for one or two nights only and regardless of season. The intention was to cover as wide an area as possible but the north-east area of Kenya is remote and difficult of access and was not covered as well as other areas.

Ecological zones

The zones into which the insect and serum sampling locations are allocated are after a classification by Pratt, Greenway & Gwynne (1966). These zones are defined by climate and described by vegetation and land use. They are summarized below.

Zone II. Climate humid to dry subhumid, moisture index not less than −10. Vegetation forests and derived grasslands and bushlands.

Zone III. Climate dry subhumid to semi-arid, moisture indices −10 to −30. Vegetation moist woodland/bushland/wooded grassland, trees typically broad-leaved *Combretum*.

Zone IV. Climate semi-arid, moisture indices −30 to −40. Vegetation dry woodland and wooded or bushed grassland, trees typically *Acacia*.

Zone V. Climate arid, moisture indices −40 to −50. Vegetation dry woodland/bushland, trees typically *Commiphora* and *Acacia*.

Zone VI. Climate very arid, moisture indices −50 to −60. Vegetation dwarf shrub grassland or dry bushed grassland, including barren land.

The zones are approximately delineated in Figs. 1 and 2. They are meant to indicate the potential of areas for use as range land by domestic and wild ungulates and as such are better suited to the purposes of this paper than vegetation maps based on physiognomic characters. The range of ecological zones in Kenya is very great. Zone II areas (except for the coastal forests) are similar in many respects to world cool temperate latitudes because of the high altitude (1700–2200 m.), although obviously insolation is higher and frosts are rare. Zone VI areas are 'semi-deserts'. Zone I is the afro-alpine zone confined to mountain slopes above 3300 m.

Bluetongue disease

Bluetongue virus has been isolated in these laboratories from field material either by sheep inoculation or directly in embryonating eggs or tissue culture (BHK cells). Laboratory records for the period 1950 to 1972 have been used to plot the distribution of clinical disease.

RESULTS

Entomology

Of the 30 species of *Culicoides* found only those 15 found in five or more of the 55 collection locations are included, in order of frequency in Table 1. Fig. 1 shows the collection locations. Numbers caught have not been shown because of the very large and erratic population fluctuations with time which make comparisons

Table 1. *Distribution of* Culicoides *species*

Species	Zones. No. of locations per zone in parentheses					Totals per species
	II (13)	III (9)	IV (18)	V (12)	VI (3)	
C. pallidipennis	9	9	11	8	1	38
C. nivosus	4	5	17	5	1	32
C. grahamii	7	5	7	3	0	22
C. milnei	9	4	7	1	0	21
C. pycnostictus	5	2	9	4	1	21
C. schultzei	3	4	5	7	0	19
C. magnus	7	3	8	0	0	18
C. praetermissus	0	1	8	7	0	16
C. cornutus	5	2	7	1	0	15
C. trifasciellus	6	2	6	1	0	15
C. kingi	0	2	6	4	2	14
C. neavei	2	5	2	1	0	11
C. similis	0	2	3	4	1	10
C. tororoensis	2	2	3	2	0	9
C. kibatiensis	3	2	3	0	0	8

Number of locations where species found are shown across the table.

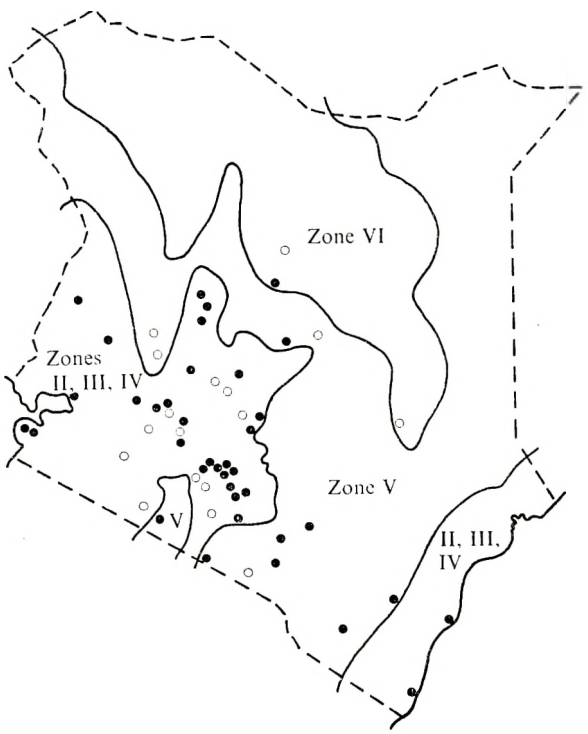

Fig. 1. Distribution of insect collection locations. ○, ●, Locations; ●, locations where *C. pallidipennis* found, ——, ecological zone boundaries; – – –, Kenya boundary.

Table 2. *Distribution of sera with antibody to bluetongue virus*

Species	Zones. No. of locations per zone in parenthesis				
	II (9)	III (7)	IV (13)	V (10)	VI (2)
Domestic cattle	90/129	72/138	47/144	62/188	18/35
Domestic sheep and goat	25/60	14/78	142/339	53/214	5/8
Coke's hartebeeste, *Alcelaphus buselaphus*	4/5	—	46/60	29/39	—
Wildebeeste, *Connochaetes taurinus*	6/10	—	42/58	27/42	—
Eland, *Taurotragus oryx*	—	—	1/7	7/17	—
Impala, *Aepyceros melampus*	1/10	—	29/91	6/13	—
Grant's gazelle, *Gazella grantii*	1/10	—	6/20	6/15	—
Thomson's gazelle, *Gazella thomsonii*	1/10	—	20/137	1/4	—
Waterbuck, *Kobus ellipsiprymnus*	—	—	10/25	1/6	—
Reedbuck, *Redunca fulvorfula*	—	11/28	0/7	—	—
Oryx, *Oryx beisa*	—	—	—	2/2	—
Buffalo, *Syncercus caffer*	—	—	7/19	—	—
Oribi, *Ourebia ourebia*	—	2/14	—	—	—

Results are shown as number positive over the sample number. Dashes indicate that no samples were taken.

between individual species at individual locations of little value. Lack of records of a species at a location means only that it was not found in these collections.

Of the species of *Culicoides* previously considered to have potential as vectors of bluetongue (Walker & Davies, 1971) *C. pallidipennis* was found at a greater number and range of sites than *C. grahamii*, *C. magnus*, *C. cornutus* and *C. tororoensis*, which were found more sporadically. In terms of numbers caught, on average, *C. pallidipennis* was also dominant.

Few species show a marked restriction to certain zones, but *C. milnei* is conspicuous for being found only once in zones V and VI. *C. nivosus* was very widespread, which was in contrast to the earlier studies. Changes in trap design could possibly account for the higher frequency of capture. However, only five specimens were found engorged with blood and none gave positive results against bovid precipitating antisera (tests performed by P. F. L. Boreham). *C. grahamii* was never caught in large numbers and only at a moderate number of locations; this contrasts with Khamala's results where *C. grahamii* was the dominant species in numbers and distribution. The greater range of sites in the present study may account for this discrepancy.

Serology

The results are summarized in Table 2 and the sampling locations are shown in Fig. 2. Of the 41 locations there were 39 at which sera gave positive results for bluetongue antibody. The sera were collected over the period 1967–72. During

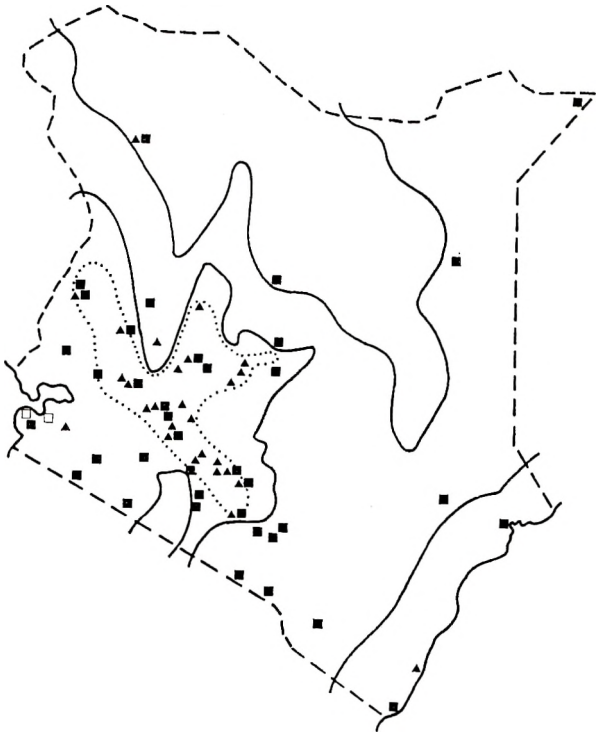

Fig. 2. Distribution of serum sample locations and clinical disease. □, ■, serum sample locations; ■, locations where sera were positive for bluetongue antibody; ▲, locations of outbreaks of clinical bluetongue disease; . . ., exotic wool sheep rearing area; ——, – – – –, as in Fig. 1.

the early part of this period there was considerable rainfall and arbovirus diseases of cattle and sheep such as Rift Valley fever and ephemeral fever were prevalent. Considerable biting activity of bluetongue-infected *Culicoides* occurred during this period, which has been confirmed by serological studies in a sentinel herd (F. G. Davies, to be published). The results taken from the earlier years gave higher percentages of positive sera than those taken in 1971–2, especially when young animals were bled. The serological results are intended to show the universality of contact with bluetongue throughout the range of ecological zones in Kenya.

Occurrence of clinical bluetongue

The distribution of locations where there have been outbreaks of clinical bluetongue disease is shown in Fig. 2. Clinical disease has occurred in all ecological zones, as might be expected from the results of the entomological and serological surveys. However, the proportions of outbreak locations per zone (zone II = 10, zone III = 6, zone IV = 9, zone V = 1, zone VI = 1) indicate that factors other than the distribution of virus and vector influence the actual appearance of clinical disease. Fig. 2 shows an approximate boundary of the current wool sheep rearing area in Kenya as derived from data in the *National Atlas of Kenya* (1970). Disease is seen almost entirely within this boundary, affecting exotic breeds of

wool sheep, with rare anomalous cases such as clinical bluetongue in introduced goats at Lodwar, the location marked in the far north-west.

DISCUSSION

The distribution of an arbovirus vector must be contiguous with the distribution of the virus. In a survey of this type negative results mean little and there are locations at which sera contained antibody to bluetongue virus and yet no *C. pallidipennis* were found. However, the results are adequate to show that *C. pallidipennis* fits the vector criterion well and that it is a very adaptable and viable species. The viability may be due to the larval habitat being in bovid dung pats, as Nevill (1968) has shown in South Africa.

The results of the serological survey demonstrate the widespread distribution of bluetongue antibody throughout Kenya. The disease occurs almost entirely in exotic sheep which are kept in a limited area. The exotic sheep appear to be more susceptible than the indigenous sheep and goats. No bluetongue disease is recognized by the pastoral tribes, such as the Maasai, who are familiar with all the other cattle and sheep or goat diseases in their areas. There is antibody to bluetongue virus in many areas of Maasai land. Bluetongue virus has been isolated from indigenous sheep, this has been adventitious however and had not been associated with any clinical signs of disease. Further work will be aimed at confirming the resistance of indigenous sheep and goats to bluetongue.

Factors other than breed susceptibility clearly influence the occurrence of disease. We suspect that the increase in vector population with amplification of the proportion infected with virus is the principal one (Walker & Davies, 1971). Further studies after a heavy period of rainfall such as was experienced in 1967–8 should serve to answer these questions.

We have no evidence of any antigenic variation amongst the bluetongue strains in Kenya. All virus strains isolated to date have been typed as one of the seven thus far recognized in Kenya (F. G. Davies, unpublished data). Further work will be aimed at typing the positive sera from different parts of Kenya from domestic and wild bovids.

No pathogenicity experiments have as yet been carried out with the game species shown to contain antibody to bluetongue virus. Plaque neutralization tests carried out by the method of Howell (1970) show that titres of antibody in game animals are comparable with those in domestic animals. They are in the range of $10^{2.5}$ to $10^{4.5}$ neutralizing indices (F. G. Davies, unpublished data). There is abundant evidence (Barzilai & Tadmor, 1972; Thomas & Trainer, 1970) that wild ruminants elsewhere sustain a viraemia of sufficient titre to infect *Culicoides* (Luedke, Jones & Jochim, 1967).

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REFERENCES

Barzilai, E. & Tadmor, A. (1972). Experimental infection of the Mountain Gazelle (*Gazella gazella*) with bluetongue virus. *Refuah Veterinarith* **29**, 45–50.

Clark, H. F. & Shepard, C. C. (1963). A dialysis technique for preparing fluorescent antibody. *Virology* **20**, 642–4.

Du Toit, R. M. (1944). Transmission of bluetongue and horsesickness by *Culicoides*. *Onderstepoort Journal of Veterinary Science and Animal Industry* **19**, 7–16.

Howell, P. G. (1970). The antigenic classification and distribution of naturally occurring strains of bluetongue virus. *Journal of the South African Veterinary Medical Association* **41**, 215–23.

Khamala, C. P. M. (1971). Ecological distribution of East African *Culicoides* as shown by light traps. *Bulletin of Entomological Research*, **60**, 549.

Khamala, C. P. M. & Kettle, D. S. (1971). The *Culicoides* of East Africa. *Transactions of the Royal Entomological Society of London* **123**, 1–95.

Luedke, A. J., Jones, R. M. & Jochim, M. M. (1967). Transmission of bluetongue between sheep and cattle by *Culicoides variipennis*. *American Journal of Veterinary Research* **28**, 457–60.

National Atlas of Kenya (1970). *Survey of Kenya*, Nairobi.

Nevill, E. M. (1968). A significant new breeding site of *Culicoides pallidipennis*. *Journal of the South African Veterinary Medical Association* **39** (part 3), 61.

Nevill, E. M. (1971). Cattle and *Culicoides* midges as possible overwintering hosts of bluetongue virus. *Onderstepoort Journal of Veterinary Research* **38**, 65–72.

Pini, A., Ohder, H., Whiteland, A. P. & Lund, L. J. (1968). Studies on the fluorescent and neutralising antibodies to bluetongue virus in sheep. *Archiv für die gesamte Virusforschung* **25**, 128–36.

Pratt, D. J., Greenway, P. J. & Gwynne, M. D. (1966). A classification of East African rangeland, with an appendix on terminology. *Journal of Applied Ecology* **3**, 369–82.

Service, M. W. (1970). A battery-operated light-trap for sampling mosquito populations. *Bulletin of the World Health Organization* **43**, 635–41.

Thomas, F. C. & Trainer, D. O. (1970). Bluetongue virus in white tailed deer. *American Journal of Veterinary Research* **31**, 271–8.

Walker, A. R. & Davies, F. G. (1971). A preliminary survey of the epidemiology of bluetongue in Kenya. *Journal of Hygiene* **69**, 47–60.

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Experimental study of a further attenuated live measles vaccine of the Sugiyama strain in Iran

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SUMMARY

After encouraging results of the mass vaccination programme in Iran, in which 5 million children in rural areas were vaccinated with the Japanese Sugiyama strain at its 82nd passage in baby calf kidney, and a progressive decrease in the incidence of measles as well as a reduction of excessive infant mortality, a further attenuated vaccine, produced with the same strain, cloned in Japan, was compared in a field trial with the parent vaccine. The new strain caused fewer reactions than the original strain. Seroconversion with a geometric mean antibody titre of 6·1 was observed in 95% of susceptible children.

INTRODUCTION

The live attenuated measles viruses now in use for measles prevention are normally propagated either in leukosis-free chick embryo cells or in primary monkey kidney cells. There are, however, strains such as Sugiyama attenuated virus adapted to calf kidney (CK) (Matumoto *et al.* 1962) or ESC and AIK strains both developed in lamb kidney cells (Chumakov *et al.* 1967; Makino *et al.* 1973). Each cell system may have several advantages as well as some shortcomings. In Iran, since 1968, the Sugiyama strain adapted to CK cells has been largely used in all rural regions where measles was the main problem. The mortality due to measles complications in those regions, estimated to be at least 10,000 per year before the mass campaign, has declined dramatically after 5 years of extensive immunization against the disease. The choice of the attenuated Sugiyama strain of measles virus was based on the following points:

(*a*) The virus is easily propagated in primary baby CK cells. Because of the repeated harvests of virus, once grown in this cell system, the production of large amount of virus at a low cost, to cover the needs of mass immunization, was possible.

(*b*) Baby CK cell is normally free of the well-known contaminants of eggs or monkey cells. Unlike ovine kidney cells which frequently show slow-growing agents with cytopathogenic effect, we have not so far observed any cytopathogenic effect of unknown origin in CK cells. Since 1968 over six million doses of the vaccine have been produced in baby CK cells at passage 82. The vaccine was well tolerated by children of 1–5 years who have shown a seroconversion rate of about 95%,

4 weeks after vaccination. The Sugiyama attenuated strain was first developed in Japan by Matumoto *et al.* (1962) and has been investigated by Myamura *et al.* (1971). According to the latter authors this strain has gradually been attenuated by passage in CK cells at low temperature, but the febrile reaction of vaccinees had not been decreased from the 70th to 86th passage. The virus from the 81st passage in CK cells was recently cloned by Hashizume and his colleagues after elution of virus from $AlPO_4$. Two batches of vaccine were prepared by these authors with the cloned virus, called 5F, after 4 or 19 further passages of the clone in CK cells. Thus the vaccines were prepared at passage 85 or 100 of the Sugiyama strain in CK cells. The vaccines were studied by these authors in a field trial with the following results:

(1) No difference was observed between the two viruses regarding neurovirulence in the monkey, *in vitro* markers, or production of reactions in susceptible children.

(2) The cloned virus did not grow at 39·2° C., at which temperature the original virus grew slightly.

(3) Febrile reactions of vaccinees inoculated with the two vaccines were decreased when compared with the parent vaccine.

(4) After inoculation of these vaccines, the rash was mostly scanty, consisting of tiny spots. The rash from the original virus was mostly maculo-papular or urticarial.

After this field trial, Dr Hashizume, Chief of Measles Unit, Chiba Serum Institute, Japan, generously supplied us with enough seed material of 5F clone to start production of our own seed lot.

The object of the present study was to compare the 5F cloned virus with the original Sugiyama strain at passage 82 in CK cells which has so far been largely used in Iran.

METHODS AND MATERIALS

Vaccines used

The Sugiyama virus at its 82nd passage in CK cells was part of a large batch of vaccine used in the country since June 1971. Each single dose of vaccine contained $10^{2\cdot7}$ TCID 50 of virus. The further attenuated vaccine was prepared with the cloned 5F passed twice in CK cells. One dose of this vaccine contained $10^{3\cdot0}$ TCID 50 of virus. Both vaccines were lyophilized and stored at $-20°$ C. before use.

Pathogensis for suckling hamster

In order to compare the virulence of the two strains for suckling hamsters, litters of five to seven baby hamsters 1–2 days old were injected intracerbrally with 0·02 ml. of undiluted virus, immediately after reconstitution of the lyophilized vaccine. Signs of illness and procedure for isolation of virus have been given before (Mirchamsy, Razavi & Ahourai, 1972).

Table 1. *Age and sex distribution of inoculated children*

Type of vaccine	Total inoculated	Sex		Age (years)		
		Female	Male	10–12 months	> 1–2	> 2–5
Sug. 82	118	54	64	22	44	52
5F 100	75	40	35	27	27	21

Table 2. *Number of children developing pyrexia*

Type of vaccine	No. of children	Fever (°C.)			Onset mean (days)	Mean duration of pyrexia (days)	Mean duration of maximum temperature (days)
		37–38	38–39	> 39			
Sug. 82	85	46 (54%)	16 (19%)	3 (3·5%)	8·11	4·22	2·6
5F 100	70	45 (64%)	4 (5·7%)	1 (1·4%)	12	1·5	1·0

Children

The study was carried out on 193 home-dwelling children in the Razi Institute and its surrounding villages. The children had not been immunized before against the disease and were without past history of clinical measles. The children were allocated to one of the two groups by the random sampling method.

Clinical observations

The temperature was recorded twice daily for 3 weeks after vaccination. The clinical reactions were recorded by trained technicians who paid regular visits to the children. Mothers were also advised to refer to the clinic of the Razi Institute when clinical manifestations were noticed. During the febrile period, each child was examined on 5 consecutive days by a physician either at home or in the clinic.

Serological testing

In order to evaluate the antibody response to vaccination, blood samples were collected immediately before inoculation and 30 days later. The blood was collected from finger pricks using paper disks as described previously (Mirchamsy, Nazari, Stellman & Esterabady, 1968). The paired sera of each child were tested simultaneously by the hemagglutination inhibition (HI) test according to Rosen's technique (Mirchamsy *et al.* 1970).

RESULTS

Clinical response

The distribution of children in each of the two groups according to sex and age is shown in Table 1. The average onset of the fever was 8–11 days for the original vaccine. This period was increased to 12 days for 5F vaccine (table 2). The pyrexia was milder for 5F vaccine in comparison with the original vaccine. As is shown

Table 3. *Incidence of rash and clinical observation*

Type of vaccine	No. of children	Rash: Mild (%)	Rash: Severe (%)	Rash: Mean onset (days)	Rash: Mean duration (days)	Koplik spot (%)	Cough (%)	Coryza (%)	Conjunctivitis (%)	Tonsillitis (%)	Convulsion (%)
Sug. 82	85	39 (46)	19 (22)	10·5	4·2	1·2	10	54	36	8	—
5F 100	70	22 (31)	2 (3)	12·5	2·1	—	6	22	5	4	—

Table 4. *Serologic responses in initially seronegative children*

Type of vaccine	No. of sera tested	Children with maternal antibody	Seroconversion convertors/vaccinated	%	GMT*
Sug. 82	85	6	76/79	96	7·0
5F 100	70	6	61/64	95	6·1

* Geometric mean titre: HI titre $\log_2$.

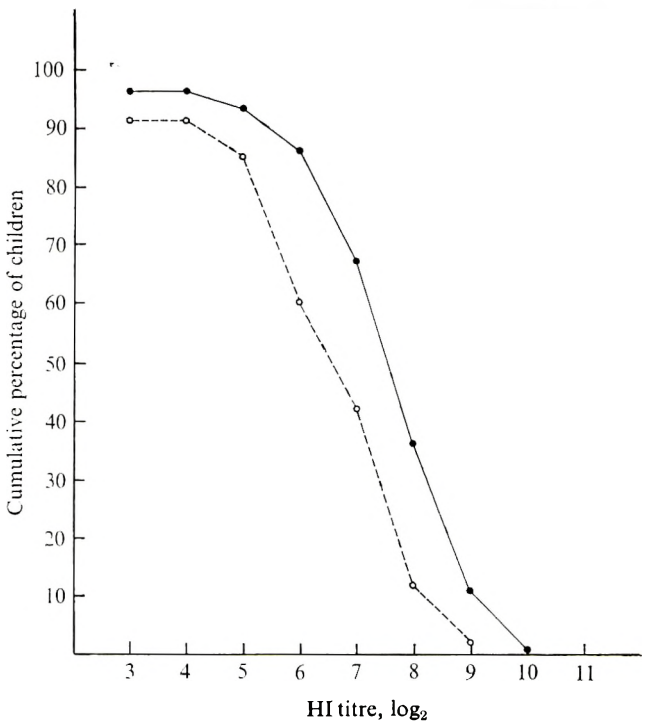

Fig. 1. HI antibody response in vaccinated children initially seronegative.
●—●, Sugiyama passage 82; ○- - -○, 5F 100 vaccine.

in Table 2, only 71 % of those given 5F vaccine reacted with fever. Of this number 64 % had a mild fever (37–38° C.), 5·7 % had shown a temperature of 38·1–39° C. Only one child had a fever of 39·5° C. Mean duration of pyrexia was 1·5 days and mean duration of maximum temperature was only 1 day.

Pyrexia was more severe in children immunized with the original vaccine. In

this group 54% of children showed a mild rise of temperature, 19% had fever of 38·1–39° C. and 3·5% a fever of over 39° C. The mean duration of pyrexia, 4·22 days, was much longer than that of the first group; the mean duration of maximum temperature was 2·6 days.

The percentage with rash was lower in children immunized with 5F vaccine. It consisted of sparse and tiny exanthema which appeared on average 12·5 days after vaccination and faded in 2 days. The morbiliform rash of the original vaccine was usually indistinguishable from natural measles, appearing about 10 days after vaccination and lasting 4 days. Koplik's spots were not observed in those given 5F vaccine. The percentage of other symptoms such as cough, coryza, conjunctivitis and tonsillitis was also reduced when 5F vaccine was used (Table 3).

Virulence for baby hamsters

The symptoms of illness in some suckling hamsters, inoculated with Sugiyama original vaccine, were observed 14–18 days after inoculation. In three successive experiments the ratio of baby hamsters showing signs of encephalitis was 1:5. Measles virus was isolated in all cases from the harvested brain in Vero cells. The suckling hamsters inoculated with 5F vaccine did not show any sign of illness and in three experiments measles virus was not isolated from their brains.

Serological findings

The seroconversion rate in both groups was 95% (Table 4). Although the cumulative titre distribution curves for the two vaccines were parallel (Fig. 1), the mean antibody titre was about 1 $\log_2$ lower in children immunized with 5F vaccine.

DISCUSSION

The Sugiyama vaccine prepared in Japan at the 73rd passage in CK cells was one of the two further attenuated vaccines which have been considered by the Japan Measles Vaccine Research Commission (Shishido, 1969) to be safe and able to be used without gammaglobulin or prior inoculation of killed vaccine. The seed material of Sugiyama virus received through the courtesy of Dr S. Hashizume of Chiba Serum Institute, Japan was at passage 78 in primary CK cells. After three passages in our laboratory in the same cell system a seed lot was prepared. Five successive batches, each of over one million doses, were then produced at passage 82 in primary young CK cells. About 6 million doses of this vaccine have so far been used throughout Iran mainly in rural regions in children of 1–5 years without any untoward reaction (Manteghi, 1971). Fever and clinical symptoms observed after use of this vaccine were mild, as we have described previously (Mirchamsy *et al.* 1970, 1971). Seroconversion also was 95–97% with a mean titre of 7·5 $\log_2$ (Mirchamsy *et al.* 1971). The only remark made by many physicians about this vaccine was that the type of rash and its occasional intensity was sometimes similar to the natural measles.

The new seed virus 5F, also kindly supplied by Dr Hashizume, has been propagated twice in our laboratory in young CK cells in order to produce a seed lot virus. A small batch of vaccine was then produced with this new seed virus and a

comparative study was undertaken in order to assess the changes which had resulted from clonage of Sugiyama attenuated virus. From data presented in this report and those already reported by Myamura *et al.* (1971) one can assume that the 5F strain of Sugiyama virus is a further attenuated strain of measles virus. The thermal reaction in vaccinees was lower in comparison with the parent vaccine, the rash was sporadic and consisted of tiny spots which faded in 2 days. The seroconversion rate was not changed and remained at 95%. The mean antibody titre was decreased to 6·1 or 1 $\log_2$ less than the mean titre in vaccinees with the parent vaccine.

The difference of 1 $\log_2$ in mean antibody titre of the two vaccines is not significant and cannot diminish the duration of immunity. The mean antibody titre of the well-known attenuated measles vaccine strains such as Schwarz, Leningrad-16, Biken CAM, ESC, and AIK strains, in different field trials, have been found to be 6·1, 6·4, 5·1, 6·3 and 6·6 respectively (Bolotovsky & Zetilova, 1968; Okuno *et al.* 1971; Shishido, 1969; Makino *et al.* 1973). It is also a fact that the high neutralizing antibody titre, normally observed 4–6 weeks after natural measles infection or after immunization with live vaccine, will decrease gradually to a low titre which may persist for many years or even during life.

The lack of pathogenicity for suckling hamster was observed in 5F cloned virus; the parent virus was slightly virulent for baby hamster.

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REFERENCES

BOLOTOVSKY, V. M. & ZETILOVA, L. P. (1968). Comparative study of the reaction-causing properties and the immunological and epidemiological effectiveness of Leningrad-16 and Schwarz live measles vaccines. *Bulletin of the World Health Organization* **39**, 293–8.

CHUMAKOV, M. P., VOROSILOVA, M. K., GRACEV, V. P., DZAGUROV, S. G. & SINEAK, K. M. (1967). Le vaccin vivant antirougeoleux de la souche très attenuée E.Sch.Ch. (Edmonston-Schwarz-Chumakov) preparé par l'Institut de poliomyelite et d'Encephalites de l'Union Soviétique. *Archives Roumaines de Pathologie Expérimentale et de Microbiologie* **27**, 17–33.

MAKINO, S., SASAKI, K., NAKAMURA, N., NAKAGAWA, M., KASAHARA, S., MIZUNOE, K., HASHIMOTO, T., EHIHARA, T., NAKAZAWA, S., OKA, H., SATO, H., WATANABE, O., NIINO, K., KOGA, F., ONUMA, M., KAGAMI, K., KIMURA, M., ISHIGURO, Y., YOSHIDA, Z. & FUGII, H. (1973). Field trial with a further attenuated live measles virus vaccine. *Japanese Journal of Microbiology* **17**, 75–9.

MANTEGHI, A. (1971). The epidemiological survey of measles in Iran following mass vaccination. *Ministry of Health Report* **49**, 1–49.

MATUMOTO, M., MUTAI, M., SABURI, Y., FUJII, R., MINAMITANI, M. & NAKAMURA, K. (1962). Live measles virus vaccine: clinical trial of vaccine prepared from a variant of the sugiyama strain adapted to bovine kidney cells. *Japanese Journal of Experimental Medicine* **32**, 433–48.

MIRCHAMSY, H., NAZARI, F., STELLMAN, C. & ESTERABADY, H. (1968). The use of dried blood absorbed on filter paper for the evaluation of diphtheria and tetanus antitoxins in mass surveys. *Bulletin of the World Health Organization*, **38**, 665–71.

MIRCHAMSY, H., SHAFYI, A., BASSALI, Y., BAHRAMI, S. & NAZARI, F. (1970). A comparative study of two live measles vaccines in Iran. *Journal of Hygiene* **68**, 101–10.

MIRCHAMSY, H., SHAFYI, A., BAHRAMI, S., NAZARI, P., MIRZADEH, M. & BASSALI, Y. (1971). Mass immunization of children in Iran with live attenuated Sugiyama measles virus adapted to calf kidney cell culture. *Japanese Journal of Experimental Medicine* **41**, 39–48.

MIRCHAMSY, H., RAZAVI, J. & AHOURAI, P. (1972). Pathogenesis of vaccine strains of measles virus in suckling hamsters. *Acta Virologica* **16**, 77–9.

MYAMURA, K., YOSHIZAWA, S., TANIA, T., SAKAI, K., HASHIZUME, S., OKUNI, H., KAWANA, R. & KIMURA, M. (1971). Further attenuated measles virus strain Sugiyama, having lower ceiling temperature, derived from Sugiyama original strain by elution from aluminum phosphate and limiting dilution of the eluate. 19*th Annual Meeting of the Society of Japanese Virologists*.

OKUNO, Y., UEDA, S., KURIMURA, T., SUZUKI, N., YAMANISHI, K., BABA, K., TAKAHASHI, M., KONOBE, T., SASADA, T., ONISHI, K. & TAKAKU, K. (1971). Studies on further attenuated. live measles vaccine. VII. Development and evaluation of CAM-70 measles virus vaccine. *Biken Journal* **14**, 253–8.

SHISHIDO, A. (1969). A field trial of further attenuated live measles virus vaccines in Japan–1968. *Japanese Journal of Medical Science and Biology* **22**, 191–200.

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Variola minor in Bragança Paulista County, 1956: lack of evidence indicating the influence of contaminated classrooms on spread of the disease*

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SUMMARY

Space-time interaction analysis was applied to data from 101 elementary school children who contracted variola minor during an epidemic in Bragança Paulista County, Brazil. One school had two and the other three shifts of students occupying the same classrooms each day. There was no evidence found for excessive numbers of cases to occur among unvaccinated students occupying the same desks or seated near the desks occupied by cases occurring during another shift. Only three cases occurred among the 31 unvaccinated students occupying desks of students with variola from other shifts. Only one of these three subsequent cases occurred at a time interval suggestive of transmission. For the three models tested there was no evidence of space-time interaction between time of onset of the disease and location of desk for pairs of students from different shifts.

INTRODUCTION

In a previous report (Klauber & Angulo, 1974), space-time interactions among 101 cases of variola minor (alastrim) in two elementary schools were analysed by Mantel's permutational procedure (1967), which is a generalization of Knox's (1963) approach. The object of that investigation was to determine the extent to which various epidemiological units, e.g. classrooms or rows of desks, patterned the school outbreak. The approach was to compare total number of pairs of cases within given time and space units to expectation. In the present study, only the number of pairs of cases where one member of the pair is from one shift and the other from another shift using the same classroom, is compared to expectation. The intent here is to obtain evidence of possible indirect contagion by contaminated desks or classrooms.

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MATERIALS AND METHODS

The study schools

The two schools where most cases of variola minor occurred during the study epidemic were described in the previous report (Klauber & Angulo, 1974). This report also described the methods of data collection, and the critical time and space units chosen before the statistical analysis were also discussed. The same cases, but different statistical procedures, are used in the present report.

For the sake of clarity, some descriptions and definitions are reproduced here. The same classroom was used daily by two groups of students in the José Guilherme (JG) School and by three groups in the Jorge Tibiriçá (JT) School. All the classes attending during the same daily period are called a *shift*. Maps of classrooms were plotted for each shift and school. Save for one classroom of the JG School, all classrooms of either school had the same dimensions. The desk occupied by each student (the same every day) was identified. Desks were paired, one contacting the other on one side. Each desk-pair was placed immediately after the other, from the front to the rear of the classroom.

Statistical methods

Space-time interaction analysis

Space-time interaction was tested by the method of Klauber (1971) using the approach of Knox (1964). That is, Z the number of close pairs in both space and time, where each pair is composed of cases from different shifts, is evaluated relative to expectation E and to variance V, computed for three different randomization models. Values of Z, E and V were computed for two pairs of shifts: *JG* School, shift 1 *vs.* shift 2 and JT School, shift 1 *vs.* shift 3. It was impossible to have any pairs within the space and time units tried for the JT School, shift 1 *vs.* shift 3 and three additional pairs were available for shifts 2 *vs.* 3, only if a 30-day time unit were used, hence, these comparisons were eliminated.

An approximate standard normal statistic, corrected for continuity is given by

$$U = (\Sigma Z - \Sigma E \pm 0{\cdot}5)/\sqrt{(\Sigma V)},$$

where the summations are over the pairs of shifts to be combined and the sign of the continuity correction, 0·5, is chosen to reduce the difference between ΣZ and ΣE.

For each pair of shifts within each school three separate randomization distributions were considered. The three randomization models correspond to hypotheses of disease spread: (1) from the later shift to the earlier shift (earlier shift random); (2) from the earlier shift to the later shift (later shift random); and (3) both (1) and (2) (both sets random). The formulae for E and V are lengthy for all three models, and the reader is referred to Klauber (1971) for the details of the computations.

Space and time units

The critical time units between onsets of illness in two cases of variola minor would be approximately 11–16 days to allow for the actual variability in the periods of infectivity and incubation. In order to compensate for possible inaccuracies in the dates of onset, the following critical time units in days were tried: 11–16, 8–19, 5–22, 2–25 and less than or equal 30.

The critical space units reported (Klauber & Angulo, 1974) are: (1) the classroom, and (2) 'within one seat', i.e. students seated side-by-side, front to back or adjacent diagonally. Actually a number of other space units were tried, but these either gave similar results to those reported or yielded too small expectations to yield normal approximations in which we had confidence.

Seating position and disease occurrence

For the units, 'seated at the same desk', 'seated side-by-side' and 'seated front to back', an alternative analysis was used. For each school and shift each student who was unvaccinated for variola was counted in a 2 by 2 contingency table according to whether or not he contracted variola minor during the epidemic, and whether or not he was 'exposed', i.e. seated within the given space unit of a previously occurring case in a different shift (Table 1). The overall significance of an association between the factor, being seated at the same desk as a previous case from another shift (or other space unit association), and development of the disease was tested by combining the results for the five shifts using the Mantel–Haenszel chi square (one degree of freedom) procedure (1963).

It should be pointed out that this second approach is not a test of space-time interaction. Each case occurring after another from a different shift within the space unit was checked for time difference in onset from that case (only pairs occurred) to see if transmission between them could possibly have occurred.

RESULTS

Same desk

An inspection of the seating maps and dates of onset indicated that 31 unvaccinated students occupied desks of students from other shifts who contracted the disease. Only three cases subsequently occurred among the 31 so 'exposed' to the index cases. The time intervals to onset of the index case were 13, 35 and 44 days, respectively. Thus, only one case conceivably could have resulted from fomites left at the desk.

The Mantel–Haenszel approach indicated no significant difference between observed versus expected cases (Table 1). The observed : expected ratio was 3 : 3·92 ($\chi^2 = 0{\cdot}06$).

Transversally attached desk

There were 37 unvaccinated students seated at desks attached to those on the left or right occupied by cases occurring during a different shift. Only one

Table 1. *Variola minor in two Bragança Paulista Schools*, 1956

(Number of cases occurring among unvaccinated students seated at desks where a case occurred previously from another shift*)

School		Shift 1 Case	Shift 1 Not-case	Shift 1 Total	Shift 2 Case	Shift 2 Not-case	Shift 2 Total
JG	'Exposed'	1	2	3	0	7	7
	'Not-exposed'	34	72	106	19	86	105
	Total	35	74	109	19	93	112

School		Shift 1 Case	Shift 1 Not-case	Shift 1 Total	Shift 2 Case	Shift 2 Not-case	Shift 2 Total	Shift 3 Case	Shift 3 Not-case	Shift 3 Total
JT	'Exposed'	0	2	2	0	13	13	2	4	6
	'Not-exposed'	30	60	90	3	84	87	12	98	110
	Total	30	62	92	3	97	100	14	102	116

* Summary $\chi^2 = 0{\cdot}06$; (observed cases)/(expected cases among 'exposed') = 3/3·92 = 0·77.

Table 2. *Variola minor in two Bragança Paulista Schools*, 1956

(Number of cases occurring among unvaccinated students seated at desks transversally adjoining a desk where a case occurred previously from another shift*)

School		Shift 1 Case	Shift 1 Not-case	Shift 1 Total	Shift 2 Case	Shift 2 Not-case	Shift 2 Total
JG	'Exposed'	0	4	4	0	11	11
	'Not-exposed'	35	70	105	19	82	101
	Total	35	74	109	19	93	112

School		Shift 1 Case	Shift 1 Not-case	Shift 1 Total	Shift 2 Case	Shift 2 Not-case	Shift 2 Total	Shift 3 Case	Shift 3 Not-case	Shift 3 Total
JT	'Exposed'	0	5	5	0	10	10	1	6	7
	'Not-exposed'	30	57	87	3	87	90	13	96	109
	Total	30	62	92	3	97	100	14	102	116

* Summary $\chi^2 = 4{\cdot}58$, $P = 0{\cdot}032$; (observed cases)/(expected cases) = 1/5·93 = 0·17.

case occurred after the index case among the 37 so 'exposed' and the onset was 64 days after that of the index case.

The Mantel–Haenszel approach yielded an expected number of cases of 5·93, which was statistically significantly different from the one observed at the 5% level; $\chi^2 = 4{\cdot}58$ (Table 2).

Table 3. *Variola minor in two Bragança Paulista Schools*, 1956

(Number of cases occurring among unvaccinated students seated at desks adjoining longitudinally a desk where a case occurred previously from another shift*)

School		Shift 1			Shift 2		
		Case	Not-case	Total	Case	Not-case	Total
JG	'Exposed'	0	5	5	0	13	13
	'Not-exposed'	35	69	104	19	80	99
	Total	35	74	109	19	93	112

School		Shift 1			Shift 2			Shift 3		
		Case	Not-case	Total	Case	Not-case	Total	Case	Not-case	Total
JT	'Exposed'	2	11	13	0	12	12	1	14	15
	'Not-exposed'	28	51	79	3	85	88	13	88	101
	Total	30	62	92	3	97	100	14	102	116

* Summary $\chi^2 = 6{\cdot}57$, $P = 0{\cdot}011$; (observed cases)/(expected cases) = 3/10·22 = 0·29.

Longitudinally adjoining desk

There were 58 unvaccinated students seated at desks directly in front of or behind desks of cases from other shifts. Three out of these 58 students had onset of variola subsequent to that of the index case. The time differences between the onsets of the index and subsequent cases were 5, 26 and 29 days.

The Mantel–Haenszel chi square was almost statistically significant at the 1 % level; $\chi^2 = 6{\cdot}57$. A total of 10·22 cases would be expected among the 58, where three were observed (Table 3).

Evidently, there is no evidence favouring the possibility that the disease was transmitted via fomites from one class period to another. Quite the contrary, if there was an effect of a desk being infected, it was to reduce the likelihood of an unvaccinated student contracting the disease, if seated at (or adjacent to) that desk.

Between-class space-time interaction

The space-time interaction tests showed no significant excess of observed pairs compared to expectation. The greatest O/E ratio was 3/2·43 = 1·24, which occurred using the later shift as the random set, a time unit of 5–22 days and the space unit, 'within one seat in any direction'. Out of the 30 O/E ratios, 25 had values less than one, confirming the impression from the previous analyses that if there was any effect, it was tendency towards fewer than expected cases occurring in different shifts, that could be possibly related. At the 5 % level (Table 4), there was only one significant difference in observed versus expected pairs 11/18·44 = 0·60. By chance, one would expect on the average 1·5 such significant differences out of 30 tests.

Table 4. *Variola minor in two Bragança Paulista Schools*, 1956

(Analysis of between-shift clustering, number of pairs of cases/expected within indicated space and time distances by sets assumed random)

		Random set		
Time unit	Space unit	Earlier shift	Later shift	Both
$11 \leqslant T \leqslant 16$ Days	Same room	10/12·14 = 0·82	10/9·55 = 1·05	10/12·23 = 0·82
	Within 1 seat	1/1·46 = 0·69	1/1·14 = 0·88	1/1·54 = 0·65
$8 \leqslant T \leqslant 19$ Days	Same room	11/18·44 = 0·60*	11/15·38 = 0·72	11/20·25 = 0·54
	Within 1 seat	1/2·49 = 0·40	1/1·56 = 0·64	1/2·55 = 0·39
$5 \leqslant T \leqslant 22$ Days	Same room	18/29·96 = 0·72	18/21·90 = 0·82	18/28·44 = 0·63
	Within 1 seat	3/3·45 = 0·87	3/2·43 = 1·24	3/3·58 = 0·84
$2 \leqslant T \leqslant 25$ Days	Same room	28/33·99 = 0·82	28/28·98 = 0·97	28/37·80 = 0·74
	Within 1 seat	3/4·75 = 0·63	3/2·92 = 1·02	3/4·76 = 0·63
$T \leqslant 30$ Days	Same room	37/43·82 = 0·84	37/36·79 = 1·01	37/47·23 = 0·78
	Within 1 seat	4/5·68 = 0·70	4/3·83 = 1·04	4/5·94 = 0·67

* Observed statistically significantly different from expectation at the 5% level.

The three randomization models yielded slightly different results. When the earlier shift cases were considered fixed, i.e. the source of disease spread, and later shift cases random, the O/E ratios averaged close to one (0·94). When the later shift cases were considered fixed and the earlier shift cases random or both sets random, all these O/E ratios were less than one and averaged 0·69.

DISCUSSION

A noteworthy finding is the very low frequency of variola minor among susceptible students occupying desks of students from other shifts who contracted variola during the study epidemic. This frequency becomes even lower when the length of the interval between the presumptive source and receptor cases is considered, unless a very long period of infectivity is admitted. In this regard, the time span covered by onsets of subsequent cases in defined social units indicate that, in real situations, the infectivity period of smallpox, particularly of variola minor, is rather limited (Dixon, 1948, 1962*a*; Anderson, Foulis, Grist & Landsman, 1951; de Salles-Gomes, Angulo, Menezes and Zamith, 1965; Angulo, Rodrigues-da-Silva & Rabello, 1967). This factual evidence contradicts the rather long periods postulated, without citation of supporting evidence, in textbooks, review articles, etc.

Further support to the above findings comes from the following facts: (*a*) a very low frequency of variola among susceptible students occupying one desk whose paired (contacting on one side) desk was occupied during another shift by a student who developed variola. This frequency becomes nil when the interval between onsets of the presumptive and receptor cases is considered; (*b*) a similar but less defined finding is made when desks placed immediately (no space between them) behind or in front of the desk occupied by a student who contracted variola are considered.

In a recapitulation, the analysis disclosed that contaminated desks, rather than increasing the likelihood of susceptible students developing the disease, showed a tendency towards fewer than expected cases occurring in different shifts, that could be possibly related. More support comes from space–time interaction analysis even though three randomization models yielded slightly different results. The object of the present study was to obtain evidence for possible contagion between students from different shifts using the same classroom. If such contagion had been evident it would not prove that the disease had been transmitted by fomites in the classroom, but rather it would admit it as a possibility. There was a tendency for students to queue outside the classroom before their shift and pass the members of the previous shift on the way to their desks. The negative findings of this study also indicate lack of evidence for this latter mode of transmission.

In the literature reviewed, only a single report of the spread of smallpox in a school was found. Although evidence of spread of variola minor in this, a primary school, was obtained, no suggestion of the influence of contaminated desks was found (Angulo, Rodrigues-da-Silva & Rabello, 1964). This intuitive examination of a small school outbreak is in full agreement with the results of the present statistical analysis. Studies resembling the present one are just intuitive examinations of the relative position of beds with cases in hospitals where smallpox was introduced (Eastwood, 1955; de Jongh, 1956; de Salles-Gomes *et al.* 1965; Angulo & de Salles-Gomes, 1967). No formal (analytical) approach was used in these studies. The possible role of fomites in those hospital outbreaks was examined without conclusive evidence of this self-suggestive mode of spread. As a matter of fact, when that role has been especially investigated in large as well as small epidemics occurring in other types of communities, negative results were also obtained (Clark *et al.* 1944; Dixon, 1948; Angulo, Rodrigues-da-Silva & Rabello, 1964). In this regard, handling bed-linen, clothing and similar objects used by or in physical contact with smallpox patients occurs in every house, hospital, etc., where patients are nursed. In spite of this enormous frequency, exceptionally few instances of spread through fomites have been reported (Corbin, 1915; Stallybrass, 1931; Ministry of Health, 1934; Parker, 1952).

In his intuitive study of a hospital-ward outbreak, Eastwood (1955) came to the conviction that fomites were not operative. Rather, aerial spread through droplet-infected dust impelled by moving the patient's beddings and clothings was the only plausible explanation for the observed spatial distribution of spread. Dixon (1962*b*), in reviewing a vast amount of published evidence on patient nursing and corpse handling, is also inclined to incriminate the infected dust rather than the body or corpse itself. In a recent study of two funeral-associated outbreaks of smallpox, Hopkins, Lane, Cummings & Millar (1971) found a 100% attack rate among corpse washers. Yet, Hopkins *et al.* also pointed out evidence suggesting that transmission might well have occurred before corpse handling. From a review of the literature, the impression is obtained that the evidence incriminating fomites is only circumstantial and that no conclusive demonstration has been made.

REFERENCES

ANDERSON, T., FOULIS, M. A., GRIST, N. R. & LANDSMAN, J. B. (1951). Clinical and laboratory observations in a smallpox outbreak. *Lancet* i, 1248.

ANGULO, J. J. & DE SALLES-GOMES, L. F. (1967). The mechanism of spread of variola minor in a hospital ward. *Hospital, Rio de Janeiro* **71**, 1037.

ANGULO, J. J., RODRIGUES-DA-SILVA, G. & RABELLO, S. I. (1964). Variola minor in a primary school. *Public Health Reports, Washington* **79**, 355.

ANGULO, J. J., RODRIGUES-DA-SILVA, G. & RABELLO, S. I. (1967). Spread of variola minor in households. *American Journal of Epidemiology* **86**, 479.

CLARK, W. G., SEILER, H. E., JOE, A., GAMMIE, J. L., TAIT, H. P. & JACK, R. P. (1944). *The Edinburgh Outbreak of Smallpox*, 1942, p. 17. Edinburgh, City of Edinburgh Public Health Department.

CORBIN, H. E. (1915). Cited by Downie, A. W. & McDonald, A. (1953). Smallpox and related virus infection in man. *British Medical Bulletin* **9**, 191.

DE JONGH, M. (1956). The alastrim epidemic in The Hague, 1953–1954. *Documenta de Medicina Geographica et Tropica* **8**, 207.

DE SALLES-GOMES, L. F., ANGULO, J. J., MENEZES, E. & ZAMITH, V. A. (1965). Clinical and subclinical variola minor in a ward outbreak. *Journal of Hygiene* **63**, 49.

DIXON, C. W. (1948). Smallpox in Tripolitania, 1946: an epidemiological and clinical study of 500 cases including trials of penicillin treatment. *Journal of Hygiene* **46**, 351.

DIXON, C. W. (1962*a*). *Smallpox*, p. 298. London: J. and A. Churchill Ltd.

DIXON, C. W. (1962*b*). *Smallpox*, pp. 300–302, 395–397. London: J. and A. Churchill Ltd.

EASTWOOD, J. (1955). Smallpox in a general hospital, 1953; a review of administration and clinical problems. *Medical Officer* **94**, 365.

HOPKINS, D. R., LANE, J. M., CUMMINGS, E. C. & MILLAR, J. D. (1971). Two funeral-associated smallpox outbreaks in Sierra Leone. *American Journal of Epidemiology* **94**, 341.

KLAUBER, M. R. (1971). Two-sample randomization tests for space-time clustering. *Biometrics* **27**, 129.

KLAUBER, M. R. & ANGULO, J. J. (1974). Variola minor in Bragança Paulista County, 1956: space-time interactions among variola-minor cases in two elementary schools. *American Journal of Epidemiology* **99**, 65–74.

KNOX, G. (1963). Detection of low level epidemicity. *British Journal of Preventive and Social Medicine* **17**, 121.

KNOX, G. (1964). Epidemiology of childhood leukaemia in Northumberland and Durham. *British Journal of Preventive and Social Medicine* **18**, 17.

MANTEL, N. (1967). The detection of disease clustering and a generalized regression approach. *Cancer Research* **27**, 209.

MANTEL, N. & HAENSZEL, W. (1963). Statistical aspects of data from retrospective studies of disease. *Journal of the National Cancer Institute* **22**, 714.

MINISTRY OF HEALTH (1934). (Cited by Downie, A. W. & McDonald, A. (1953). Smallpox and related virus infections in man. *British Medical Bulletin* **9**, 191.)

PARKER, W. S. (1952). Smallpox; the first few days of the Brighton outbreak, 1950. *Journal of the Royal Sanitary Institute* **72**, 105.

STALLYBRASS, C. O. (1931). *The principles of epidemiology and the process of infection*, pp. 329, 338. London: Routledge & Sons.

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Studies on the pathogenicity of *Acholeplasma axanthum* in swine

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SUMMARY

Acholeplasma axanthum sp. was isolated from the lung of swine with catarrhal pneumonia. Clinical symptoms of respiratory disease, gross and histological lesions of pneumonia, as well as serological response were produced by intranasal inoculation of 'miniature pigs' with the supernatant of lung suspension containing *Acholeplasma axanthum* and by a 48 hr. broth culture of the strain.

A similar picture of disease was observed in animals held in contact with the animals inoculated with untreated lung suspension. *Acholeplasma axanthum* was isolated from the nasal cavity, lung and peribronchial lymph nodes 7–41 days after inoculation. No lesions were observed after inoculation of pigs with the supernatant of lung suspension pretreated with oxytetracycline or chloroform, and no successful isolation of *Acholeplasma axanthum* could be achieved after this treatment.

INTRODUCTION

Up to the present the following, still existing *Mycoplasma* and *Acholeplasma* species have been grown from swine: *A. granularum* (Switzer, 1953), *M. hyorhinis* (Switzer, 1955), *M. hyopneumoniae* (*suipneumoniae*) (Mare & Switzer, 1965; Goodwin, Pomeroy & Whittlestone, 1965, 1967), *M. gallinarum* and *M. iners* (Taylor-Robinson & Dinter, 1968), *A. laidlawii* (Taylor-Robinson & Dinter, 1968; Stipkovits, Schimmel & Varga, 1973), *M. hyosynoviae* (Ross & Karmon, 1970), *M. flocculare* (Meyling & Friis, 1972) and *A. axanthum* (Stipkovits, Varga & Schimmel, 1973).

Among these species *M. hyopneumoniae* (*suipneumoniae*) (Goodwin *et al.* 1965), *M. hyorhinis* (Gois, Valicek & Sovadina, 1968), *M. hyosynoviae* (Ross & Duncan, 1970) and *A. laidlawii* (Dzu *et al.* 1971) proved to be pathogenic.

This paper presents data about the recent isolation and studies on the pathogenicity of *Acholeplasma axanthum*.

MATERIALS AND METHODS

Animals

Four- to six-month-old specific pathogen free (SPF) miniature pigs originating from Minnesota and reared on the farm of our institute were used in the experiments.

Artificial infection

A 10 % suspension prepared from the lung of a conventional pig suffering from catarrhal pneumonia and originating from a farm heavily infected with enzootic pneumonia was used for intranasal inoculation of animals as follows: four animals (nos. 6–9) were given 3 ml. supernatant of untreated lung suspension, two pigs (nos. 16, 17) received 3 ml. suspension pretreated with oxytetracycline (2000 μg./ml.) for 1 hr. at 37° C., two animals (nos. 14, 15) were injected with 3 ml. suspension pretreated with 20 % chloroform for 1 hr., two pigs (nos. 10, 11) were infected with 3 ml. of 5-times cloned 48 hr. broth culture of *A. axanthum* (no. 112/BA3-sc 5/B3) (3×10^6 CFU), which had been isolated from the lung suspension mentioned above. Two animals (nos. 12, 13) were kept in close contact with animals nos. 6–9 injected with untreated supernatant of lung suspension and two animals (nos. 18, 19) were left as controls. Animals were examined for clinical symptoms periodically and for the presence of mycoplasmas in the nasal cavity 1–2 weeks after injection. Experimental pigs were killed at various times after inoculation. Post-mortem examination, including histological studies using haemalum-eosin, resorcin-fuchsin staining and Van Gieson's and Gömöri's techniques was performed, as well as mycoplasma isolation from the nasal cavity, lung and peribronchial lymph nodes.

Examination for mycoplasma and acholeplasma

Nasal swabs and various dilutions of lung and lymph node suspensions were inoculated into liquid media described by Hayflick (1965) and by Goodwin *et al.* (1965) for cultivating at least 3 weeks. Growth of micro-organisms was checked by periodical plating. Isolates were cloned 3 times and examined biochemically (Stipkovits *et al.* 1973) and studied in growth inhibition test using the following antisera: *A. granularum* (Friend), *M. hyorhinis* (PG29), *M. hyosynoviae* (AMRC/C104), *M. hyopneumoniae* (NCTC 10127), *M. gallinarum* (PG16), *M. mycoides* var. *mycoides* (PG1), *M. bovigenitalium* (PG11), *M. bovirhinis* (PG43), *M. agalactiae* var. *agalactiae* (PG2), *M. agalactiae* var. *bovis* (Donetta), *A. modicum* (Squire PG49), *M. alkalescens* (PG51), group 7 (Leach PG50), group L (Al-Aubaidi, B 144P), *M. gateae* (CS), *A. axanthum* (ATCC 25176), *M. mycoides* var. *capri* (PG3), *M. pneumoniae* (MAC), *M. fermentans* (PG18), *M. arthritidis* (PG6), *M. hominis* (PG21), *M. orale* I (CH 19299), *M. orale* II (CH 20247), *M. salivarium* (PG20), *M. gallisepticum* (PG31).

Serological examination

Serum samples collected from animals before and 3 weeks after inoculation (except animals nos. 6 and 7, from which serum was obtained 1 and 2 weeks after inoculation respectively) were examined by the indirect haemagglutination test (Stipkovits, 1964). *Acholeplasma axanthum* culture was sedimented, the sediment was washed three times at 10,000 *g* for 40 min. in PBS and diluted to one-twentieth of the original volume of broth culture. The antigen was treated ultrasonically for 5 min. (15 amp. MSE. u.P.U.) and used for sensitization of trypsin-treated chicken erythrocytes. The serum samples were adsorbed with chicken erythrocytes before testing.

RESULTS

Clinical observations

Animals (nos. 6–9) inoculated with untreated supernatant of lung suspension and with broth culture of *Acholeplasma axanthum* (nos. 10, 11), as well as pigs (nos. 12, 13) kept in contact with infected animals, showed temperature elevation of 1·0–1·4° C. during 2–3 days and very slight clinical symptoms of respiratory disease (deep breathing, coughing) after 1–3 weeks. None died. There were no clinical changes in animals inoculated with the supernatant treated with oxytetracycline (nos. 16, 17) or chloroform (nos. 14, 15), or in the control pigs (nos. 18, 19).

Gross lesions

In our experiment no lesions were found in animals nos. 6 and 7 killed 1–2 weeks after inoculation. In animals inoculated with untreated supernatant of lung suspension and slaughtered 3–4 weeks after injection (nos. 8, 9) catarrhal pneumonia was observed. Similar lesions were found in pigs nos. 10 and 11 inoculated with *A. axanthum* broth culture and in the contact animals (nos. 12, 13), but in the former macroscopic lesions were slight. Animals inoculated with treated supernatant of lung suspension (nos. 14–17) did not show any alterations in the lung. All other organs examined were free from lesions.

Histological picture

In the lungs of animals (nos. 6–13) inoculated with untreated supernatant of lung suspension (nos. 6–9) and broth culture of *A. axanthum* (nos. 10, 11) and of pigs in contact with infected animals (nos. 12, 13), histological lesions of the interstitium (peribronchial lymphoid hyperplasia, peribronchiolitis, perivasculitis, intralobular interstitial pneumonia) and epithelium of bronchioli and alveoli (vacuolization, necrosis, desquamation of cells, proliferation of alveolar cells, alveolar macrophage reaction) were demonstrated (Table 1, Plates 1–3).

Lesions produced by pure culture of *A. axanthum* were not as severe as those in animals infected with untreated supernatant.

There were no lesions of the bronchiolar and alveolar wall in animals inoculated with oxytetracycline- and chloroform-treated supernatant of lung suspension, or in the control pigs. Very slight alterations of the interstitium were observed in

Table 1. *Histological lesions in the lung of pigs inoculated with untreated and treated lung suspensions and with* Acholeplasma axanthum *broth cultures intranasally*

		Lesions of the interstitium				Lesions of epithelium of bronchioli				Lesions of alveoli			
Designation of animals	Gross lesions in the lung	Peribronch. lymph. hyperplasia	Peribronchiolitis	Perivasculitis	Intralobul. interst. pneum.	Vacuolization	Necrosis	Desquamation	Proliferation	Alveolar cell proliferation	Alveolar macrophage	Neutrophyl granulocyta	Exsudate in alveoli
6	0	1	2	2	2	3	0	1	0	1	0	0	0
7	0	1	1	1	1	3	0	2	1	1	0	0	0
8	2	2	3	3	2	2	2	3	0	1	1	2	1
9	3	3	3	3	2	1	3	3	1	3	3	2	1
12	2	1	2	2	1	2	1	2	2	3	3	3	1
13	2	1	2	2	1	2	3	1	2	2	2	2	1
10	2	1	3	2	1	3	0	1	1	1	1	0	0
11	1	1	3	2	2	1	2	2	0	1	1	0	0
14	0	0	1	0	2	0	0	0	0	0	0	0	0
15	0	0	1	1	1	0	0	0	0	0	0	0	0
16	0	0	0	0	2	0	0	0	0	0	0	0	0
17	0	0	0	0	1	0	0	0	0	0	0	0	0
18	0	0	1	0	2	0	0	0	0	0	0	0	0
19	0	0	1	0	1	0	0	0	0	0	0	0	0

Notes: 0–3 = degree of severity and extension of lesions.

Table 2. *Isolation of* Acholeplasma axanthum *from artificially infected pigs, and their serological response*

Method of infection	Pig no.	*Acholeplasma* isolation: Before inoc.	*Acholeplasma* isolation: After inoculation: 1 week	*Acholeplasma* isolation: After inoculation: 2 weeks	Time killed after inoc. (days)	*Acholeplasma* isolation after death from: Nasal cavity	Lung	Peribronch. lymph nodes	Serum titres
Intranasal untreated lung suspension	6	—	—	—	7	—	—	—	—
	7	—	axan.	—	14	axan.	—	—	—
	8	—	axan.	axan.	21	axan.	axan.	axan.	1/4
	9	—	—	laid.	28	axan.	axan.	—	1/64
Contact animals	12	—	axan.	axan.	36	—	axan.	—	1/4
	13	—	—	—	36	—	axan.	—	1/16
Intranasal cloned broth culture of *A. axanthum*	10	laid.	—	axan.	41	—	axan.	—	1/32
	11	—	—	—	41	—	axan.	axan.	1/16
Intranasal chloroform-treated lung suspension	14	—	—	laid.	43	—	—	—	—
	15	—	laid.	—	29	laid.	—	—	—
Intranasal oxytetracycline-treated lung suspension	16	—	—	laid.	30	—	—	—	—
	17	—	—	laid.	43	—	—	—	—
Control animals	18	—	—	laid.	57	—	—	—	—
	19	—	—	—	57	laid.	—	—	—

axan. = *A. axanthum*; laid. = *A. laidlawii*.

the control animals and in pigs inoculated with oxytetracycline- and chloroform-treated material, but these lesions were not as severe as those in animals receiving material containing live *A. axanthum*.

Isolation of Acholeplasma axanthum

From the lung suspension used for inoculation only *A. axanthum* sp. was isolated. The examination of the suspension for the presence of other *Acholeplasma* and *Mycoplasma* species and for bacteria gave negative results. Virus isolation performed in swine kidney tissue cultures during four blind passages also gave negative result. Data of reisolations of *A. axanthum* strains from inoculated animals are shown in Table 2.

Acholeplasma axanthum could be isolated from most of the samples obtained from animals injected with untreated supernatant of the lung suspensions and broth cultures of *A. axanthum*, as well as from the contact pigs, whereas this species was not present in other animals. Some of the experimental animals were contaminated with *Acholeplasma laidlawii* strains identical with saprophytic *A. laidlawii* reference strains in the biochemical tests (Stipkovits, Schimmel & Varga, 1973). No other *Mycoplasma* and *Acholeplasma* species were detected, although the technique used was suitable for detecting all *Mycoplasma* and *Acholeplasma* species of swine (including *M. hyopneumoniae*).

Serological examination

A serological response was seen in animals inoculated with material containing live *A. axanthum* and in the contact animals, but not in the pigs nos. 14–19 (Table 2).

DISCUSSION

The present paper demonstrates another successful isolation of *Acholeplasma axanthum* from the lung and peribronchial lymph nodes of a pig from a farm with catarrhal pneumonia (Stipkovits, Varga & Schimmel, 1973). By inoculation of animals with untreated supernatant of lung suspension containing *A. axanthum* mild clinical symptoms, definite gross lesions and histological changes of pneumonia were produced. Lesions of the same type were found in the lung of animals held in contact with pigs inoculated with untreated supernatant of lung suspension, confirming the ability of *A. axanthum* to spread by contact. Microscopic and macroscopic lesions which developed in the lung after infection of animals with broth culture of *A. axanthum* were similar to, but weaker than, those of contact animals. This observation could be explained by the possibility that the pathogenicity of *A. axanthum* might have decreased during the passages performed in artificial medium; thus it could produce slighter lesions only.

The negative results of inoculating animals with oxytetracycline- and chloroform-treated material are due to the killing effect of these substances on *Mycoplasma* and *Acholeplasma* species as demonstrated by Stipkovits, Schimmel, Molnár & Somos (1971) and Bögel, Berchthold, Brunner & Klinger (1962).

On the basis of clinical symptoms of respiratory disease, and pathological lesions in the lung of inoculated animals, the presence of *A. axanthum* in organs of pigs and their serological response to *A. axanthum* present in the material used for inoculation, a pathogenic role of this species might be supposed in the aetiology of pneumonia in swine. Specific pathogen free miniature pigs seem to be sensitive for the demonstration of such a pathogenic effect, although the animals are contaminated by saprophytic *A. laidlawii* species.

REFERENCES

Bögel, K., Berchthold, M., Brunner, H. & Klinger, L. (1962). Suitability of tissue culture techniques for the isolation and characterization of a cytopathic mycoplasma strain in the bull. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* (Abt. I, Orig.) **185**, 423–39.

Dzu, N. M., Pustovar, A., Bathke, W., Hubrig, Th., Krausse, H. & Schimmel, D. (1971). Zur Ätiologie und Diagnose der Mykoplasmose der Schweine. I. Experimentelle Infektionen mit Mykoplasmen und Pasteurellen. *Monatshefte für Veterinärmedizin* **26**, 169–81.

Gois, M., Valicek, L. & Sovadina, M. (1968). *Mycoplasma hyorhinis*, a causative agent of pig pneumonia. *Zentralblatt für Veterinärmedizin*. Reiche B **15**, 230–40.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1965). Production of enzootic pneumonia in pigs with a mycoplasma. *Veterinary Record* **77**, 1247–9.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1967). Characterization of *Mycoplasma suipneumoniae*: A mycoplasma causing enzootic pneumonia in pigs. *Journal of Hygiene* **65**, 85–96.

Hayflick, L. (1965). Tissue cultures and mycoplasmas. *Texas Report of Biology and Medicine* **23** (Supplement 1), 285–303.

Mare, C. J. & Switzer, W. P. (1965). New species: *Mycoplasma hyopneumoniae*. A causative agent of virus pig pneumonia. *Veterinary Medicine* **60**, 841.

Meyling, A. & Friis, N. F. (1972). Serological identification of a new porcine mycoplasma species, *Mycoplasma flocculare*. *Acta Veterinaria Scandinavica* **13**, 287–9.

Ross, R. F. & Duncan, J. R. (1970). *Mycoplasma hyosynoviae* arthritis of swine. *Journal of the American Veterinary Medical Association* **157**, 1515–18.

Ross, R. F. & Karmon, J. A. (1970). Heterogeneity among strains of *Mycoplasma granularum* and identification of *Mycoplasma hyosynoviae* sp.n. *Journal of Bacteriology* **103**, 707–13.

Stipkovits, L. (1964). Indirect haemagglutination test for chickens infected artificially with *Escherichia coli*. *Avian Diseases* **8**, 637–48.

Stipkovits, L., Schimmel, D., Molnár, L. & Somos, P. (1971). Sertésböl és szarvasmarhából izolált mycoplasma törzsek antibiotikum érzékenysége. (Antibiotic sensitivity of mycoplasma strains isolated from swine and cattle.) *Magyar Állatorvosok Lapja* **26**, 516–21.

Stipkovits, L., Varga, L. & Schimmel, D. (1973). Isolation of *Acholeplasma axanthum* from swine. *Acta Veterinaria Academiae Scientiarum Hungaricae* (in the Press).

Stipkovits, L., Schimmel, D. & Varga, L. (1973). Studies on *Acholeplasma* strains isolated from swine. *Acta Veterinaria Academiae Scientiarum Hungaricae* (in the Press).

Switzer, W. P. (1953). Studies on infectious atrophic rhinitis of swine. II. Intraperitoneal and intranasal inoculation of young pigs with a filterable agent isolated from nasal mucosa of swine. *Veterinary Medicine* **48**, 392.

Switzer, W. P. (1955). Studies on infectious atrophic rhinitis. IV. Characterization of pleuropneumonia-like organisms isolated from the nasal cavities of swine. *American Journal of Veterinary Research* **16**, 540–44.

Taylor-Robinson, D. & Dinter, Z. (1968). Unexpected serotypes of mycoplasma isolated from pigs. *Journal of General Microbiology* **53**, 221–9.

EXPLANATION OF PLATES

All sections are stained with H.E.

PLATE 1

Fig. 1. Peribronchiolar lymphocytic infiltration. Magnification, × 54. Pig no. 10.

Fig. 2. Perivascular lymphohistiocytic infiltration. Magnification, × 346. Pig no. 11.

Fig. 3. Thickening of the alveolar wall due to histiolymphocytic infiltration (intralobular interstitial pneumonia). Magnification, × 139. Pig no. 11.

PLATE 2

Fig. 4. Vacuolization of bronchiolar epithelial cells. Magnification, × 1350. Pig no. 10.

Fig. 5. Severe infiltration in the propria of bronchiolar mucosa (see below). Epithelium lost definition over the infiltrated area. Magnification, × 346. Pig no. 12.

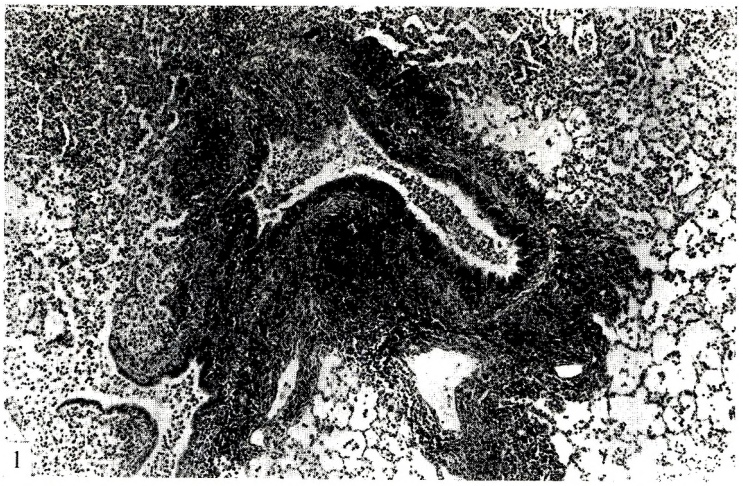
Fig. 6. Bronchiolar epithelium is desquamated, the propria is infiltrated with lymphocytes and histiocytes coming from the peribronchiolar connective tissue (arrow). Magnification, × 346. Pig no. 7.

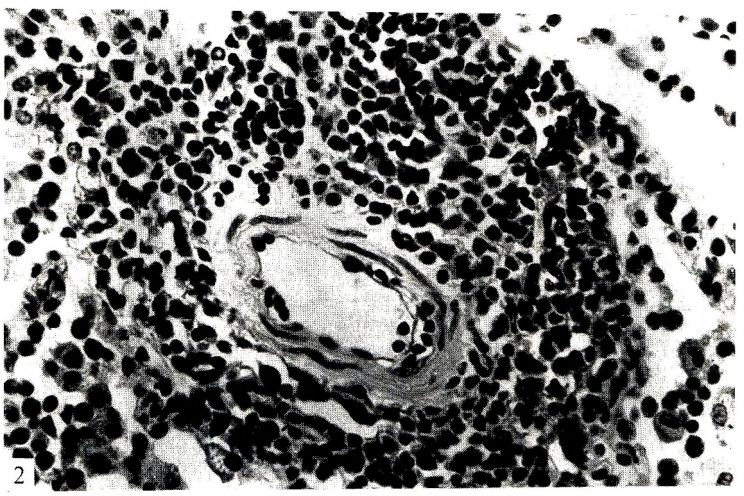
PLATE 3

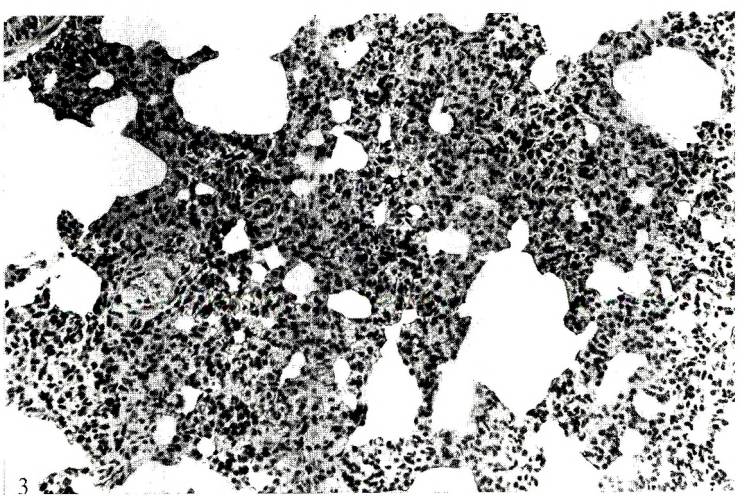
Fig. 7. Vacuolized alveolar cells. Magnification, × 1350. Pig no. 8.

Fig. 8. Desquamated, non-vacuolized alveolar cells in the alveolar lumen. Magnification, × 1350. Pig no. 9.

Fig. 9. Alveolar macrophages, lymphocytes and neutrophil granulocytes in the alveolar lumen. Magnification, × 1350. Pig no. 8.







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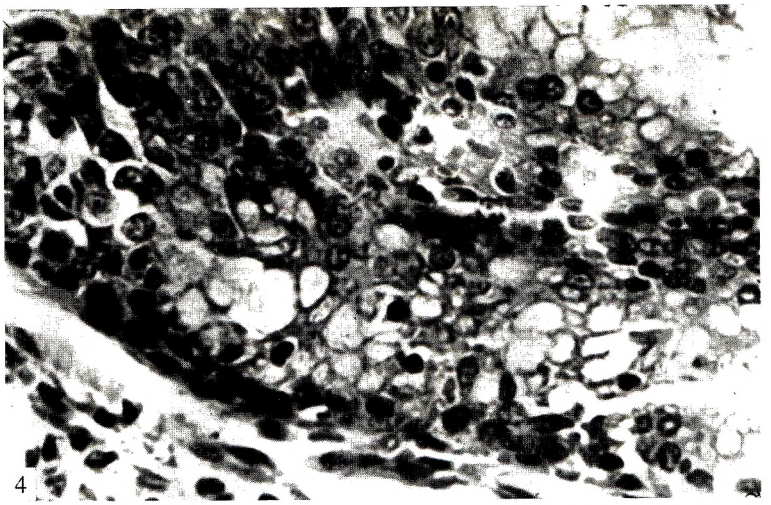
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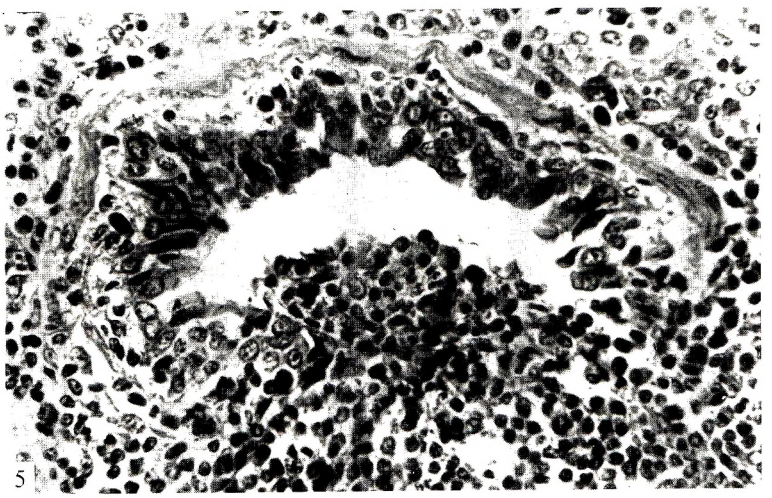
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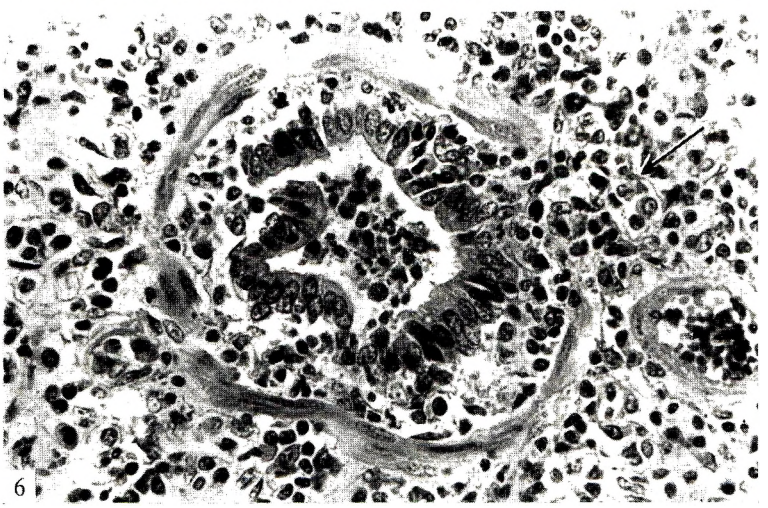
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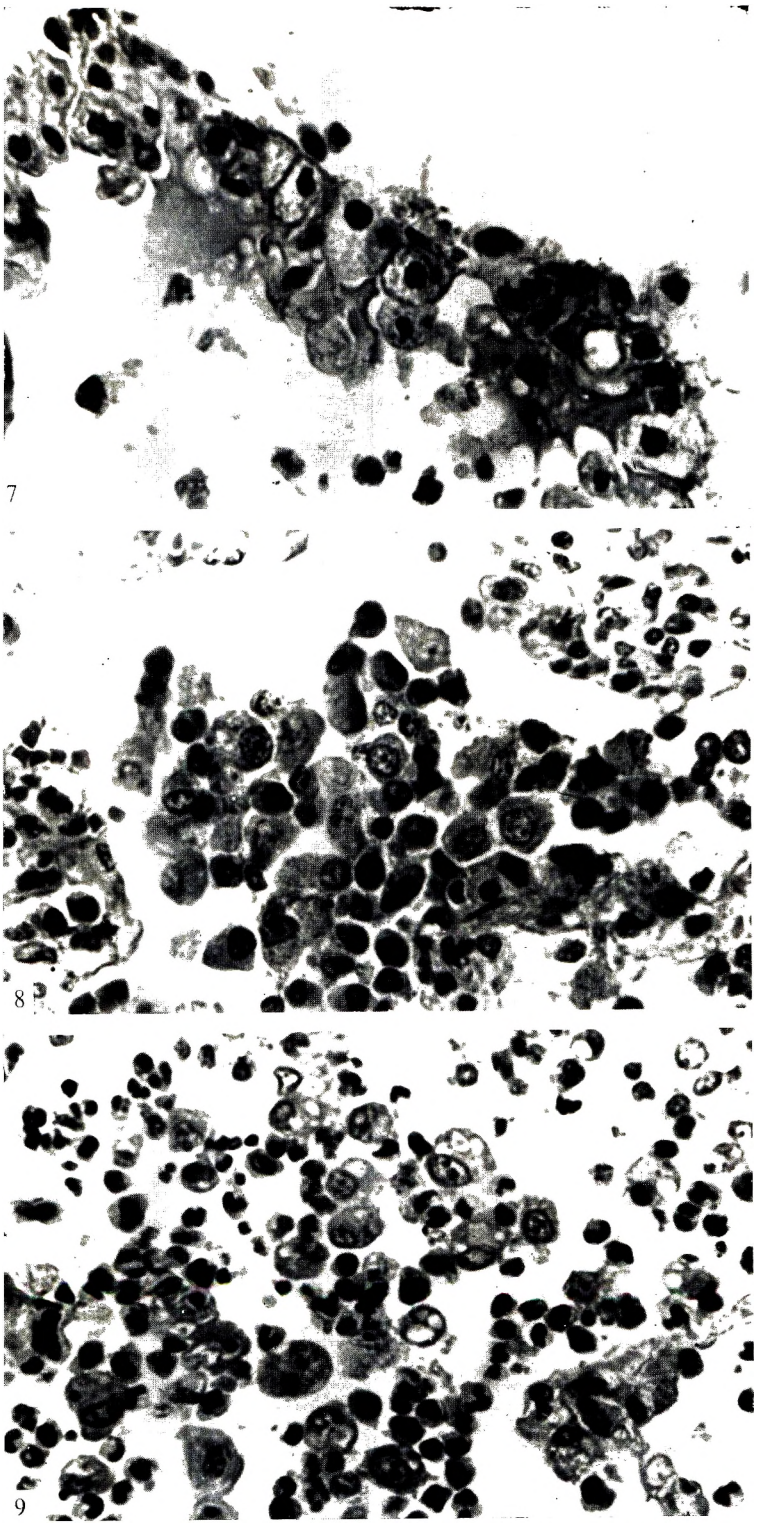
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Interactions of TRIC agents with macrophages: effects on lysosomal enzymes of the cell

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SUMMARY

Two changes were observed in the acid phosphatase of macrophages that had ingested infective TRIC organisms: the proportion of extralysosomal enzyme rose, while the total amount in the cells fell. Both effects were directly related to the number of organisms ingested and increased with time. When macrophages were inoculated with more than 50 organisms per cell, changes were obvious within a few hours; with 2–10 organisms per cell changes were detectable only after 18 hr. or more. Enzyme appeared in the culture medium as the amount in the cells decreased. Ingestion of organisms killed by heat or treated with antibody did not induce such changes. In infected BHK-21 cells, no changes in acid phosphatase were detected at any stage of the developmental cycle of the organism.

INTRODUCTION

In people suffering from trachoma, damage to the epithelial cells of the conjunctiva is often severe and it may result, at least in part, from pathological changes in macrophages in the exudate (Taverne & Blyth, 1971). Furthermore, intravenous injection of chlamydiae into mice causes toxic and sometimes lethal reactions that are associated with rapid injury to the reticulo-endothelial system, in particular to macrophages (Schoenholtz, 1962).

Macrophages inoculated *in vitro* with the agents of trachoma or inclusion conjunctivitis (TRIC agents) undergo a cytotoxic reaction and die. Vacuolation was observed in the cytoplasm of the cells before changes in the permeability of the cell membrane were detected (Taverne & Blyth, 1971). Similar effects caused by bacterial endotoxins have been related to their action on lysosomes, with the subsequent release of lysosomal enzymes (Weissmann & Thomas, 1962). Thus it seemed likely that the toxic activity of TRIC agents might also be mediated through the cell's lysosomes, particularly in view of the contrast observed by electron microscopy between events occurring in macrophages and those in

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BHK-21 cells that have ingested TRIC agents (Lawn, Blyth & Taverne, 1973); in macrophages the organisms entered lysosomes; but in BHK-21 cells, in which they multiply without inducing cytotoxic effects, the organisms developed outside the lysosomal system.

We therefore tested the hypothesis that the ingestion of TRIC organisms by macrophages is followed by alterations in the activity of lysosomal enzymes in the cells; and using acid phosphatase as an indicator, we investigated the possibility that such changes might be used as the basis of a quantitative test for the toxicity of TRIC organisms for macrophages.

METHODS

Methods for the culture of BHK-21 cells and macrophages, and for the preparation of suspensions of TRIC agents grown in yolk sacs and their titration in BHK-21 cells, have been described in detail (Taverne & Blyth, 1971).

TRIC agents

The fast-killing variant (Reeve & Taverne, 1963) of strain TRIC/2/SAU/HAR-2/OT (Murray *et al.* 1960) was used as suspensions made either from infected yolk sacs or from infected BHK-21 cells. The latter were prepared by disrupting cells 42–46 hr. after infection by shaking them with glass beads or by sonication in an ultrasonic cleaning bath. In experiments with cockerel antiserum, the fast-killing variant of MRC-4 (Jones, 1961) was used.

Macrophage cultures

Peritoneal exudate cells from CS1 mice which had not received peritoneal irritants were seeded into flat-bottomed plastic tubes (Conway tubes, Turner-Stayne Laboratories, Bishop Auckland, Co. Durham). Glass coverslips were included only when examination under the microscope was required. Cultures were used after overnight incubation at 35° C. in Eagle's MEM medium (Wellcome Reagents Ltd.) containing 40 % horse serum in an atmosphere of 5 % CO_2 in air. They usually then contained $2–5 \times 10^5$ cells per tube.

Inoculation of cultures

The culture medium was replaced with 1 ml. of Eagle's MEM medium supplemented with 10 % horse serum (MM) containing the required number of TRIC organisms; the tubes were centrifuged at 600 ***g*** for 30 min. at 37° C. The medium was then replaced with fresh MM.

Antiserum against TRIC agents

A cockerel was given six intraperitoneal injections at weekly intervals of a suspension of MRC-4*f*, each equivalent to one infected yolk sac. It was bled 8 days after the last injection.

Measurement of acid phosphatase activity in cell cultures

To determine the total amount of acid phosphatase (ACPase) activity in the cells, the medium was replaced with 2 ml. of 0·05 M citrate buffer pH 5·0 containing 0·003 M *p*-nitrophenol phosphate (Sigma Corporation) and 0·05 % Triton X 100 (Sigma Corporation). To determine the amount of extralysosomal ACPase, the medium in replicate cultures was replaced with 2 ml. of substrate in buffer without Triton X 100. The tubes were then incubated at 37° C. for 30 min. after which the enzyme reaction was stopped by adding 2 ml. N-NaOH. This also lysed the cells so that the *p*-nitrophenol formed by the action of the enzyme was released into the supernatant fluid; its optical density (OD) was measured at 405 nm. All tests were done on groups of three replicate cultures and activities were expressed in terms of mean OD 405. In some experiments, the culture medium was transferred to clean tubes and assays of the total and extralysosomal ACPase activity were done as above.

Measurements of ACPase activity in yolk-sac suspensions

Duplicate volumes of suspension were incubated with *p*-nitrophenol phosphate in citrate buffer containing Triton X 100 and the reaction was stopped with NaOH as above; OD 405 readings were made against a blank containing an equivalent amount of yolk-sac material, prepared by mixing 2 ml. substrate in buffer containing Triton X 100 with 2 ml. NaOH and then adding 1 ml. of the yolk-sac suspension.

β-Glucuronidase assays

Since mouse macrophages contain 10 times less β-glucuronidase than ACPase, overnight cultures in small plastic flasks (Falcon Plastics Ltd.) were used. The medium from duplicate flasks, each containing 3×10^6 macrophages, was replaced with 2 ml. of 0·001 M *p*-nitrophenol glucuronide (Sigma Corporation) in 0·05 M citrate buffer pH 5·0 or 2 ml. of substrate in buffer containing 0·05 % Triton X 100. The flasks were incubated at 37° C. for 1 hr., the reaction was stopped with 2 ml. of 0·1 M glycine in 0·1 M-NaCl at pH 10·0 and OD 405 was determined.

RESULTS

Previously we demonstrated that macrophages inoculated with TRIC organisms underwent a cytopathic effect, the severity of which depended both on the number of infective organisms ingested by each cell and the time after inoculation at which the cultures were examined (Taverne & Blyth, 1971). To relate these observations to lysosomal changes, groups of cultures were inoculated with various numbers of organisms per cell and intracellular ACPase activity was measured immediately before and at intervals after centrifugation. In control cultures, that had received either various concentrations of a normal yolk-sac (NYS) suspension or fresh culture medium, the total amount of ACPase per 10^6 cells gave a mean OD 405 of 2·99 (S.E. ±0·14); it did not alter significantly during

Table 1. *Changes in ACPase activity in macrophages inoculated with TRIC organisms*

Hours after end of centrifugation	Control cultures: Total* ACPase	Control cultures: Extra-lysosomal ACPase†	Inoculated cultures: Infective organisms per cell	Inoculated cultures: Total* ACPase	Inoculated cultures: Extralysosomal ACPase†
Before centrifugation	0·933	0·067		—	—
0	1·037	0·098		0·887	0·205
1½	—	—		0·707	0·356
3	1·006	0·095	200	0·564	0·292
4½	—	—		0·511	0·218
6	1·111	0·106		0·415	0·194
0	1·050	0·065		0·893	0·069
3	—	—	50	0·860	0·069
5	—	—		0·756	0·219
0	0·620	0·117		0·632	0·138
6	—	—		0·530	0·088
12	0·558	0·113	10	0·394	0·108
18	—	—		0·346	0·088
24	0·781	0·093		0·412	0·076

* Mean OD405: assayed in presence of Triton X100.

† Mean OD405: assayed in absence of Triton X100.

In the experiment with 200 organisms per cell, control cultures received an equivalent concentration of normal yolk-sac suspension; otherwise the organisms used were grown in BHK-21 cells and control cultures were incubated with fresh medium.

experiments lasting 6 hr., although over longer periods it sometimes showed a slight increase. In these control cultures the average proportion of enzyme outside the lysosomes was 10·5 % (S.E. ±0·74) of the total; a rise to 25 % or more was taken as a sign that the cultures were unhealthy and results from experiments in which they were used were ignored.

Similar measurements with infected cultures revealed that the total amount of ACPase in the cells diminished during the 24 hr. period of observation after infection; the more organisms per cell, the sooner the diminution was observed. The results of representative experiments with three different multiplicities of infection are given (Table 1).

When the total amount of enzyme present in inoculated cells was expressed as a percentage of that in control cultures at the time of inoculation (or, in longer experiments, in control cells at the time of sampling) the loss of enzyme observed with time was clearly demonstrated. The rate of loss depended on the multiplicity of infection, as shown for the first 6 hr. after incubation with 50–500 infective organisms per cell (Fig. 1) and during 24 hr. with 2 or 10 organisms per cell (Fig. 2). Accompanying the decreases in the total amount of ACPase, changes in the amount of extralysosomal enzyme were also observed, in that during the first 2–6 hr. after infection there was sometimes more free enzyme present than in control cells (Table 1). These effects were induced by four strains of TRIC agent tested, whether the organisms were cultured in BHK-21 cells or in chick

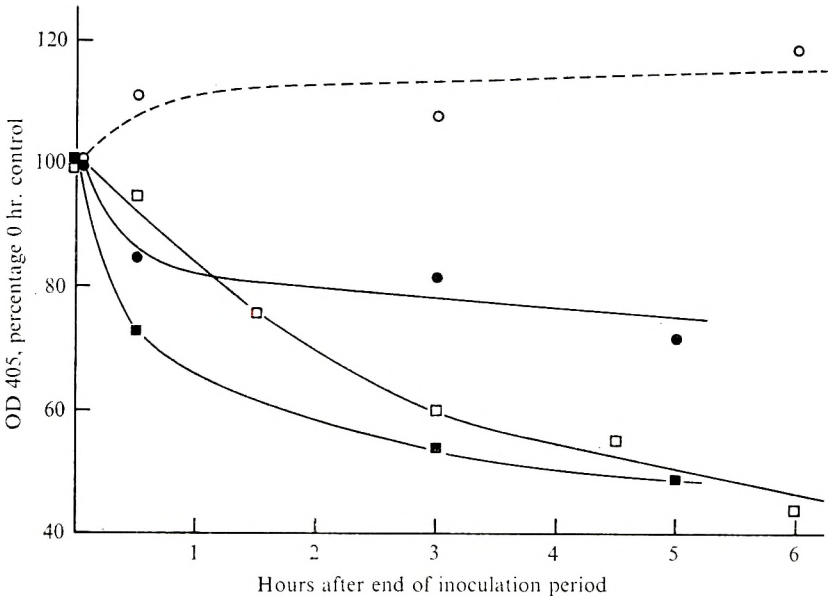

Fig. 1. Decrease of ACPase activity with time, in macrophages inoculated with large numbers of TRIC organisms. ○, Normal yolk sac; ●, 50 organisms per cell; □, 200 organisms per cell; ■, 500 organisms per cell.

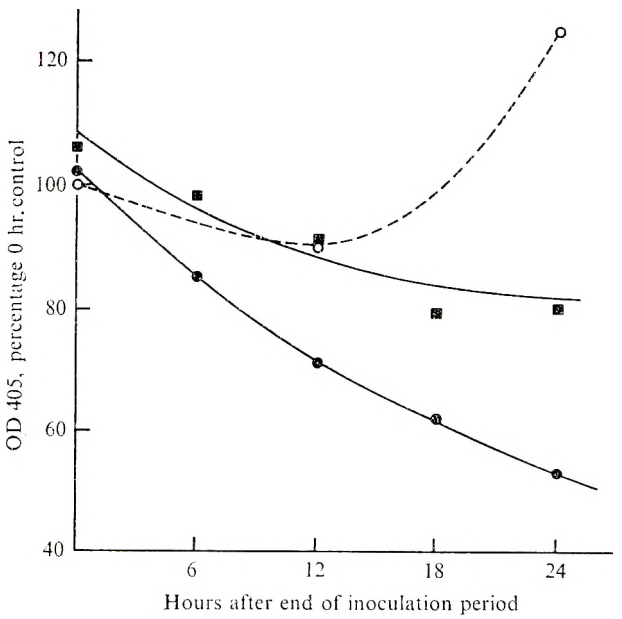

Fig. 2. Decrease of ACPase activity with time, in macrophages inoculated with small numbers of TRIC organisms. ○, Medium only; ■, two organisms per cell; ●, 10 organisms per cell.

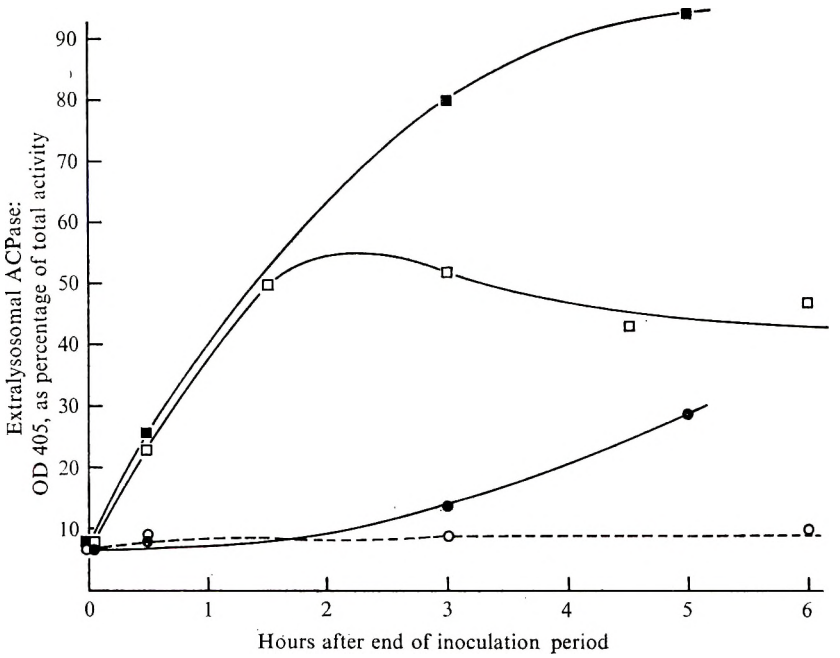

Fig. 3. Increase in extralysosomal ACPase activity with time, in macrophages inoculated with large numbers of TRIC organisms. ○, Normal yolk sac; ●, 50 organisms per cell; □, 200 organisms per cell; ■, 500 organisms per cell.

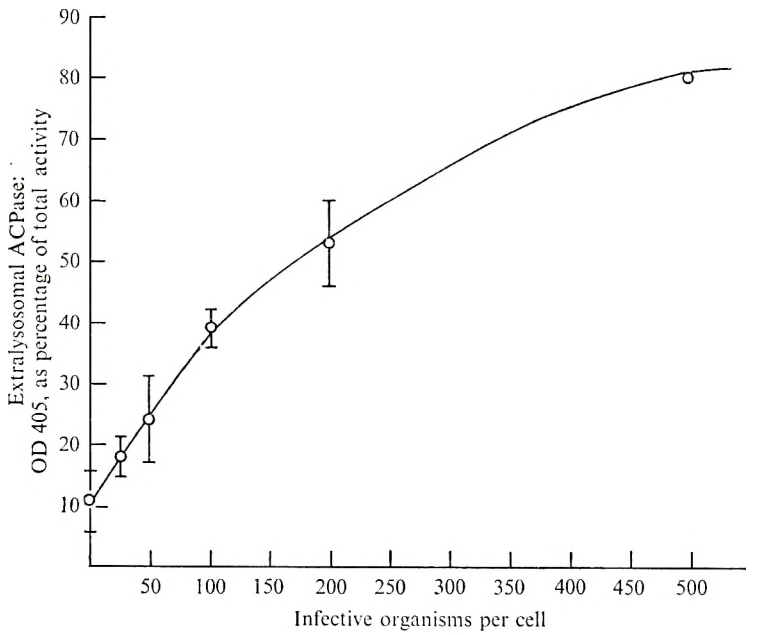

Fig. 4. Increase in extralysosomal ACPase activity with number of TRIC organisms in macrophages 3 hr. after inoculation. ϕ, Mean rate with standard deviations.

embryo yolk sacs. The changes in the amount of extralysosomal enzyme are more obvious when expressed as a percentage of the total activity present in the cells at each time (Fig. 3). With 500 organisms per cell the concentration of free enzyme rose steadily until virtually all the enzyme remaining in the cells was free 5 hr. after centrifugation; with 200 organisms the proportion free reached a maximum of about 50 %, 3 hr. after centrifugation. In cultures inoculated with less than about 50 organisms per cell the proportion free was not significantly different from that in the controls during the first 5 hr. after inoculation, and rarely rose above 30 % of the total during the next 18 hr. Thus at any time from 1 to 3 hr. after inoculation there was a direct relationship between the amount of ACPase released from the lysosomes (but remaining within the cells) and the number of organisms inoculated per cell; this is illustrated for the 3 hr. sample (Fig. 4).

ACPase activity in culture medium

Since it seemed likely that the decrease in the total amount of ACPase in macrophages that had ingested viable TRIC organisms might be due to leakage of the enzyme from the cytoplasm into the extracellular fluid, ACPase activity in the culture medium was measured at various times after the inoculation of different numbers of organisms. Yet again, the enzyme activity showed a relationship between dose and time after inoculation. For instance, with 64 organisms per cell, about 20 % of the total activity in the culture was found in the medium 2 hr. later; after 20 hr. this increased to 40 %, whereas with two organisms per cell, only 16 % was found in the medium 24 hr. after inoculation.

ACPase activity in the culture medium measured in the presence of Triton X 100 was frequently lower than in its absence; indeed on some occasions there was apparently no activity although it was still measurable in the absence of the detergent. When medium from cultures inoculated 2 hr. previously with 200 infective organisms per cell was centrifuged at 8000 ***g*** for 10 min. – conditions in which lysosomes are deposited – about 75 % of the ACPase activity remained in the supernatant fluid.

β-Glucuronidase activity in macrophages

To verify that results obtained with ACPase were representative of other lysosomal enzymes, macrophage cultures were inoculated with 10 TRIC organisms per cell and both β-glucuronidase and ACPase activities were assayed in the presence of Triton X 100 24 hr. later.

As with ACPase, the amount of β-glucuronidase diminished with time, although to a lesser extent: 24 hr. after infection the cells contained 59 % of the β-glucuronidase activity and 27 % of the ACPase activity present at the end of the inoculation period.

Effect on ACPase activity of organisms killed by heat

Heat-killed TRIC organisms are not toxic to mice when injected intravenously, and except in massive doses, are not cytotoxic for macrophages in culture

Table 2. *Protection by specific antiserum against the action of TRIC agents on the acid phosphatase of macrophages*

No. of organisms per cell	Serum	Total ACPase: % of that in uninoculated cells	% extra-lysosomal ACPase
None	Normal	100	5
	Antiserum	83	8
100	Normal	38	36
	Antiserum	82	12
1000	Normal	36	60
	Antiserum	86	21

Organisms were incubated with a 1/4 dilution of serum at 37° C. for 30 min. before inoculation; assays were done 4 hr. later.

(Taverne & Blyth, 1971). Various concentrations of organisms – up to 200 per cell – that had been killed by incubation overnight at 56° C. were centrifuged onto macrophage cultures and ACPase activity was measured at intervals up to 6 hr. after centrifugation. With a dose of 80 or more organisms per cell there was an insignificant decrease in the amount of enzyme present (to a mean of 90 % of control values) and some release of free enzyme (mean 21 % of total), but there was no increasing effect with time.

Protection by specific antiserum

To establish that changes observed in the ACPase of macrophages were caused specifically by the TRIC organisms in the inoculum, suspensions containing 1×10^7 or 1×10^8 infective organisms were incubated with an equal volume of fowl antiserum diluted 1/4 at 37° C. for 30 min. The mixtures were then used to inoculate macrophage cultures at a concentration (based on the original infectivity of the suspension) of 1000 or 100 organisms per cell; ACPase was assayed 4 hr. later (Table 2). In macrophages of control cultures that received 100 organisms per cell the amount of ACPase activity fell to 38 % of that in uninoculated cultures, and 36 % of the activity present was extralysosomal; but in those that received the same dose of organisms incubated with antiserum both the total amount and the proportion free were the same as in uninoculated cells. Even in cultures inoculated with 1000 organisms per cell no significant changes were caused by organisms treated with antibody. The antiserum had a neutralization titre of 1/12 in terms of a 50 % reduction of inclusion count in a neutralization test in BHK-21 cells (Blyth & Taverne, 1974); a 1/3 dilution protected 50 % of mice against the toxic effect of 4 LD50 injected intravenously. Similar results were obtained with an antiserum prepared in mice.

Preliminary experiments were done on the behaviour of macrophages from mice immunized with TRIC organisms by intravenous injection 10 days earlier. Inoculation of these macrophages with infective organisms induced greater changes in both the total amount of ACPase and in the proportion that was

Table 3. *The relative amounts of ACPase activity and infective organisms deposited by centrifugation through various concentrations of sucrose*

Sample	Molarity of sucrose	Centrifugation		ACPase* activity	Infectivity†	OD 405/ 10^6 IFU
		× *g*	min.			
Unpurified yolk sac	None	—	—	585	$2{\cdot}6 \times 10^8$	2·25
	None	8,000	30	156	$3{\cdot}1 \times 10^8$	0·5‡
Suspension of sample marked‡ in 0·25 M sucrose	0·25 M	8,000	20	45	$1{\cdot}2 \times 10^8$	0·36
	0·5	8,000	20	15	$4{\cdot}5 \times 10^7$	0·33
	1·0	8,000	20	9	$3{\cdot}9 \times 10^7$	0·23
	0·25	10,000	60	69	$5{\cdot}1 \times 10^8$	0·14
	0·5	10,000	60	24	$5{\cdot}4 \times 10^8$	0·044
	1·0	10,000	60	3	$2{\cdot}4 \times 10^8$	0·013

* OD 405/ml. adjusted to volume of original sample.
† Inclusion forming units (IFU)/ml. adjusted similarly.

extralysosomal than in cells from unimmunized mice. It thus appeared that these cells were sensitized to the action of the organisms, but, paradoxically, the protection afforded by antiserum was greater with cells from immunized than from normal mice.

ACPase activity in yolk-sac suspensions

In later experiments on changes in the ACPase of macrophages after the ingestion of TRIC organisms the effects reported above were not always reproduced. Inoculation with some infected yolk-sac suspensions was followed by an increase in the total amount of ACPase in the cells, instead of a decrease. Sometimes the amount rose as high as 150 % of the control immediately after centrifugation, and this value was maintained for at least 5 hr. There was also an increase above that expected in the extralysosomal enzyme. Further investigation revealed that the suspensions giving anomalous results themselves contained significant quantities of ACPase.

By chance, the suspensions used earlier had possessed very high infectivity titres ($> 5 \times 10^9$ infective organisms/ml.) so that although they too contained ACPase, it had apparently been diluted out enough not to interfere with measurements of the enzyme activity in macrophages, even when the multiplicity of infection was high. Suspensions of organisms made from infected BHK-21 cells usually contained relatively less ACPase activity than yolk-sac suspensions, and as they also possessed high infectivity titres results obtained with them generally agreed with those of the early experiments. No simple relationship could be found, however, between the amount of activity in a suspension and increase in activity in the macrophage cultures after inoculation with that suspension. Attempts were therefore made to separate the enzyme activity of the suspension from the organisms.

Since the last stage of purification of yolk-sac suspensions was centrifugation at 8000 *g* for 15 min., the enzyme activity must have been sedimented with the organisms. It must therefore still have been attached to lysosomal membranes, although the lysosomes themselves cannot have been intact since more than 90 %

Table 4. *The effect of purified and unpurified TRIC organisms on the ACPase of macrophages inoculated with* 200 *organisms per cell*

Yolk-sac suspension	ACPase activity in inoculum*	Total ACPase in cells†		Extralysosomal ACPase in cells‡	
		0 hr.	4 hr.	0 hr.	4 hr.
Before treatment	136	144	124	56	46
After purification§	5	104	85	27	23

* OD 405 units per ml.

† Total activity in cells measured in presence of Triton X100 expressed as percentage of uninoculated controls.

‡ Activity in absence of Triton X100 expressed as percentage of total activity in those cultures.

§ Centrifugation through 1·0 M sucrose at 10,000 *g* for 60 min.

of the activity was detected by incubation with *p*-nitrophenol phosphate in the absence of Triton X 100. The particles bearing ACPase were, however, separable from infective TRIC organisms by centrifugation through dense solutions of sucrose. To determine the best conditions for separation, an unpurified 20 % suspension of infected yolk sac was centrifuged at 8000 *g* for 30 min. and the pellet was resuspended to the original volume in 0·25 M sucrose in phosphate buffered saline pH 7·4. One ml. volumes of this suspension were layered onto 40 ml. volumes of buffer each containing a different concentration of sucrose and centrifuged at 4° C., either at 8000 *g* for 20 min. or 10,000 *g* for 60 min. The deposits were resuspended in 0·25 M sucrose in buffer and the ACPase activity was assayed and related to the number of infective organisms determined by titration in BHK-21 cells (Table 3).

Although centrifugation of crude yolk-sac material in the absence of sucrose removed much ACPase activity, the higher the concentration of sucrose the greater the separation of ACPase and infectivity achieved. Centrifugation at 8000 *g* through 1·0 M sucrose did not deposit all the organisms, but all were deposited with the least ACPase contamination per infective organism when centrifuged through 1·0 M sucrose at 10,000 *g* for 60 min.

To verify that when ingested by macrophages TRIC organisms purified in this way behaved like organisms from infected cell cultures – or in yolk-sac suspensions of high titre – and did not induce an increase in the total activity of ACPase, macrophage cultures were inoculated with a suspension that had been centrifuged through 1·0 M sucrose for 60 min.; duplicate cultures received the same suspension, untreated, as a control. The multiplicity of infection for the untreated suspension was calculated to be 200 infective organisms per cell and the purified suspension was used at an equivalent dilution. ACPase in the cells was assayed at the end of the inoculation period and 4 hr. later (Table 4). As before, the untreated suspension caused an increase both in total and extra-lysosomal enzyme activity. By contrast, the purified material caused no significant increase in total activity at 0 hr.: the results obtained at 4 hr., however, suggest that the multiplicity of infection of the purified suspension was lower

than the estimated 200 organisms per cell (Unfortunately, the infectivity titrations on this inoculum failed because the BHK-21 cells were unhealthy). Nevertheless, our findings indicate that centrifugation through sucrose provides a useful method for removing ACPase activity from suspensions to be used in experiments of this kind. It should be noted, however, that in preliminary experiments suspensions purified in this way tended to lose infectivity if stored at $-70°$ C. in 0·25 M sucrose in buffer.

Effect of TRIC organisms on ACPase of BHK-21 cells

By contrast with the toxic effect of TRIC organisms on macrophages, no cytopathic effects are seen during the growth cycle of the organism in BHK-21 cells, apart from the developing inclusion. To discover if lysosomal reactions were also different, cultures were infected with 1, 10, 50 or 100 organisms per cell and the total amounts of ACPase and extralysosomal portion were monitored at intervals during the growth cycle. No significant changes in enzyme activity were observed. Even an inoculum of 450 infective organisms per cell caused no changes in ACPase activity 2 hr. after infection, although by this time 60% of the activity was extralysosomal in macrophages inoculated with the same dose of organisms.

DISCUSSION

In earlier work on the effect of ingested particles on the lysosomes of macrophages, the cells were usually disrupted mechanically, the suspension was centrifuged to deposit lysosomes and enzyme activity was assayed in both deposit and supernatant fluid. In this way, Cohn & Wiener (1963) detected a difference in the distribution of ACPase between the lysosomal and non-lysosomal fractions obtained 2 hr. after macrophages had ingested as few as 15 heat-killed *Escherichia coli*. These workers concluded, with some caution, that although the enzyme was apparently released from the lysosomes, it was likely that it was merely redistributed into secondary lysosomes, and that as these were bigger and perhaps more fragile they broke up during the process of cell disruption. In experiments not reported here we ensured that our method of assay of ACPase was as sensitive as those that involved mechanical disruption of cells and also that during incubation with enzyme substrate at pH 5·0 the cells remained intact. Using our system of assay we therefore repeated the experiment of Cohn & Wiener with macrophages that had ingested 15 heat-killed *E. coli*; the proportion of extralysosomal ACPase was no different from that in control cultures. We conclude that the ingestion of such dead bacteria does not induce the release of lysosomal enzymes into the cytoplasm of the macrophage, and that the presence even of dead bacteria in the lysosomes probably makes them more susceptible to mechanical disruption.

By contrast, ingestion of infective TRIC organisms by macrophages leads to profound changes in the cell's lysosomal enzymes. Measurements on one such enzyme, acid phosphatase, indicate that the extralysosomal activity increases *pari passu* with a decrease in the total amount of activity in the cells; analogous

results were obtained with another lysosomal enzyme, β-glucuronidase. That these changes are caused by the organisms themselves is shown by the protective effect of specific antiserum against TRIC agents; the dependence of the changes upon infectivity is shown by the lack of reactivity of heat-killed organisms. The alterations in hydrolase activity closely parallel the morphological damage reported previously. For both phenomena, the time of onset and the severity of the observed changes depend on the number of organisms ingested. Both also show the same degree of sensitivity; 50 or more organisms per cell cause dramatic morphological changes within a few hours, but when a few organisms are ingested by each cell these changes are not manifest until the next day.

By contrast with the lysosomal reactions that occur in macrophages that have ingested TRIC organisms, no leakage was observed from the lysosomes of BHK-21 cells at any stage during the multiplication cycle of the organism, even with heavy multiple infection. This contrast in behaviour between the two types of cell can be explained by the observation that, whereas in macrophages TRIC organisms enter secondary lysosomes and are degraded, in BHK-21 cells they multiply in inclusions that never fuse with lysosomes (Lawn *et al.* 1973).

Our findings are best explained by the hypothesis that, in macrophages, infective TRIC organisms interact with and damage the lysosomal membrane; the consequent release of the contents in soluble form, first into the cytoplasm of the cell and then into the culture medium causes the morphological damage. The absence of cytotoxicity of TRIC agents for the cell in which they multiply contrasts with the effects of the agent of psittacosis multiplying in L cells (Kordova, Wilt & Sadiq, 1971). Cytopathic effects were reported which were accompanied by the release of lysosomal enzymes towards the end of the growth cycle. It is likely that the cell lysis was related to the development of the new generation of organisms; there is no evidence that lysosomes were directly involved. That TRIC agents do not induce these effects may be related to their tendency to remain within a circumscribed inclusion which can remain intact even after extrusion from the cell (Blyth, Taverne & Garrett, 1971). In virus infections too, effects on lysosomes, cell lysis and the manner of release may be related. Wolff & Bubel (1964) found that with vaccinia – which leaks from cells slowly without lysis – there was no alteration of lysosomal enzyme activity; whereas with poliovirus there was rapid release of both virus and lysosomal enzymes, associated with lysis of the host cell.

The simplicity and reproducibility of our technique makes it useful as a routine method for measuring the toxicity of suspensions of TRIC agents, in preference to tests based on the death of mice and calculation of LD 50. If large numbers of organisms are available (> 50 per cell), assay of extralysosomal activity provides a measure of toxicity within a few hours. This rapid test is no more sensitive than assay by intravenous injection in 10 g. mice since the inoculum required for the macrophage culture – about 10^7 live organisms – is equivalent to 1 LD 50. Suspensions containing fewer organisms can, however, be assayed *in vitro* if measurements are made of the total amount of activity in macrophages about 24 hr. after inoculation.

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REFERENCES

BLYTH, W. A., TAVERNE, J. & GARRETT, A. J. (1971). Trachoma inclusions separated from cells. In *Trachoma and Related Disorders. Proceedings of a Symposium Held in Boston, Mass. August* 1970 (ed. R. L. Nichols), pp. 79–87. Amsterdam: Excerpta Medica.

BLYTH, W. A. & TAVERNE, J. (1974). Neutralization of TRIC organisms by antibody: enhancement by antisera prepared against immunoglobulins. *Journal of Hygiene* **72**, 129.

COHN, Z. A. & WIENER, E. (1963). The particulate hydrolases of macrophages. II. Biochemical and morphological response to particle ingestion. *Journal of Experimental Medicine* **118**, 1009–20.

JONES, B. R. (1961). Trachoma and allied infections. *Transactions of the Ophthalmological Society of the United Kingdom* **81**, 367–78.

KORDOVA, N., WILT, J. C. & SADIQ, M. (1971). Lysosomes in L cells infected with *Chlamydia psittaci* 6BC strain. *Canadian Journal of Microbiology* **17**, 955–9.

LAWN, A. M., BLYTH, W. A. & TAVERNE, J. (1973). Interactions of TRIC organisms with macrophages and BHK-21 cells observed by electron microscopy. *Journal of Hygiene* **71**, 1–14.

MURRAY, E. S., BELL, S. D. JR., HANNA, A. T., NICHOLS, R. L. & SNYDER, J. C. (1960). Studies on Trachoma. 1. Isolation and identification of strains of elementary bodies from Saudi Arabia and Egypt. *American Journal of Tropical Medicine* **9**, 116–24.

REEVE, P. & TAVERNE, J. (1963). Observations on the growth of trachoma and inclusion blennorrhoea viruses in embryonate eggs. *Journal of Hygiene* **61**, 67–75.

SCHOENHOLTZ, W. K. (1962). Experimental Studies on Lethal and Immune Mechanisms in Massive *Bedsonia* Infections. PhD Dissertation, University of California, Berkeley, California.

TAVERNE, J. & BLYTH, W. A. (1971). Interactions between trachoma organisms and macrophages. In *Trachoma and Related Disorders. Proceedings of a Symposium Held in Boston, Mass. August* 1970 (ed. R. L. Nichols), pp. 88–107. Amsterdam: Excerpta Medica.

WEISSMANN, G. & THOMAS, L. (1962). Studies on lysosomes. 1. The effects of endotoxin, endotoxin tolerance and cortisone on the release of acid hydrolases from a granular fraction of rabbit liver. *Journal of Experimental Medicine* **116**, 433–50.

WOLFF, D. A. & BUBEL, H. C. (1964). The disposition of lysosomal enzymes as related to specific viral cytopathic effects. *Virology* **24**, 502–5.

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