Vol. 68, No. 3 September 1970

THE JOURNAL OF HYGIENE

(Founded in 1901 by G. H. F. Nuttall, F.R.S.)

CAMBRIDGE UNIVERSITY PRESS

Bentley House, 200 Euston Road, London, N.W.1

American Branch: 32 East 57th Street, New York, N.Y.10022

Subscription per volume £7 *net* ($23.00) (4 parts)

Single parts 40*s. net* ($6.50)

The Journal of Hygiene

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The Journal of Hygiene is published quarterly.

Single parts 40*s*. net ($6.50 in the U.S.A.) plus postage.

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Cambridge University Press

Bentley House, 200 Euston Road, London, N.W. 1

32 East 57th Street, New York, N.Y. 10022

J. Hyg., Camb. (1970), 68, 343
Printed in Great Britain

Bactericidal properties of Tego 103 S and Tego 103 G

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(*Received* 30 *January* 1970)

SUMMARY

The bactericidal activity of Tego 103S was compared with that of chlorhexidine in ethanol, and Tego 103G with Halamid. The activity was determined on *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Achromobacter anitratus* by various methods. Tego 103S in 1% solution was less active than chlorhexidine 0·5% in 70% ethanol, and Tego 103G in 1% solution was less active than Halamid 0·3%. The presence of serum did not noticeably influence the activity of the Tego preparations.

INTRODUCTION

Tego preparations (Th. Goldschmidt Ltd., Chemical Industries, Essen, Germany) are ampholytic surface-active compounds. Tego 103S and Tego 51 contain dodecyl-di (aminoethyl)-glycine. Tego 103G and Tego 51B are mixtures of compounds with alkyl radicals of different chain lengths. Tegolan is an emulsion of the monoaminopropyl-amino butyric acid derivate. They are variously recommended for disinfecting the skin (Tego 103S and Tegolan) and surfaces particularly in hospitals (Tego 103G) and in the meat and dairying industries (Tego 51 and Tego 51B). Papers concerning bactericidal properties of Tego 103S and Tego 103G are not frequent and the findings about these properties of the preparations are not uniform. Brausz (1953) and Perkins, Darlow & Short (1967) found Tego 103S and Tego 103G effective. However, Naumann (1952) found bactericidal activity more apparent than real. After Dold & Gust (1957) isolated living *Pseudomonas fluorescens* from solutions of Tego, changes have been made in the composition of Tego compounds (Goldschmidt A. G., 1967). Tego 103G, indeed, causes morphological changes in *Ps. aeruginosa* (Lickfeld, 1965), but data about the bactericidal properties are lacking.

In this paper the activities of Tego 103S in 1% solution and of Tego 103G in 1% solution are compared with chlorhexidine 0·5% in 70% ethanol and Halamid (paratoluol-sulphon-chloramid-Na) 0·3%, respectively.

MATERIALS AND METHODS

Organisms and method of culture

The test organisms used were freshly isolated strains of *Ps. aeruginosa* (our phage and pyocine type 12), *Staphylococcus aureus* (phage type 29/52/80) and

Achromobacter anitratus (laboratory no. 1493). Each culture was grown in 10 ml. of nutrient broth No. 2 (Oxoid Ltd, London), enriched with 0·5 % glucose, for 18 hr. at 37° C. The final viable counts for the three organisms were 2·8, 3 and 31 million/ml. respectively, as determined by the pour-plate method. Each culture was then centrifuged and the pellet washed twice with sterile water and finally resuspended in sterile water to the same opacity as before.

The solid media used were glycerol agar (glycerol, 1 %, w/v; Difco proteose-peptone, 0·5 %; K_2HPO_4, 0·04 %; $MgSO_4.7H_2O$, 2 %; $FeSO_4.7H_2O$, 0·003 %; Bacto-agar, 1 %; pH 7·1–7·2), Blood agar Base (Oxoid) and Endo agar (Oxoid) for *Ps. aeruginosa*, *St. aureus* and *Achr. anitratus*, respectively. For agar cylinders we used a consolidated agar (1·8 %).

For neutralization of the carry-over of Tego 103S and Tego 103G we included 0·5 % (w/v) of lecithin in 3 % (w/v) of Tween 80 in the media (Die Deutsche Gesellschaft für Hygiene und Mikrobiologie, 1958). For chlorhexidine and Halamid we used 1 % (w/v) of Lubrol W and 0·5 % of lecithin (Sykes, 1965), and 0·5 % (w/v) of sodium thiosulphate and 0·5 % of Tween 80 (Kayser & van der Ploeg, 1965), respectively. The neutralizing solution, used in a membrane filter test, was prepared by adding the neutralizers to quarter-strength Ringer's solution.

Germicides

The two Tego preparations were both used at a concentration of 1 % in water, as recommended by the manufacturers. The water used had a total hardness of 13·75. The test solutions had a temperature of 22° C. and pH of 7·4. The influence of organic material was determined by adding 10% of rabbit serum.

Tests

The activities of the germicides were determined in three ways by a suspension test, a membrane filter test (Hirsch, 1950) and by surface tests. Each test was done five times.

Suspension test

In the suspension test 0·15 ml. of a culture was added to 2·5 ml. of germicide and at intervals 0·25 ml. was subcultured into tubes containing 7 ml. of nutrient broth No. 2 containing 0·2 M sucrose. For testing Tego 103S and chlorhexidine in ethanol these intervals were $\frac{1}{2}$, 1, $2\frac{1}{2}$, 5, $7\frac{1}{2}$ and 10 min.; for Tego 103G and Halamid they were 5, 10, 15, 20, 30, 45 and 60 min. The sucrose broth contained suitable inhibitors (see above). After incubation at 37° C. for 48 hr. the tubes were subcultured on solid media.

Membrane filter test

A Sartorius filter apparatus SM 16114 (Sartorius-Membranfilter GmbH, Göttingen, Germany) with type SM 11009 membranes (Sartorius) was used, except for chlorhexidine in ethanol when SM 11106 membranes (Sartorius) were used.

The filters were sterilized and washed before use (Taylor & Burman, 1964). The test cultures, 0·15 ml., were added to 2·5 ml. of germicide and, after the same intervals as were used in the suspension test, 10 ml. of a neutralizing solution was

added. The mixture was then membrane-filtered. The membrane was washed through with 40 ml. of the neutralizing solution and finally cultured on a solid medium. After 48 hr. at 37° C. the number of colonies was counted by means of a modified Quebec counter.

Surface Tests

Surface tests were done with a swabbing test and an agar cylinder method (Kuipers, 1968). The activity of Tego 103S and of chlorhexidine in ethanol were determined on the skin of the forearm of members of the laboratory staff. A small volume, 0·1 ml., of one of the test cultures was spread evenly on the forearm at six marked spots, each with an area of 10 cm.^{2}. A germicide was spread over the contaminated parts of the skin. Tests were made after intervals of $\frac{1}{2}$, 1, $2\frac{1}{2}$, 5, $7\frac{1}{2}$ and 10 min.

Tego 103G and Halamid were tested in a similar way on a polyvinylchloride (PVC) floor, using culture volumes of 0·3 ml. spread over areas of 64 cm.^{2}. Tests were made after intervals of 5, 10, 15, 20, 30, 45 and 60 min.

Swabbing test. The marked spots on the skin and floor were wiped with sterile cotton-wool swabs moistened with nutrient broth No. 2 (Oxoid). The swabs were inoculated in tubes containing 7 ml. of nutrient broth No. 2 containing 0·2 M sucrose and the appropriate neutralizers. After incubation for 48 hr. at 37° C. the tubes of sucrose broth were subcultured on solid media.

Agar cylinder method. Impressions of the marked spots on the skin and the floor were made with agar cylinders with a surface of 10 and 64 cm.^{2} respectively. The agar contained the appropriate inhibitors for the germicides. After incubation for 48 hr. at 37° C. the number of colonies on the slices were counted with a modified Quebec counter.

RESULTS AND DISCUSSION

Tego 103S did not kill *Ps. aeruginosa*, *St. aureus* and *Achr. anitratus* in a suspension test within 10 min. (Table 1). In a membrane filter test and an agar cylinder test 99·97% of these organisms were not killed within 10 min. (Table 2 and Table 3).

An antiseptic should have an immediate effect within a short time of contact, as the reference antiseptic, chlorhexidine in ethanol, did. In the membrane filter test, when 10% of serum was added to the test solution of chlorhexidine in ethanol we found longer times to kill 99·97% of test organisms (Table 2). Evidently the neutralization in this testing method is less effective than in the suspension test and in the surface tests (Chiori, Hambleton & Rigby, 1965).

Tego 103G in a suspension test killed *Ps. aeruginosa in* 60 min. or less, but failed to kill *St. aureus* in 60 min. (Table 1). The times required to kill 99·97% of these organisms was also 60 min. or more (Table 2). Tego 103G was less active than Halamid, the reference disinfectant, in the three tests. In a suspension test and a membrane filter test Tego 103G and Halamid showed equal activity against *Achr. anitratus*, but in the *in vivo* test the activity of Halamid on this organism was greater (Table 3).

The influence of 10 % of serum on the bactericidal properties of Tego 103S could not be determined in intervals up to 10 min. Therefore we made the same tests under the same conditions with Tego 103 in intervals up to 60 min. There

Table 1. *Action of Tego 103S, chlorhexidine in ethanol, Tego 103G and Halamid on test organisms in the suspension test*

Germicide	Concn of test solution (%)	Time (min). to kill *Ps. aeruginosa*	*St. aureus*	*Achr. anitratus*
Tego 103S	1 (v/v)	> 10	> 10	> 10
Tego 103S + serum	1 (v/v)	> 10	> 10	> 10
Chlorhexidine in ethanol	0·5 (w/v) / 70 (w/v)	0·5	0·5	0·5
Chlorhexidine + serum in ethanol	0·5 (w/v) / 70 (w/v)	1	1	1
Tego 103G	1 (v/w)	45	> 60	15
Tego 103G + serum	1 (v/v)	60	> 60	15
Halamid	0·3 (w/v)	30	5	15
Halamid + serum	0·3 (w/v)	60	10	30

Table 2. *The times required (min.) to kill 99·97 % of the test organisms by Tego 103S, chlorhexidine in ethanol, Tego 103G and Halamid in the membrane filter test*

Germicide	Concn of test solution (%)	Time (min). to kill 99·97 % of *Ps. aeruginosa*	*St. aureus*	*Achr. anitratus*
Tego 103S	1 (v/v)	> 10	> 10	10
Tego 103S + serum	1 (v/v)	> 10	> 10	> 10
Chlorhexidine in ethanol	0·5 (w/v) / 70 (w/v)	2	0·5	0·5
Chlorhexidine + serum in ethanol	0·5 (w/v) / 70 (w/v)	10	7·5	2·5
Tego 103G	1 (v/v)	> 60	> 60	20
Tego 103G + serum	1 (v/v)	> 60	> 60	20
Halamid	0·3 (w/v)	30	20	20
Halamid + serum	0·3 (w/v)	45	30	30

Table 3. *The times required (min.) to kill 99·97 % of the test organisms on skin by Tego 103S and chlorhexidine in ethanol and on* PVC *floor by Tego 103G and Halamid*

Germicide	Concn of test solution (%)	Time (min.) to kill 99·97 % of *Ps.* aeruginosa	*St. aureus*	*Achr. anitratus*
Tego 103S	1 (v/v)	> 10	> 10	> 10
Chlorhexidine in ethanol	0·5 (w/v) / 70 (w/v)	1	0·5	0·5
Tego 103G	1 (v/w)	> 60	> 60	> 60
Halamid	0·3 (w/v)	60	10	10

was no great change in the end-points or in the times required to kill 99·97% of the organisms when 10% of serum was added to the test solution.

Tego 103G with 10% of serum was more effective than Halamid with serum, tested against *Achr. anitratus* (Tables 1, 2). The influence of 10% of serum on the activity of Tego 103G on *Ps. aeruginosa* and *St. aureus* was tested at intervals up to 120 min. and no change was found in the end-points or in the times required to kill 99·97% of the organisms.

The findings of Herrmann & Preusz (1949) that Tego preparations were more effective against Gram-negative organisms than against *St. aureus* were supported by some tests we made with Tego 103S and Tego 103G, each used in a concentration of 2%.

We have not tested the activity of the Tego preparations on *Escherichia coli* or on *Proteus vulgaris*. The trade literature according to the statements of Wallhäuser & Schmidt (1967) gives very confused findings. For example, the bactericidal activity of Tego 103S on these organisms at a concentration of 0·5% appears greater than at 1%.

In spite of changes that have been made in the composition of the Tego compounds, the bactericidal properties of Tego 103S and Tego 103G are slight. Tego 103G has moreover the disadvantage that eczema may appear after skin contact (Sanderink & Singelenberg, 1963).

We are indebted to Mr A. Feringa, Mrs H. F. van der Linden-Bonnema and Miss W. Kuiper for valuable assistance.

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J. Hyg., Camb. (1970), **68**, 349
Printed in Great Britain

Studies on the serological relationships between avian pox, sheep pox, goat pox and vaccinia viruses

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(*Received* 2 *February* 1970)

SUMMARY

By using neutralization, complement fixation and immunogel-diffusion tests, it has been demonstrated that cross-reactions occur between various avian pox viruses and between sheep pox and goat pox viruses. No such reactions were demonstrated between avian pox viruses and vaccinia virus or between avian pox and sheep pox and goat pox viruses. Furthermore, no serological relationship was demonstrable between vaccinia virus and sheep pox and goat pox viruses.

INTRODUCTION

The antigenic relationships within the vaccinia-variola group of viruses have been studied by many workers. It has been shown that vaccinia, variola, alastrim, cow pox and ectromelia viruses are antigenically related (McCartney & Downie, 1948; Downie & Macdonald, 1950; Gispen, 1955; Woodroofe & Fenner, 1962). The antigenic relationships of contagious pustular dermatitis virus and pig pox virus to vaccinia virus have also been reported (Webster, 1958; Datt & Orlans, 1958). However, very little is known about the serological relationship of sheep pox or goat pox viruses to vaccinia or avian poxes. It has been reported that fowl pox and vaccinia viruses are antigenically unrelated (Ledingham, 1931; Burnet & Lush, 1936; Tsubahara & Kato, 1961; Harada & Matamato, 1962), but Takano (1948), by cross-protection tests in rabbits, and Takahashi, Kameyama, Kato & Kamahora (1959), by complement fixation tests and by fluorescent antibody staining techniques, demonstrated strong antigenic relationships between these two viruses. Woodroofe & Fenner (1962) also found that nucleoprotein (NP) antigen extracted from vaccinia or myxoma viruses appeared to contain a group antigen which was common to a wide variety of pox viruses.

This paper describes the study by neutralization, complement fixation and gel-diffusion tests of immunological relationships among avian and mammalian poxes. The avian viruses studied were fowl pox, pigeon pox, canary pox and duck pox and the mamalian viruses were sheep pox, goat pox and vaccinia.

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MATERIALS AND METHODS

Virus strains

Egg-adapted fowl pox, pigeon pox and canary pox virus strains were obtained from the Indian Veterinary Research Institute (I. V. R. I.) Mukteswar, India, the Commonwealth Serum Laboratories, Melbourne, Australia and the National Institute of Animal Health, Japan, repectively. A virus strain labelled as duck pox isolated by Rao (1965) from an outbreak in ducks from Andhra Pradesh (India), was also obtained in the form of scabs. In our experiments, the strain was neither infective to ducks nor could be adapted on chorio-allantoic membrane (CAM) of developing chick embryos. The scabs were therefore used as such in the immunogel-diffusion test.

A strain of vaccinia virus was obtained from Dr B. M. Gupta, Central Drug Research Institute, Lucknow, India.

Sheep pox, Jaipur and goat pox, Mukteswar strains, were obtained from the virus laboratory of I.V.R.I. Mukteswar. These strains have been maintained by regular passages in their respective hosts.

Preparation of virus suspension

The infected CAM of egg-adapted strains of fowl pox, pigeon pox, canary pox and vaccinia viruses were extracted with McIlvaine buffer pH 7·2 (citric acid phosphate–phosphate buffer 0·004 M). Suspensions were centrifuged at 2500 rev./min. for 15 min. and sediments were washed three times with buffer. The supernatants so obtained were centrifuged at 10,000 rev./min. for 1 hr. in a refrigerated centrifuge. Sediments were resuspended in McIlvaine buffer and were kept frozen in small volumes. Viruses were titrated separately in developing chick embryo by the CAM route, the chorioallantoic membranes were dropped in the manner described by Beveridge & Burnet (1946).

For the experiments in which the feather follicle method of inoculation was employed, dry scabs or CAM were ground and suspended in 50 % glycerine phosphate-buffer solution (pH 7·2).

Sheep pox or goat pox nodules, preserved in glycerine phosphate buffer, were washed well in McIlvaine buffer and then macerated in a pestle and mortar with the help of neutral glass powder and resuspended in McIlvaine buffer. The suspension was lightly centrifuged. Antibiotics were added to the supernatant to give a final concentration of penicillin and dihydrostreptomycin of 1000 i.u. and 100 μg./ml. respectively. The treated virus suspensions were stored in the refrigerator overnight and tested for sterility before inoculation. Whenever virus suspensions were partially purified before inoculation, the following procedure was adopted:

A 20 % suspension of infected CAM was made in 0·004 M McIlvaine buffer and was treated with 'Genetron 226' (CF_2Cl, $CFCl_2$) on the lines described by Datt (1964) with slight modifications.

Preparation of antisera

Normal control serum was collected from all experimental animals and birds before their inoculation with infective material.

Egg-adapted fowl pox virus in fowls

Fowl pox egg-adapted virus was introduced by the feather follicle method into the thighs of two White Leghorn chickens aged 8 weeks. There was a swelling of the follicles on the third day after inoculation and lesions coalesced by about the eighth day. Fifteen days after infection, each bird was given an intravenous injection of 0·5 ml. of partially purified fowl pox virus suspension. A second similar injection was administered to each bird after a further 7 days. The birds were bled for the collection of serum 1 week after the final injection.

Egg-adapted fowl pox virus in pigeons

Partially purified egg-adapted fowl pox virus was administered to two healthy pigeons by the feather follicle route. A fortnight after initial infection each pigeon was inoculated intraperitoneally with a 0·5 ml. dose of the infective material. Each bird received a similar inoculation 10 days after the first and this was followed, after a further 6 days, by an intravenous inoculation of the infective material at the same dose. Seven days after this final injection, the pigeons were bled and serum was collected.

Egg-adapted pigeon pox and canary pox viruses in fowls and pigeons

Immune sera against these two viruses were prepared on similar lines to those described above except that the first inoculation of canary pox virus was given subcutaneously as this virus, in initial experiments, failed to produce lesions in fowls and pigeons by the feather follicle route.

Egg-adapted vaccinia virus in rabbits

The egg-adapted vaccinia virus was first adapted into rabbits. The material obtained from such rabbits was inoculated into two healthy rabbits intradermally on either side of the flanks. After recovery, the rabbits were given three additional injections of partially purified vaccinia virus intravenously. Rabbits were bled 8 days after the last injection.

Egg-adapted vaccinia virus in fowls

Fowls were immunized by the method of McCartney & Downie (1948) except that the virus was partially purified with fluorocarbon before injecting the fowls.

Sheep pox virus in sheep and goat pox in goats

Two healthy sheep and two healthy goats, 8–12 months old, were selected. The abdominal region of each was shaved, cleaned thoroughly and inoculated intradermally with 0·2 ml. of infectious material in each of 20 sites. Sheep pox virus was

administered at a dilution of 10^{-3} (titre of the virus $10^{6.5}$/ml.) and goat pox virus at a dilution of 10^{-2} (titre of the virus 10^5/ml.). Sera were collected 3 weeks after inoculation.

Serological tests

Neutralization test

Preliminary trials were conducted to standardize the test. Selection of a suitable diluent for titration of the virus, the optimum temperature at which the virus and serum mixture should be held and the optimum period of interaction between virus and serum were determined. A known dilution of virus suspension in McIlvaine buffer, pH 7·2, containing *ca.* 700 (600–800) pock-forming units (pk.f.u.) per ml. was added to an equal volume of serially diluted antiserum. The mixture was allowed to react for 4 hr. at room temperature. Five 12-day-old embryonated eggs were inoculated on the CAM with 0·1 ml. of virus and serum mixture containing *ca.* 35 pk.f.u. The highest dilution of serum neutralizing more than 50 % of the pk.f.u. was taken as the titre of the serum.

Complement fixation test

Direct. The technique as reported by Uppal & Nilakantan (1966) in respect of sheep pox antigen-antibody systems was followed.

Indirect. The principle of this test was similar to that described by Rice (1948). Serial twofold dilutions of heat inactivated fowl sera in 0·2 ml. volumes were made. To each dilution of serum was added 0·1 ml. of antigen. The antigen dilution used was the highest dilution which had given complete fixation in the antibody titration. The system was kept overnight at 4° C. This was followed by the addition of 0·2 ml. of complement (2 M.H.D.) and 0·1 ml. of inactivated pigeon or rabbit serum (1 unit). After mixing, the test was kept in a water bath at 37° C. for 90 min.; 0·2 ml. of sensitized sheep red blood cells were then added to each of the tubes which were further incubated for 30 min. Suitable controls of antigen, antiserum and complement were included. The highest dilution of fowl serum showing 50 % haemolysis was taken as the titre of the serum. In the initial stage, 50 % suspensions of infected C.A.M. showing confluent lesions were made in veronal buffer and centrifuged at 3000 rev./min. for 15 min. The supernatant was titrated by box titration and was used as an antigen.

Gel-diffusion test

The Ouchterlony double gel-diffusion technique reported by Uppal & Nilakantan (1967) with sheep pox antigen and antibody was followed with slight modifications. Both absorbed and unabsorbed antisera were used. Sera were absorbed with freeze-dried uninfected and infected tissue powder. Wherever CAM was used as an antigen, the membranes showing confluent lesions were triturated in an equal volume of McIlvaine buffer and were used as antigens. Similarly duck pox scabs were suspended in McIlvaine buffer. The method of preparation of sheep pox or goat pox antigen was as described by Uppal & Nilakantan (1967).

RESULTS

Neutralization tests

The results of cross-neutralization tests in respect of fowl pox, pigeon pox and canary pox virus are shown in Fig. 1. The antisera showed their maximum neutralizing power against homologous strains of virus and varying degrees of neutralization could be demonstrated against each of the other strains. The avian pox viruses

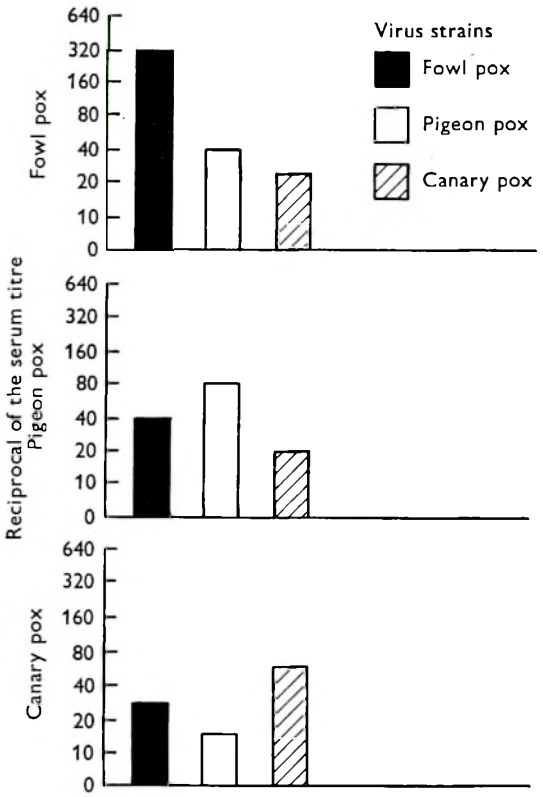


Fig. 1. The neutralization of fowl pox, pigeon pox and canary pox by homologous and heterologous antisera.

were not neutralized by the vaccinia, sheep pox and goat pox sera. The vaccinia virus was neutralized by antisera prepared against it both in fowls (1/640) and in rabbits (1/2560). It was not neutralized, however, by any of the avian pox, sheep pox, or goat pox antisera.

Complement fixation tests

The results of cross-complement fixation tests performed with homologous and heterologous systems using antisera against different avian and mammalian pox antigens are presented in Tables 1 and 2. Among avian poxes, there was cross-fixation between fowl pox, pigeon pox and canary pox viruses but no cross-fixation could be shown with vaccinia virus.

Vaccinial antisera fixed complement with the homologous antigen but there was no fixation when the antigen was prepared either from sheep pox or goat pox viruses. Both sheep pox and goat pox antisera showed relatively high titres with their homologous antigens and lower titres with heterologous antigens. Neither sheep pox or goat pox antisera showed any cross-fixation with vaccinia antigens.

Table 1. *Indirect cross-complement fixation test with vaccinia, fowl pox, pigeon pox and canary pox viruses and their immune sera*

	Antisera			
Antigen	Fowl pox	Pigeon pox	Canary pox	Vaccinia
Fowl pox	64	16	8	—
Pigeon pox	16	32	8	—
Canary pox	8	8	32	—
Vaccinia	—	—	—	128

Results are expressed as the reciprocals of the serum titres.

Table 2. *Direct cross-complement fixation tests with vaccinia, sheep pox and goat pox viruses and their immune sera*

	Antisera		
Antigen	Vaccinia	Sheep pox	Goat pox
Vaccinia	1280	—	—
Sheep pox	—	80	20
Goat pox	—	10	40

Results are expressed as the reciprocals of the serum titres.

Gel-diffusion tests

The results of gel-diffusion tests are presented in Table 3. Fowl pox antisera produced two precipitin lines against fowl pox antigen, one each against pigeon pox, canary pox and duck pox and none against vaccinia, sheep pox and goat pox antigens (Figs. 2, 3). Pigeon pox and canary pox antisera each gave one precipitin line with pigeon pox, canary pox, fowl pox and duck pox antigens but no precipitation lines were visible when these antisera were diffused against vaccinia, sheep pox and goat pox antigens. Similarly, duck pox antigen, diffused against fowl pox, pigeon pox and canary pox antisera, showed one precipitin line of identity but showed none when diffused against vaccinia, sheep pox and goat pox antisera (Figs. 4, 5).

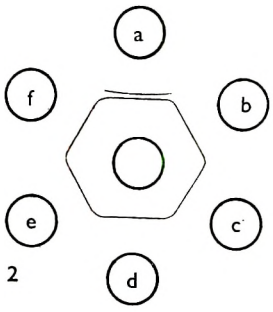
Three precipitating lines were observed when vaccinia antiserum was diffused against vaccinia antigen but there were no precipitin lines when vaccinia antiserum was diffused against fowl pox, canary pox, pigeon pox, sheep pox or goat pox (Fig. 6).

Both sheep pox and goat pox antisera exhibited two precipitin lines when diffused against their homologous antigens and one line when diffused against their heterologous antigens. No lines were observed when these antisera were diffused against vaccinia, fowl pox, duck pox, canary pox and pigeon pox antigens (Figs. 7, 8).

Table 3. *Formation of precipitin lines with homologous and heterologous avian and mammalian pox groups of antigen-antibody systems*

	Antisera					
Antigen	Fowl pox	Pigeon pox	Canary pox	Vaccinia	Sheep pox	Goat pox
Fowl pox	2	1	1	—	—	—
Pigeon pox	1	1	1	—	—	—
Canary pox	1	1	1	—	—	—
Duck pox	1	1	1	—	—	—
Vaccinia	—	—	—	3	—	—
Sheep pox	—	—	—	—	2	1
Goat pox	—	—	—	—	1	2

Results indicate the number of precipitin lines developed.



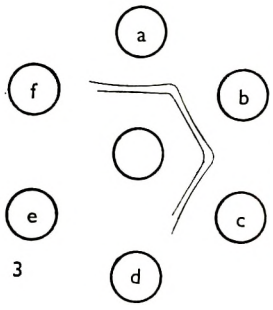
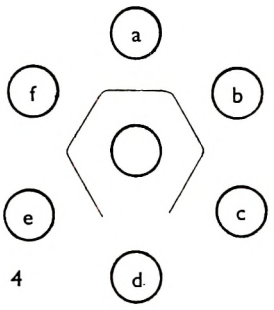


Fig. 2. Fowl pox antiserum in the central well and antigens (a) fowl pox; (b, d) pigeon pox; (c, e) duck pox and (f) canary pox.

Fig. 3. Fowl pox antiserum in the central well and antigens (a–c) fowl pox; (d) vaccinia; (e) sheep pox and (f) goat pox.



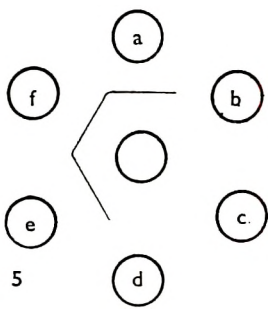


Fig. 4. Pigeon pox antiserum in the central well and antigens (a, e) pigeon pox; (b) canary pox; (c) duck pox; (d) vaccinia and (f) fowl pox.

Fig. 5. Duck pox antigen in the central well and antiserum (a) pigeon pox; (b) vaccinia; (c) goat pox; (d) sheep pox; (e) canary pox and (f) fowl pox.

Fowl pox, pigeon pox, canary pox and duck pox antisera, absorbed with uninfected chorioallantoic membranes, showed the same number of bands as were seen before such absorption. Similar antisera, when absorbed with infected membranes, showed no lines (Fig. 9). Similar results were obtained with vaccinia, sheep pox and goat pox absorbed antisera.

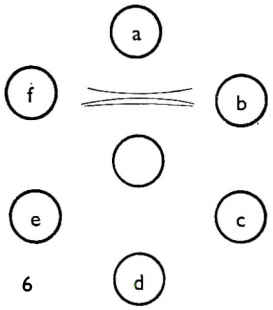


Fig. 6. Vaccinia antiserum in the central well and antigens (a) vaccinia; (b) fowl pox; (c) pigeon pox; (d) canary pox; (e) sheep pox and (f) goat pox.

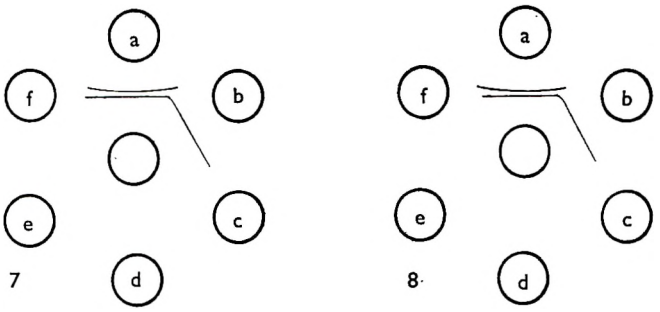


Fig. 7. Sheep pox antiserum in the central well and antigens (a) sheep pox; (b) goat pox; (c) vaccinia; (d) fowl pox; (e) pigeon pox and (f) canary pox.

Fig. 8. Goat pox antiserum in the central well and antigens (a) goat pox; (b) sheep pox; (c) vaccinia; (d) fowl pox; (e) pigeon pox and (f) canary pox.

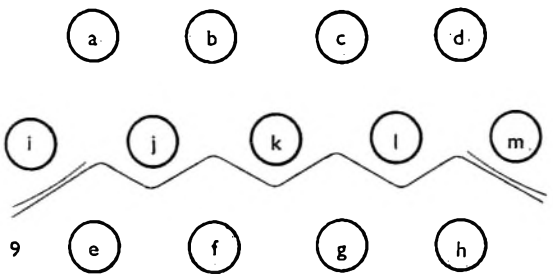


Fig. 9. Antigens in the central wells (i, m) fowl pox; (j, l) pigeon pox and (k) canary pox. Absorbed antisera with infected CAM in the top peripheral wells (a, d) fowl pox (b) pigeon pox and (c) canary pox. Absorbed antisera with uninfected CAM in the bottom peripheral wells (e, h) fowl pox (f) pigeon pox and (g) canary pox.

DISCUSSION

Uppal (1963) employing both complement fixation and immunogel-diffusion tests, reported that different strains of sheep pox virus (Jaipur, Bengalor and Mysore) isolated in India were antigenically related. In the present study it has been shown that sheep pox and goat pox viruses are also serologically related. With sheep pox or goat pox antigens, two precipitin lines were observed when diffused against their homologous antisera. The later findings are in agreement with the early observations of Uppal (1963); Sharma, Nilakantan & Dhanda (1966); Uppal & Nilakantan (1967) and Sen & Datt (1968) but are contrary to those of Bhambani & Murty (1963), who could not demonstrate any precipitin lines when sheep pox antigen was diffused against either sheep pox or goat pox convalescent sera. Nevertheless these workers considered sheep pox and goat pox viruses to be antigenically related on the basis of results obtained in gel-diffusion tests with antisera prepared in rabbits against goat pox scabs obtained from a natural outbreak of goat pox. Uppal & Nilakantan (1967), however, found that antisera prepared in rabbits against sheep pox scabs, even when partially purified with fluorocarbon, gave as many as three non-specific lines in gel-diffusion tests due to normal components of sheep skin or other tissues and they therefore regarded this method of study as unsatisfactory. In the present work, therefore, antisera were prepared against goat pox and sheep pox viruses in their natural, susceptible host. While the results indicate relationship between sheep pox and goat pox viruses, they further indicate that these two show no serological cross-reaction with either vaccinia virus or with avian pox viruses.

Woodroofe & Fenner (1962), obtained an NP antigen by alkaline extraction from vaccinia and myxoma viruses which appeared to contain a group antigen common to all pox viruses he examined. As they did not include sheep pox and goat pox viruses in their studies it would be interesting to investigate whether this group antigen is in fact common to them.

The lack of serological relationship between fowl pox and vaccinia as demonstrated by neutralization (Burnet & Lush, 1936), gel-diffusion (Gispen, 1955; Tsubahara & Kato, 1961) and complement fixation tests (Harada & Matamato, 1962; Woodroofe & Fenner, 1962) has been confirmed in the present study by concurrent application of all these serological tests. It was also found that pigeon pox, canary pox and duck pox viruses were unrelated to vaccinia virus.

The results indicate that there exist serological relationships between fowl pox, pigeon pox, canary pox and duck pox viruses. These findings are contrary to those of Miyamoto (1959) who claimed that there was no immunological affinity between canary pox virus and the viruses of fowl pox and pigeon pox. This claim, however, was based on the failure of cross-protection tests in immunized birds. The present findings are in agreement with those of Burnet & Lush (1936), who reported relationship between canary pox and fowl pox by neutralization tests, with those of Harada & Matamato (1962) who showed cross-reactions with avian poxes by the complement fixation test, and with Tsubahara & Kato (1961), who found at least

one common antigenic component in fowl pox, pigeon pox and canary pox viruses by the immunogel-diffusion test.

The authors wish to thank the former Director, Mr M. R. Dhanda, and the present Director, Dr C. M. Singh, for providing the necessary laboratory facilities for carrying out the present work.

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Vaccination against Hong Kong influenza in Britain, 1968–9

A report to the Medical Research Council Committee on Influenza and other Respiratory Virus Vaccines*

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(*Received* 4 *February* 1970)

SUMMARY

Studies of the effect of Hong Kong (HK) influenza vaccine were made in adults and children in Great Britain during 1968 and 1969. The vaccines were administered intramuscularly and also by intranasal spray. The serum antibody response was studied in 284 subjects. Most developed rising titres to vaccine given intramuscularly and few to vaccine given intranasally. Deoxycholate-split vaccine was as potent as conventional whole virus vaccine. Antibody titres were maintained for months. Over 4000 subjects in factories, offices and schools were observed during the epidemic. The incidence of disease was not significantly reduced by either form of vaccination. A survey was made of epidemics in boarding schools in which some of the pupils had been vaccinated, in six with commercial polyvalent vaccine and in five with HK; there was a lower incidence of influenza in two schools vaccinated 2 or 4 weeks earlier with HK vaccine.

INTRODUCTION

While the new Hong Kong (HK) virus was spreading in the Far East and in the tropics, influenza virus vaccines to protect the population were being prepared in this country. Attempts were also planned by our committee to assess the value of both standard and recently introduced measures in the face of the expected epidemic of a new serotype.

The 'standard' measures were the use of parenteral killed vaccines made from (1) the pre-Hong Kong influenza A 2 serotype A2/Eng/12/64, and (2) the new strain. The 'recent' measures were (1) the parenteral injection of virus split by sodium dodecyl sulphate (SDS), and (2) the administration of 'standard' HK vaccine by nasal spray.

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It was decided to evaluate the results by measuring haemagglutination-inhibiting antibodies in volunteers' sera, and in terms of protection against disease in the course of the expected influenza epidemic.

MATERIALS AND METHODS

Vaccines

Standard vaccines. The commercial polyvalent (CP) vaccine contained in 1 ml. 3000 HA units of the strain A2/Eng/12/64, 6000 HA units of A2/Eng/76/66 and influenza B strains. The HK vaccine contained 7000 HA units of A2/Eng/344/68 which was serologically identical with HK strains isolated in the Far East. The vaccines were prepared at Evans Medical and supplied by British Drug Houses.

'*Split vaccine*'. was prepared at the Commonwealth Serum Laboratories, Melbourne, Australia, by the method of Laver (1961), and supplied by Burroughs Wellcome.

Administration

One ml. of vaccine was administered by intramuscular injection into the arm or by spraying into the external nares with a hand-spray or a De Vilbiss apparatus. It was thought that both apparatuses deposited most of the spray in the nasal cavity whence it would be carried to the pharynx by ciliary activity.

Serological tests

In most studies of antibody response blood was collected at the time of vaccination and 2–3 weeks later. Antibody was usually measured by haemagglutination inhibition (HI) using four doses of A2/Eng/12/64 (Asian) or A2/Eng/344/68 (HK) virus. The procedures were those of the W.H.O. method. Sera were treated overnight with 4 or 5 volumes of cholera filtrate, virus and antiserum were kept in contact for 1 h, and 1 % human group O red cells were used.

Diagnostic tests

In most of the studies of protection some of the patients were proved to have influenza by laboratory tests. Viruses were isolated in monkey kidney cultures from nasal or throat swabs and all were identified as HK serotype; paired sera were titrated by complement fixation (CF) test against the S antigen.

Plan of trials

Trials were combined to investigate simultaneously several of the problems outlined in the introduction. All trials were, as far as possible, conducted by a blind technique and a trial scheme found acceptable for many studies is outlined in Table 1. This ensured that all volunteers received both an intranasal spraying and an intramuscular injection, that each received some material of possible benefit to him, and that the effects of intramuscular HK virus could be determined by comparing groups A and C and of intranasal HK virus by comparing groups A and B.

Schools 1 and 2 would not agree to this scheme and so boys were vaccinated more or less at random, with either intranasal or intramuscular HK vaccine. One school, number 3, did agree.

Assessment of results

The records of sickness and absence of factory and office workers were examined and illnesses were classified, as far as possible, as influenza-like, or other respiratory diseases. Such illnesses occurred over a long period and those recorded during a period when influenza was known to be circulating, January to March 1969 inclusive, were used for analysis. In schools 1 and 3 there were short, clear-cut epidemics and almost all of a group of typical cases were shown to be infected with HK virus; there was a low-grade epidemic in number 2 and no epidemic in a fourth. All patients had an acute febrile illness with respiratory symptoms compatible with epidemic influenza.

The serological results reported are based on studies of 79 students, 109 industrial employees in the Midlands, 71 M.R.C. office staff and 25 employees in an oil refinery; and 90 factory workers were included in a special challenge study. Studies of protection were made on 1425 children in three schools, 3048 employees in two large firms (Reed Paper Group and Imperial Chemical Industries) and the M.R.C. office staff.

Table 1. *Outline of trial plan*

Group	Material given	
	Intramuscularly	Intranasally
A	Polyvalent vaccine	Saline
B	Polyvalent vaccine	Hong Kong Vaccine
C	Hong Kong vaccine	Saline

1 ml. was given in each case by each route.

RESULTS

Trials of antigenicity of vaccines

Vaccines were given to students in Oxford and the serological results are summarized in Table 2 and Fig. 1. These show that volunteers had little or no antibody against HK virus before vaccination; that although significant rises in titre of antibody against HK virus were stimulated by polyvalent vaccine in 44% of the volunteers, they were stimulated by HK virus vaccines in 85% of volunteers and rose to higher titres. The 'split' vaccine was slightly more potent than the standard vaccine. The sera were also titrated against A2/Eng/12/64. The HK vaccines stimulated antibody against that virus almost as frequently as the polyvalent vaccine did against the 1964 strain, although the final titres were somewhat lower. This point is analysed in more detail by Hobson *et al.* (1970). There was no troublesome local or general reaction to the vaccine, and the analysis of diary cards issued to the volunteers showed that the frequency and duration of local soreness was the same in those who had split and whole virus vaccine—about one-third were affected on the day of vaccination and none by 2 days later.

Table 2. *Serum antibody responses against HK virus in volunteers given intramuscular vaccines*

Subjects	Vaccines	No. in group	No. showing: Antibody before vaccination ⩾10	No. showing: ⩾ 4-fold rise in titre after vaccination	GM* titre after vaccination
Students	CP*	25	9 (36 %)	11 (44 %)	34 (31–35)†
	Split HK*	27	7 (26 %)	24 (89 %)	169 (166–172)
	Whole HK	27	8 (30 %)	22 (81 %)	131 (129–134)
Factory employees	CP	37	16 (43 %)	13 (35 %)	36 (33–38)
	Split HK	37	19 (51 %)	25 (67 %)	133 (130–136)
	Whole HK	35	24 (69 %)	29 (83 %)	102 (99–104)

*In these and subsequent tables CP = commercial polyvalent, HK = Hong Kong, GM = geometric mean. †Confidence limits 95 %.

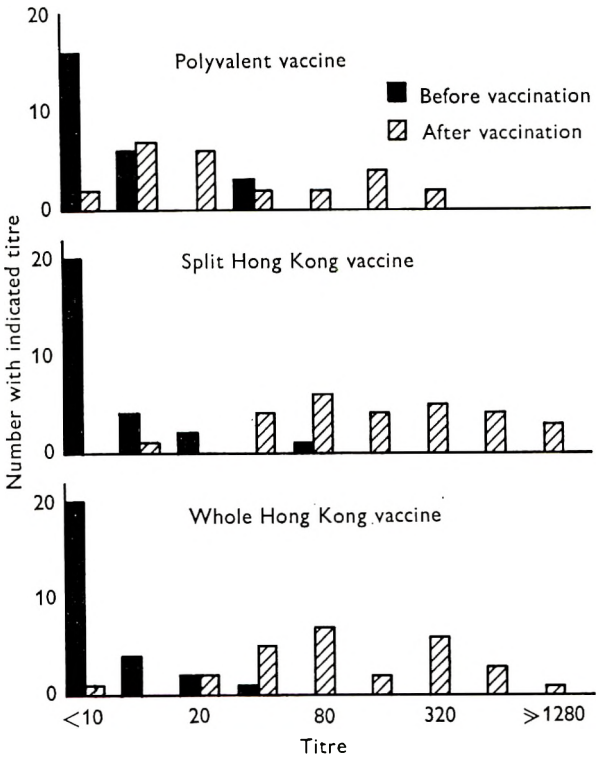

Fig. 1. Distribution of serum antibody titres in subjects, mainly medical students, before and after being given intramuscular vaccines.

Similar results were obtained on vaccinating industrial employees in the Midlands and are summarized in Table 2. In this group relatively fewer responded to split vaccine than to whole virus vaccine.

The sera from groups of volunteers vaccinated as shown in Table 1 were also tested. As shown in Fig. 2. and Table 3, the addition of intranasal HK vaccine

Table 3. *Serum antibody responses against HK virus in office staff given intramuscular and intranasal vaccines*

Group	Vaccine schedule IM*	Vaccine schedule IN*	No. in group	No. showing: Antibody titre ⩾ 10 before vaccination	No. showing: ⩾ 4-fold rise in titre after vaccination	GM titre after vaccination
A	CP	Sal*	28	5 (18 %)	6 (21 %)	17 (15–20)
B	CP	HK	19	6 (32 %)	5 (26 %)	17 (14–19)
C	HK	Sal	24	5 (21 %)	19 (79 %)	78 (76–82)

*In these and subsequent tables IN = intranasal, IM = intramuscular, Sal = saline.

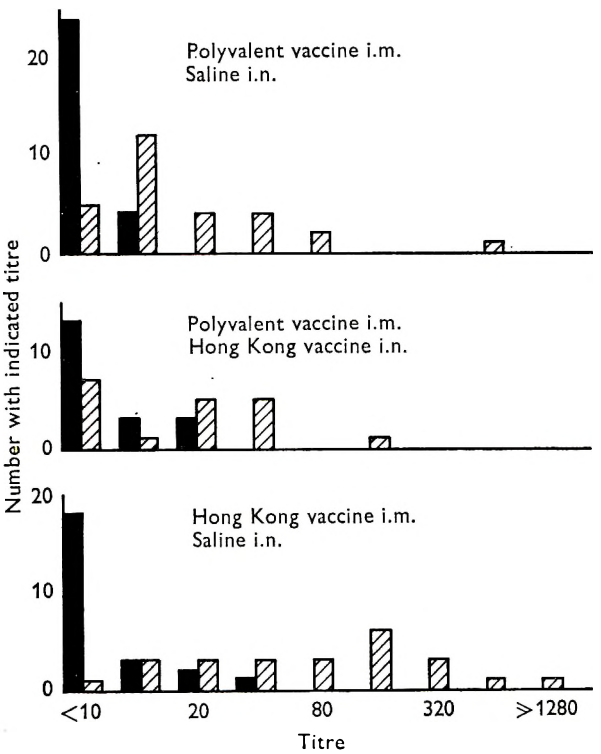

Fig. 2. Distribution of serum antibody titres in office workers vaccinated according to the scheme shown in Table 1. The titres were unaffected by intranasal administration of vaccine.

had no effect on the circulating antibody response to intramuscular vaccine. This was further supported by a trial in an oil refinery in which 25 volunteers each received intranasal HK vaccine alone—2 of 25 showed a fourfold rise in antibody titre and the others showed no change.

Certain sera were titrated by neutralization tests in tissue culture and the results showed that there was a close correlation between neutralizing and HI antibody titres. The titres of HI antibody were, nevertheless, substantially lower after

vaccination than after typical attacks of influenza seen in schoolboys, which varied from 320 to ⩾ 1280. Further analysis of the serological responses of one industrial group are reported elsewhere (Hobson *et al.* 1970).

Trials of protection

The results of attempts to demonstrate protection in two schools are shown in Table 4. In another school no epidemic occurred. There was no convincing evidence of protection by either procedure, and none in another school in which intranasal vaccine was given after the epidemic had begun.

Table 4. *Results of attempt to demonstrate protection against epidemic influenza in boarding schools*

	Results of virological tests on patients		Unvaccinated		Intramuscular HK vaccine		Intranasal HK vaccine	
School	Swabs positive	Sera positive	Total	No. ill	Total	No. ill	Total	No. ill
1	14/14	16/17	182	47 (26%)	198	37 (19%)	195	33 (17%)
2	8*	1*	96	4 (4·2%)	280	3 (1·1%)	271	1 (0·4%)

*The majority of boys with respiratory infection in this school were tested for virus infection throughout the term.

Table 5. *Results of protection trials of intranasal and intramuscular HK vaccine and intramuscular polyvalent vaccine*

			Frequency of influenza-like disease							
	Vaccine schedule		School 3 epidemic		I.C.I. Northwich 15. xi. 68–15. v. 69		Reed paper Group 2. i. 69–31. iii. 69		M.R.C. Head office	
Group	IM	IN	Total	No. ill	Total	No. ill	Total	No. ill	Total	No. ill
A	CP	Sal	25	17 (68%)	545	16 (2·9%)	351	15 (4·3%)	22	4 (3)*
B	CP	HK	25	13 (52%)	491	14 (2·8%)	331	8 (2·4%)	31	5 (0)*
C	HK	Sal	24	15 (62%)	508	8 (1·6%)	322	8 (2·5%)	27	4 (1)*
Unvaccinated			129	76 (59%)	500	5 (1%)	—	—	—	—

*Number of cases of disease in which influenza A was confirmed by serology.

Table 5 shows the results from school 3 and from the factories—in all these the trial procedure was as shown in Table 1. There was a slightly lower frequency of influenza in one or both of the groups of HK vaccinated subjects in the factories,

but the total incidence was low; the disease may not have been due to the influenza virus, as in the small parallel study in the M.R.C. office only a fraction of the cases diagnosed clinically as influenza were confirmed by serological tests. In the school in which a short epidemic of proved influenza with a high incidence occurred, there was no evidence of protection.

Finally, one group of factory workers was vaccinated and then challenged with an attenuated live influenza vaccine 6 months later after the epidemic was over; the challenge virus and methods have been described elsewhere (Beare, 1970). Circulating antibodies were stimulated and the titres were like those shown in Fig. 2. They were well maintained (Table 6). The challenge was apparently rather light, since a relatively small proportion of unvaccinated subjects became infected, but there was no evidence of protection except for a possible reduction of infection in those given intramuscular HK vaccine.

Table 6. *Persistence of antibody in vaccinated factory employees and results of challenge with attenuated* A2/*HongKong*/1/68

	Vaccine schedule		Changes in antibody titre from 2 weeks after vaccination to before challenge (7 months)			Rising antibody titres after challenge
Group	IM	IN	⩾ 4-fold rise	No change	⩾ 4-fold fall	
A	CP	Sal	0/12	12/12	0/12	4/21
B	CP	HK	1/9	6/9	2/9	2/17
C	HK	Sal	3/11	6/11	2/11	1/20
Unvaccinated			—	—	—	3/21

All volunteers available were challenged (column 6), but from only some of these were serial blood specimens collected (columns 3–5).

Experience in boarding schools

In addition to these studies we were kindly provided with a detailed analysis of influenza epidemics in boarding schools, mostly for boys (Fig. 3). In five of these no vaccine had been given, in six polyvalent vaccine, and in five substantial numbers had received HK vaccine; the vaccines were offered to all children but the few in whom it was contra-indicated for medical reasons, and those whose parents agreed were injected. The total of children observed was 5387 of whom 1197 were not vaccinated, 2754 were vaccinated with CP and 1436 with HK vaccine. In general, only children admitted to sanatoria with a febrile illness clinically compatible with influenza were included, and in all cases the aetiology of the epidemic was confirmed by virus isolation, antibody titration or both, and these tests were performed in the local Public Health Laboratory. The evaluation of illness was better than that in the trials in adults although there had been no attempt at random selection of subjects. In these, as in the schools mentioned above, the incidence was similar in most age groups and houses, although in several cases preparatory departments attached to the main schools apparently escaped infection. All but one of the epidemics occurred in January and February 1969, and in the five unvaccinated, or virtually unvaccinated schools, the incidence

ranged from 27% to 82%. In the six schools in which polyvalent vaccine was given the incidence ranged from 12% to 54%, and in all but one the incidence in the vaccinated was almost exactly the same as that in the unvaccinated pupils. One school had an early epidemic in 1968 and a second epidemic at the beginning of 1969 in which a further 48 boys were affected, this bringing the total incidence to over 20%. Finally, in five schools substantial numbers received intramuscular HK vaccine. In one the vaccine was given as the epidemic was beginning and the lack of protection is not surprising. There is an impression that there was a reduction of incidence where vaccination occurred 2–4 weeks before exposure, but no firm conclusion can be drawn.

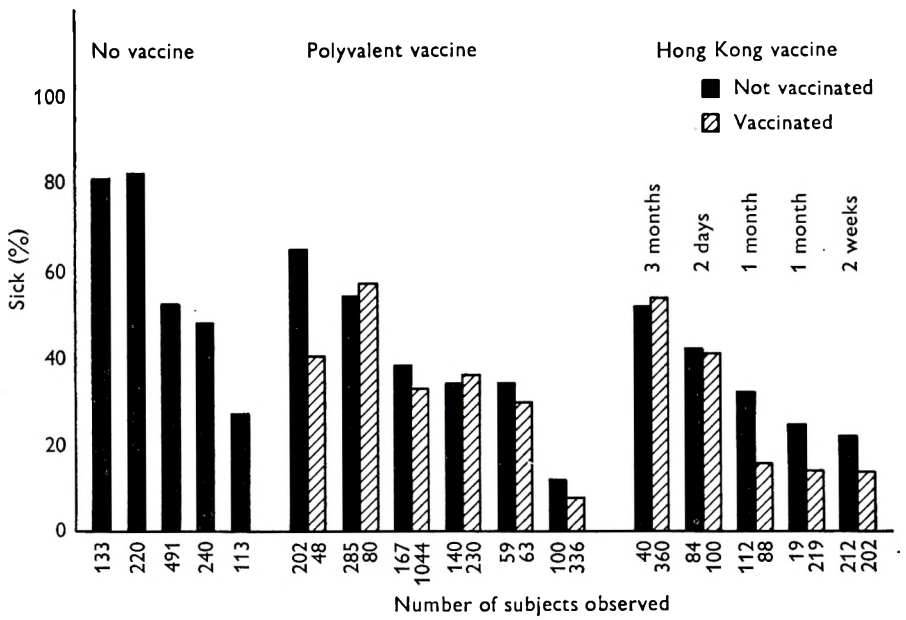

Fig. 3. Frequency of influenza in schools in which polyvalent vaccines or Hong Kong vaccines had been given before the occurrence of a proven epidemic of influenza A 2 Hong Kong. The interval between vaccination and the beginning of the epidemic is shown for schools where the HK vaccine was given.

DISCUSSION

It was a real disappointment to have found so little evidence for protection in these studies and it seems wise to consider some possible reasons for this.

In spite of the need for haste the HK vaccines which were made reached the standard specifications and were able to stimulate circulating antibody. In this respect they seemed to perform better than the first Asian-strain vaccines, possibly because the virus grew better and the antigenic shift was not quite as great this time. We know little of the 'quality' of the circulating antibody produced but it was able to neutralize virus. It has been suggested that antineuraminidase antibody may be important, but it is likely that both polyvalent and Hong Kong vaccines would have stimulated this.

There is ample evidence in the literature that parenteral killed virus vaccine can protect (Hobson, 1967), but most of the relevant studies took place at least a year or so after the emergence of a radically new serotype, so that the subjects had probably had some antigenic experience of the antigen given or one rather like it. The only exceptions are those studies performed in the Asian influenza epidemic of 1957, but in these the vaccine was given shortly before the epidemic arrived, and, in our own study, after the epidemic had begun (Committee, 1958). It is therefore possible that the serological response was of short duration, and that protection might have waned during the gap of 2–4 months which elapsed before exposure took place in our studies this year. However, we have good evidence that our adult volunteers retained their circulating antibodies for at least 7 months, but it is still possible that antibody in the nasal secretion did not last as long as this. Antipoliovirus secretory antibody produced by killed virus in the colon persists less than 2 months and not as long as that induced by infection (Ogra & Karzon, 1969). There is evidence which is now widely accepted that the titre of local antibody, which probably mediates immunity against respiratory viruses, need not be directly related to circulating antibody titres, particularly after parenteral injection of killed vaccines. We think that in order to get useful protection under such circumstances it may well be necessary to give substantially more antigen. There is evidence (A. H. Griffith, unpublished, and F. Warburton, unpublished) that two injections of the vaccine used did not increase the level of circulating antibody above that obtained after one injection. However, after a large dose of vaccine, antigen might in one way or another reach the nasal mucosa and there stimulate antibody-producing cells; such large doses could now be given in the form of pure concentrated virus. A similar effect might be produced by using oil-adjuvant vaccine.

We were also disappointed at the results of giving the vaccine by intranasal spray. The results of Waldman, Mann & Small (1969) suggested that this would stimulate better immunity than the same antigen given intramuscularly, although in these experiments more antigen was given up the nose than into the arm. Using the same amount of antigen by each route it is clear that there was no protection in our studies and this must mean that the intranasal route was not *much* more effective. Other experiments have been done using influenza B antigens, in which intramuscular vaccine appeared to protect against an experimental infection with a live influenza B virus; in these, one dose of intranasal vaccine had no prophylactic effect, while two doses may have had some (Beare *et al.* 1969). It is clear that it would be of value to have measurements on the amount of antigen required to stimulate comparable amounts of nasal antibody when given by the intranasal and by the intramuscular routes.

We wish to thank the following for carrying out field work: Dr M. Ashley-Miller, Dr C. P. Chivers, Dr D. Hobson, Dr R. A. Gordon-Smith, Dr H. Howells, Dr L. Tyler, Dr M. C. S. Kennedy, Dr C. Veys, Dr J. H. Pennington, Dr B. Juel-Jensen, Dr F. O. MacCallum, Dr F. H. Lee, Surg. Capt. P. de Bec Turtle, Dr D. M. Baker, Dr J. P. Sparks, Dr S. E. Reed. We are most grateful to the Public Health

Laboratory Service for supplying information about school epidemics and to the many school medical officers who sent details of vaccination and influenza in the schools for which they were responsible.

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Printed in Great Britain

A comparison of monovalent Hong Kong influenza virus vaccine with vaccines containing only pre-1968 Asian strains in adult volunteers

A report to the Medical Research Council Committee on Influenza and other Respiratory Virus Vaccines*

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(*Received* 20 *February* 1970)

SUMMARY

A total of 1601 adult industrial workers were vaccinated with either monovalent inactivated vaccine of the Hong Kong strain of influenza A virus, or with polyvalent vaccine containing only pre-1968 Asian viruses. Serological investigations on a random sample of volunteers showed that 53/56 (95%) given Hong Kong vaccine developed a significant rise in specific haemagglutination-inhibiting antibody; final titres were 1/48 or greater in 39 (70%) and the GMT (geometric mean titre) was 96·5. After polyvalent Asian vaccine, 40/67 (60%) also produced antibody against Hong Kong virus, but only 21 (31%) had final titres of 1/48 or above, and the GMT rose only to 14·1. An intranasal spray of the Hong Kong vaccine in addition to injected Asian vaccine gave no additional increase in antibody.

Each type of vaccine stimulated a recall of pre-existing antibody against Asian viruses. The possible significance of heterologous responses to the two vaccines is discussed.

The incidence of clinical influenza in the trial population was sporadic, and the infection rates were too low to allow any accurate estimate of the protective efficiency of the two vaccines.

INTRODUCTION

In the summer of 1968 the epidemic of influenza which began in Hong Kong spread rapidly through populations in which protective antibodies against Asian (A 2) strains of influenza virus were already widely distributed. The causative virus

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isolated from outbreaks in many parts of the world, including Britain, was an influenza type A strain with marked antigenic differences from Asian or any other foregoing epidemic subtype. In laboratory tests with animal antisera, the haemagglutinin (HA) of the Hong Kong virus and Asian viruses showed little cross-reactivity, but the viral enzyme neuraminidase was antigenically similar in both kinds of virus. Hence, Hong Kong (HK) strains have been regarded as an extreme variation within the A 2 family of viruses rather than a new subtype A 3 (Coleman *et al.* 1968). However, all available evidence from previous epidemics and from influenza vaccine trials (reviewed by Hobson, 1967) suggests that, although antibody directed against the viral HA is correlated with immunity to infection, haemagglutination-inhibiting (HI) antibody against one subtype of influenza A confers no protection against strains with different HA antigens. Thus it was urgently necessary to determine whether an entirely new vaccine formulation containing HK strains would be essential to limit future epidemic risks, or whether already available commercial vaccines containing A 2 strains isolated in 1964–6 could be relied upon to stimulate antibody effective against HK virus. In human adult populations with a broad previous experience of the various influenza A subtypes, it seemed possible that A 2 vaccines might invoke a wider range of immunity than in experimental animals.

Accordingly, the Medical Research Council initiated a series of clinical trials in which the serological responses of adult volunteers to either commercial polyvalent (CP) vaccine of A 2 + B viruses or monovalent HK vaccine could be compared, and their protective effect against infection be evaluated in a double-blind procedure. In the present trial of this series, 1601 adult volunteers from the management and industrial personnel of the Mond Division of Imperial Chemical Industries Ltd. were given one or other of the vaccines by intramuscular injection; approximately half of the volunteers given CP vaccine also received an intranasal spray of inactivated HK virus to test the possibility that local stimulation by antigen might induce a higher degree of protection against infection than does parenteral inoculation (Waldman *et al.* 1968).

The effectiveness of each vaccine in producing or enhancing circulating HA antibody against each type of virus was estimated in approximately 11% of the population. Clinical histories were taken from all volunteers absent through sickness over the period 18 November 1968 to 30 April 1969, and each respiratory illness was classified, before the double-blind vaccine code was broken, into the various clinical syndromes characterized by Stuart-Harris (1965). In addition, all sickness absence due to respiratory disease in the four main factories from which the trial population was drawn, with a total payroll of 3580, was recorded from week 45 of 1968 (2 weeks before vaccination of the trial group) to week 20 of 1969.

MATERIALS AND METHODS

Volunteers

A total of 1601 adult workers of both sexes, with an age range of 16–64 years, volunteered to enter the trial and were allocated to one of three groups by random

numbers. The age and sex distribution, and the scatter of volunteers through the various factory sites, were similar in each group.

Vaccines

Formalin-inactivated influenza virus vaccines were obtained from British Drug Houses Ltd. CP, the polyvalent vaccine (Admune aqueous), contained, per 1·0 ml. dose,

	HA units
A2/Eng/12/64	3000
A2/Eng/76/66	6000
B/Eng/5/66	3000
B/Swiss/265/67	3000

HK vaccine (Admune Mono 68) contained 7000 HA units of A2/Eng/344/68 per 1·0 ml. dose.

Individual doses of the vaccines for intramuscular injection (i.m.) and pools of HK vaccine or sterile normal saline for intranasal instillation (i.n.) by fine hand-spray set to deliver metered doses of 1·0 ml. were prepared and letter-coded in another laboratory, and a double-blind procedure was maintained throughout the trial.

Each volunteer received both an injection and an intranasal spray as follows:

Group	Intranasal	I.m.
A	Saline	CP
B	HK	CP
C	Saline	HK

Serological procedures

Paired samples of sera were taken on the day of vaccination and 14 days later from 183 volunteers previously selected by random numbers. There were no significant differences in the number selected from groups A, B and C, in the age and sex distribution in these groups or in the distribution of prevaccine antibody titres against HK or earlier A2 viruses.

The procedure for HI tests differed from the standard W.H.O. method (Report, 1953) only in certain details; sera, pretreated with cholera filtrate (Philips Duphar) were diluted in M/50 phosphate buffered saline pH 7·6 and incubated with 4 HA units of non-inactivated virus for 1 hr. at room temperature before adding a 1% suspension of human group O Rh-negative erythrocytes.

Preliminary HI tests with A2/Eng/12/64 and A2/Eng/76/66 gave identical titres with either human and immune rabbit sera. Hence, only the former strain was used in the main titrations shown in the text.

The original seed cultures of A2/Eng/12/64 (A64 virus) and A2/Eng/344/68 (HK virus) from which the trial vaccines had been prepared were kindly provided by Dr D. C. Breeze, Evans Medical Ltd., Speke, Liverpool. Eleven-day chick embryos were inoculated with 10^4 egg infective doses (EID 50) of seed virus, and infected allantoic fluids were harvested after 48 hr. incubation at 36° C. A single working pool of each strain was used throughout.

Rabbit antisera were prepared by six serial intravenous injections of seed cultures of the test viruses at 3 to 4-day intervals. Neutralization tests were performed by incubating serial dilutions of antisera with 10^3 EID 50 of virus for 1 hr. at room temperature before inoculating each mixture into 4–6 eleven-day chick embryos. Spot HA tests on each egg were made after 48 hr. incubation at 36° C.

Clinical assessment

All persons with sickness absence were seen by one of us (C.P.C.) and the type of illness was categorized as indicated above. Where possible, paired sera were obtained in the acute stage of the illness and 14 days later. Throat swabs were taken for attempted virus isolation by courtesy of Dr J. O'H. Tobin, Public Health Laboratory, Manchester.

RESULTS

Serological investigations

Titrations with specific animal antisera

Hyperimmune rabbit sera prepared against A 64 and HK viruses were titrated with each virus in HI tests and in neutralization tests in chick embryos. The results (Table 1) confirm the marked antigenic differences between these strains: less than 5% cross-reactivity was shown by either antiserum against the heterologous virus.

Table 1. *The activity of specific animal antisera on A2/Eng/12/64 (A64 virus) and A2/Eng/344/68 (HK virus)*

Rabbit antiserum	HI titres		Neutralization titres	
	HK virus	A 64 virus	HK virus	A 64 virus
Anti HK	3072	96	2560	100
Anti A 64	96	3072	30	3200

Titrations with human sera

Despite the specific behaviour of HK and A 64 viruses with animal antisera, rises in HI antibody titre were detected by both agents in many volunteers, apparently regardless of the schedule of vaccination. The serological data presented below have therefore been analysed to determine the frequency and possible significance of cross-reactions induced by the two vaccines.

The production of HK antibody

Prevaccination sera from 54/183 (29%) volunteers reacted with HK virus; almost all HI titres were at 1/6 only, and were equally distributed amongst the 3 trial groups (Table 2). There was no apparent correlation between the presence of prevaccine HK antibody and prevaccine titres of A 64 antibody.

After vaccination 131/183 (71%) showed a 4-fold or greater rise in HK antibody, i.e. immune responses were not confined to those given homologous HK vaccine.

Table 2. *HI antibody titres against HK influenza virus* (A2/*Eng*/344/68) *after vaccination with homologous* (*HK*) *and heterologous* (*CP*) *influenza A virus vaccines*

Group	Vaccine schedule		No. of persons in group	No. with pre-vaccine antibody	No. with ⩾ 4-fold rise in antibody	No. with final titre ⩾ 48	Geom. mean titres	
	IN	IM					Pre-vaccine	Post-vaccine
A	Saline	CP	67	18 (27 %)	40 (60 %)	21 (31 %)	1·8	14·1
B	HK	CP	60	19 (31 %)	38 (63 %)	23 (38 %)	2·1	21·2
C	Saline	HK	56	17 (30 %)	53 (95 %)	39 (70 %)	2·0	96·5

In this and subsequent tables: HI = haemagglutination-inhibition; IN = intranasal; IM = intramuscular; CP vaccine = A2/Eng/12/64 (A 64) and A2/Eng/76/66; HK vaccine = A2/Eng/344/68 (HK)

Table 3. *HI antibody titres against influenza virus A2/Eng/12/64 after vaccination with heterologous* (*HK*) *and homologous* (*CP*) *influenza A virus vaccines*

Group	Vaccine schedule		No. of persons in group	No. with pre-vaccine antibody	No. with ⩾ 4-fold rise in antibody	No. with final titre ⩾ 48	Geom. mean titres	
	IN	IM					Pre-vaccine	Post-vaccine
A	Saline	CP	67	60 (90 %)	59 (88 %)	63 (94 %)	17·4	220·0
B	HK	CP	60	58 (97 %)	50 (83 %)	55 (92 %)	23·1	215·6
C	Saline	HK	56	51 (91 %)	31 (55 %)	47 (84 %)	24·0	108·5

Table 4. *The effect of CP and/or HK vaccines in producing homologous or heterologous HI antibody responses in individual volunteers*

Group	Vaccine schedule		No. of volunteers with ⩾ 4-fold rise in antibody after vaccine				
	IN	IM	Both HK + A 64	HK only	A 64 only	Neither	Totals
A	Saline	CP	35	5	24	3	67
B	HK	CP	33	5	17	5	60
C	Saline	HK	31	22	0	3	56
Totals			99	32	41	11	183

However, the greatest response was found in group C, where injected HK vaccine induced a 48-fold rise in geometric mean titre (GMT) overall, and the conversion rate to titres of 1/48 or greater was 70 %. In group B the intranasal spray of HK vaccine given in addition to injected CP vaccine gave results little or no better than in group A given CP vaccine alone. In both groups less HK antibody was induced than in group C, e.g. the GMT rose 7·7-fold in group A and 10-fold in group B, and the conversion rate to titres of 1/48 or above was only 31 % for group A and 38 % for group B.

The production of A 64 antibody

Antibody against A 64 virus was found before vaccination in 169/183 (92 %) volunteers (Table 3). Individual titres varied from 1/6 to 1/384 but the GMT did not differ significantly from group to group.

The recall of antibody to the Asian strain by CP vaccine was greater than by heterologous HK vaccine. In groups A and B the GMT increased by 12-fold and 9-fold respectively and more than 80 % of volunteers showed a 4-fold or greater rise in titre. On the other hand, the GMT of group C increased only by 4·5-fold and only 55 % showed a 4-fold or more increase in titre.

The independence of immune responses to CP and HK vaccines

The results shown in Tables 2 and 3 make it unlikely that A 64 and HK viruses were merely inducing or measuring the same antibody with differing efficiency. Further evaluation of individual responses (Table 4) confirmed that A 64 and HK antibodies were independently variable. Thus, in group C, given only HK vaccine, 22/56 (27 %) showed a 4-fold or greater increase only in HK antibody, and none had an increase in A 64 antibody alone. In groups A and B, on the other hand, CP vaccine produced an isolated increase in HK antibody in only 10/127 (7·9 %) volunteers, whereas A 64 antibody alone increased by 4-fold or more in 41/127 (32 %). Even in the 99 volunteers who developed a simultaneous increase in both antibodies there appeared to be some specificity of response, i.e. there was no consistent relationship between the height of the HK and A 64 antibody responses. HK antibody increased by more than 16-fold in 25/35 (65 %) of group C, but only in 22/64 (34 %) in groups A and B.

The effect of neuraminidase antigen in HI tests

The viral neuraminidase of the HK strain is antigenically similar to that of the foregoing Asian strains (Coleman *et al.* 1968), and thus both types of vaccine used in the present trial induced, in addition to the HI antibody responses already described, a common enzyme-inhibiting antibody in each group of volunteers (G. C. Schild, personal communication).

It thus seemed possible that the haemagglutination-inhibition observed when HK virus was titrated against sera from volunteers given heterologous CP vaccine might not be the result of cross-reacting antibodies against the HA antigen, but attributable to uptake by the virus particles of common anti-neuraminidase antibody, with steric hindrance to haemagglutination.

Accordingly, pairs of sera from groups A and C which showed a high degree of cross-reactivity in HI tests were re-titrated against a genetic recombinant virus A/3/1/A (kindly provided by Dr G. C. Schild, World Influenza Centre, London) which contains only the neuraminidase antigen of HK virus, but the HA antigen of fowl plague virus. None of 20 serum pairs from group A volunteers who had

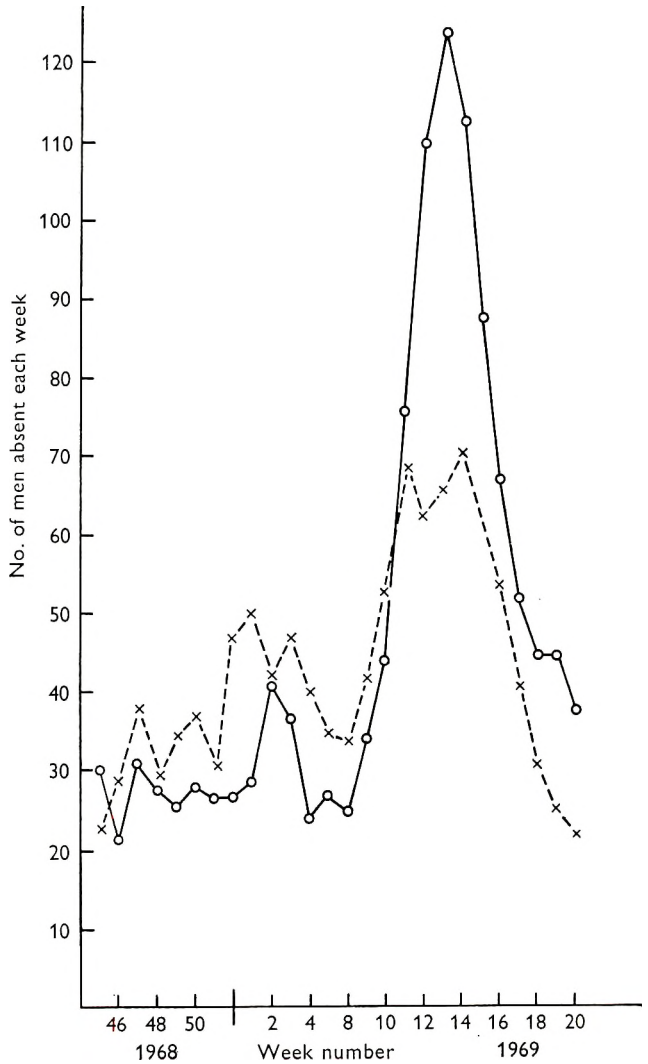

Fig. 1. Sickness absence caused by respiratory infections in the general factory population from which volunteers for influenza vaccination trial were drawn. Combined figures for four main factory sites only, for week 45, 1968, to week 20, 1969. Number on payroll, 3580. O—O, Upper respiratory tract infections; × – – ×, lower respiratory tract infections.

shown a 4- to 8-fold increase in HI titres against the heterologous HK virus gave any detectable HI with A/3/1/A virus. Similarly none of 25 serum pairs of group C volunteers who had shown 4- to 8-fold increases in HI titres to the heterologous A 64 virus showed any HI activity against the A/3/1/A recombinant.

It thus seems improbable that the heterologous antibody responses to the vaccines demonstrable in HI tests were the result of interference by common anti-neuraminidase antibody.

Clinical investigations

There were no untoward effects of the vaccination procedures. In the general factory population (Fig. 1) the incidence of both upper and lower respiratory tract infections began to rise after week 8 of 1969, and the total peak incidence was 190/3580 (5%) in week 13. This did not fall to the 8th week level again until week 18, 2 weeks before the conclusion of the trial. These weekly total figures include all types of respiratory disease, including exacerbations of chronic lower respiratory tract disabilities, and influenza was not the predominant clinical syndrome in any week. However, illnesses clinically diagnosed as influenza occurred sporadically between weeks 8 and 20 of 1969; in week 11 two strains of influenza virus antigenically identical with HK virus were isolated from men reporting sick, and in both cases a 4-fold or greater rise in HK antibody occurred over the courses of their illness. In a further 9/20 other persons with acute respiratory disease, paired sera also showed an increase in HK antibody; the first of these was in week 9 and the last in week 18, 1969. None of these confirmed cases of influenza was a member of the trial groups.

Table 5. *Sickness absence caused by respiratory infections in volunteers inoculated with HK or CP influenza vaccines (week* 45, 1968 *to week* 20, 1969)

Trial group	No. in group	No. of men with respiratory illness	Working days lost	No. of men with 'clinical influenza'	Working days lost by 'influenza'
A	545	82	481	16	88
B	491	80	494	14	77
C	508	77	473	8	52

Table 6. *The incidence of clinical influenza in weeks* 11–14, 1969, *in volunteers previously inoculated with HK or CP influenza vaccines*

Trial group	No. in group	No. of men ill	Sickness rate (%)	Mean duration of illness (days)
A	545	6	1·1	7·7
B	491	7	1·4	5·0
C	508	3	0·59	4·7

Respiratory illness amongst the volunteers (Table 5) followed the pattern of the general factory population. The incidence of respiratory illness throughout the winter was investigated in 1544 of the original 1601 volunteers; the remainder left employment or their work took them to other Divisions of the Company before the completion of the trial period. No schedule of vaccination had any significant effect on the total number of men with respiratory illnesses or on the mean duration (6 days). Illnesses clinically diagnosed as influenza occurred in only 38/1544 (2·5 %) volunteers; 16 of these were in group A given only CP vaccine

and 8 in group C given only HK vaccine, i.e. an influenzal rate of 2·9% with a mean duration of illness of 5·5 days in group A, and a rate of 1·7% with a duration of 6·5 days in group C.

A dilution effect, by non-specific 'influenzal' illnesses not caused by influenza A viruses, could possibly have obscured the true picture in these data covering the whole winter. Hence, the clinical influenza attack rate was calculated for each trial group over the period weeks 11–14 1969 when the presence of HK in the general factory population had been proven (Table 6). With such low rates of influenzal illness statistical evaluation would have little meaning, and the slight apparent advantage of HK vaccine to group C in both the short- and long-term survey may be merely adventitious.

DISCUSSION

Monovalent HK vaccine, composed of inactivated A2/Eng/344/68 virus, was more efficient in producing serum HI antibody against the new epidemic strain than was CP vaccine containing A2/Eng/76/66 and A2/Eng/12/64 viruses, the only preparation generally available in autumn 1968. Of those injected with HK vaccine, 95% showed a 4-fold or greater rise in HK antibody titre, and the mean post-vaccine titre in this group C was 96·5. In contrast, although CP vaccine injected alone in group A gave a 4-fold rise in HK antibody in 60% of volunteers, most of their post-vaccine titres were low, and the mean titre of the whole group was only 14·1. As far as specific serum antibody was concerned, the intranasal spray of HK vaccine given to group B achieved nothing. The possibility of induction of nasal antibody, particularly IgA, by this procedure was not investigated, and the clinical attack rates of influenza in all groups were too low for any protective advantage of such local immune stimulation to be inferred.

The apparent cross-immunization against HK virus by vaccines containing A 64 is somewhat surprising in view of the apparently distinct antigenic constitution of the haemagglutinin of these strains in laboratory tests with animal antisera. However, this heterologous response was considerably less than the homologous response to HK vaccine, as indicated above, and may not have the same significance as far as immunity to HK infection is concerned. Most of the volunteers were over 35 years of age, with a wide range of antibodies against foregoing epidemic strains of influenza A. It seems probable that with such an immunological background the antibody response would be less narrowly specific than in laboratory animals not previously exposed to influenzal antigens, and that the serum reactions against HK virus induced by CP vaccine may represent 'poor fit' heterologous immunoglobulins with kinetics of neutralization quite different from those of true homologous immunoglobulins induced by HK vaccine. The HI test gives no true measure of 'goodness of fit' or avidity of antibody to HA antigen, and the clinical rates of infection in the present trial were too low for the protective quality of HK antibodies in groups A and C to be compared.

The enhancement of heterologous A 2 antibodies by HK vaccine is in agreement with many earlier experiences. Newly emerging antigenic variants may recall antibody induced by earlier epidemic strains which have been experienced

by human populations, either because the new strain continues to carry small amounts of antigens predominant in earlier strains, or by mal-recognition of a new stimulus by previously committed clones of immunocytes.

Although monovalent HK vaccine may thus help to maintain a pre-existing immunity against A 2 viruses, it is doubtful if it would induce A 2 antibodies in those lacking previous experience. In the present trial, 12 volunteers were without prevaccine antibody to Asian viruses; 6/7 developed such antibody after CP vaccine, but 0/5 after HK vaccine.

Preliminary observations on sera from the present trial with a sensitive enzyme-inhibition test have shown (G. C. Schild, personal communication) that anti-neuraminidase antibody was produced by both types of vaccine. The significance of such antibody in reducing the spread of influenza virus infection has been suggested in animal experiments (Schulman, Khakpour & Kilbourne, 1968) but the low attack rates in the present trial did not allow this aspect to be investigated further.

It is clear therefore from the present trial that an inactivated polyvalent vaccine containing only pre-1968 strains of Asian viruses can no longer be relied upon to produce uniformly distributed high levels of serum HI antibody against current epidemic strains of influenza virus. In the past it has always appeared that such vaccines (i.e. which fail to induce specific HI antibody) would fail to protect in the field (Hobson, 1967). A monovalent vaccine of HK virus, however, did stimulate high titres of specific HI antibody in almost all volunteers and would seem to be the essential basis of future vaccine formulations over the next few years, unless another new antigenic variant of influenza should emerge. If, however, Hong Kong and Asian strains should continue to coexist a polyvalent vaccine of both types of virus would probably be necessary to maintain a sufficient breadth of immunity.

We are grateful to the staff and volunteers in Mond Division, I.C.I. Ltd., for their wholehearted co-operation in making this trial possible, and it is a pleasure to thank members of the Medical Department, Mond Division, and of the Department of Medical Microbiology, University of Liverpool, for their skilful organization of the vaccination sessions.

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Printed in Great Britain

The irreducible minimum in cross-infection control and isolation nursing

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(Received 16 February 1970)

SUMMARY

This is a survey of 10 years experience in trying to find out how to control hospital sepsis economically. We appear to have obtained reasonable improvements in hygiene by applying the teaching which has been given to students since the time of Leonard Colebrook. For our investigations we used only routine methods.

The order of importance of the factors in reducing cross-infection would appear to be: (1) single rooms for all septic cases and for those requiring protective isolation; (2) a sister supported in complete authority over anyone entering the unit; (3) a simple, inflexible drill to introduce an impermeable layer between the nurse or doctor and the patient—gloves and apron provide this; (4) overshoes and barrier mats; (5) hygienic disposal of linen; (6) an efficient wet dusting and floor cleaning system. Anything beyond this must be justified by saving of nursing time or some factor other than prevention of infection.

PRELIMINARY SURVEY

When the then Ministry of Health asked that Control of Infection Officers should be appointed, Dr John Ackroyd and I looked into local conditions and our findings formed the basis of later work. A record of all cross-infection was made and ward was compared with ward and surgeon with surgeon. Later a study was done of the working conditions in all wards. It might be supposed that the correlation would be between the lack of convenience and the sepsis rates—there was none. Some of the worst wards had the best records. The most eccentric surgeon, often quoted as an excuse for bad practice and clinical behaviour, was found to be faultless when judged by post-operative sepsis. It was also embarrassing to find that the private block had no cross-infection, at a time when the main hospital had had 23 fatal and 460 other cross-infections in the year. The ward assessment had been subjective but we were able to apply more accurate measurement when standards were published for hospital building and for offices.

We marked each ward or department against the standards suggested by Florence Nightingale (many of the buildings are of her period), and in those matters not dealt with by her we used the minimal standards for hospitals (Building Note no. 4). For staff facilities we followed the Bill, afterwards the Offices and Factories Act, 1963. Taken together these gave 14 heads for judging a ward, ranging from bed spacing and sterilizing facilities to changing rooms and staff

lavatories. Each was marked out of 10 without any weighting. The lowest score was 56 (40%); even our most recent ward, less than 30 years old, fell short of requirements.

There were two interesting parallels however. Where sepsis was prevalent, members of the nursing staff were being referred to the Student Health Service for psychiatric help, and the Medical Social Workers were receiving various complaints from patients' relatives. This suggested that good morale was more important than the physical environment. Since the private beds appeared immune, although mainly supplied with temporary staff and having inferior theatre facilities, the main objective of our policy became the provision of isolation cubicles in the main hospital, which had none at all.

It is not possible to arrange material in chronological order since many points were investigated in parallel and the results applied as opportunity and money was found.

SHEDDING AND SPREADING INFECTION

Dirt, alive and dead

Popular anxiety about cross-infection was followed by letters both in the lay and medical press pointing out flaking paint and other evidence of decorative neglect and claiming that this was responsible. There was confusion here between general untidiness and the presence of pathogenic bacteria, and limited resources might have been wrongly directed. To discover where the true danger was, we sampled areas of 100 cm^2. with a swab moistened in a tube of 2 ml. of nutrient broth, using masks cut in X-ray film (for conveying the findings to the cleaning staff this is conveniently about the size of a slice of toast). The swab was then shaken in the remaining broth and the organisms present counted by plating out.

Results showed that the area of greatest risk was always the floor. Horizontal surfaces at other levels, locker tops, window sills, etc., were about one-tenth as affected, while vertical surfaces, whether absorbent bed curtains or painted walls, were of insignificant importance.

For example, when a patient with a fatal *Staphylococcus aureus* infection was being nursed, the wooden floor gave total counts of 30,000, with his pathogenic strain 3000, per 100 $cm.^2$. The counts fell off rapidly up the wall until, level with his head, a total of 15 organisms was found, none his own strain. We therefore direct our main effort in cleaning to horizontal surfaces and to the dust in the air.

Dirt in our area is itself relatively harmless. We were fortunately in possession, in the laboratory, of a cupboard that had remained unmoved for over 20 years. The dust was 1·0 cm. thick and weighed amounts could be taken. It was surprising to find counts as low as 100 per g., all spore-bearers. The dust was acid, pH 4·0, and probably self-disinfecting.

Studies on air-borne particles had shown that the infective particle was much larger than single bacteria, 11–14 μ (Lidwell, Hoble & Dolphin, 1959). Using the skin shedders in our wards, Davies & Noble (1962) were able to demonstrate that the infected skin squame was the element responsible for transmission from patient to patient.

The extent of skin carriage is illustrated by a patient during a widespread outbreak. Normal persons shed between 100 and 2000 organisms while undressing and redressing in a cubicle. This patient while merely taking off and replacing his pyjamas shed 30,000 staphylococci of the epidemic strain. About 0·1 g. skin scales could be recovered from his pyjamas per hour.

Hospital linen

The role of sheets and blankets has been assumed to be to provide fluff as a vehicle of transport for pathogenic organisms; the fact that it is the skin squame that is responsible does not alter the importance of the linen. We found that instructions, aimed to prevent the theft of linen during the war, were still being followed. The night staff in wards and theatres was tipping all soiled linen, even that from heavily infected patients, onto the floor, sorting it into piles and then packing it in canvas bags for transport; no separate clothing was provided for this work and the bags were not sterilized. Wound dressing was done in the contaminated ward environment so created.

We substituted plastic bags, red for infected and foul linen, and white for the rest. Counting was stopped; losses of linen have not increased as it is not at this stage that they occur, as it is clean linen that is taken—who wants foul?

Sucking and blowing

In our Children's hospital Bate had demonstrated the spread of intestinal pathogens by a vacuum cleaner (Bate & James, 1958). Redesigning of these tools followed his report and now all sweepers are fitted with disposable paper lining bags. We endeavoured to get rid of all alternative forms of dry sweeping. This required a prolonged battle against the maids by the Domestic Supervisor. No sooner had all brooms been confiscated from the wards than they transferred them from other areas. These were in turn removed, only to be replaced by more from as far away as the nurses' homes and the laboratories. Only after 3 months was it possible for colleagues in the staff ward to be spared the sight of mops and brushes being shaken from upstairs windows and the dust floating down into the maternity ward.

Bate pointed out to us that suction cleaning carried with it the risk inherent in the venturi effect, the exhaust creating a cloud of dust behind the machine. We use extension hoses for all procedures, keeping the sweeper itself outside the ward as long as possible and moving it on to a cleaned patch of floor thereafter.

Once we had realized that all machines that sucked also blew, we directed our attention to all pumps, aspirators, etc. A suction line had been built into the operating theatres. When suction was required, in removing blood and pus from the peritoneum for example, the nozzle and tube were connected through a collecting bottle to this line. There was no filter in the system. The pump was sited at the most inaccessible position on the roof. We sampled the exhaust, and collected on a moistened disk, 2·5 cm. in diameter, exposed for 1 min. at a distance of 30 cm. 440,000 *S. aureus* of an epidemic strain. We had established a continuous culture of staphylococci with diluted blood as nutrient. By siting the pump

up-wind from the window of the sterilizing room it was possible to feed back into the theatre an aerosol of these organisms. The problem was dealt with by introducing formalin into the system and, as soon as possible, providing filtered air to the suite.

Even the provision of filtered air is not fool-proof. One of the staff required to change filters objected to the climb onto the roof. He economized his efforts by cutting hard-board to fit the carriers. When this was discovered, he justified himself by saying that in any case the ordinary filters only got dirty.

On other occasions selective culture systems were established. An eye infection with a Gram-negative organism drew our attention to the antiseptic hand-washing cream. The organism was present in the cream-dispenser's reservoir in pure culture. The dispenser was connected to a foot-pump which drew in air at floor level and over a period of months a resistant organism had been introduced. It is difficult to convince staff that containers for antiseptics must be heat-sterilized before refilling (in the case of liquid-soap dispensers mounted over sinks this is virtually impossible). The equipment was heat-sterilized and the technician devised a simple cotton-wool filter and inserted this into the input tubing. However, the same mistake was repeated not only at other hospitals but in two of our own theatres in the group, from a failure in reporting widely enough.

A similar selective culture system was established in the drains of a new theatre, which was constructed over a ward, without much space between its floor and the ceiling below. Although to be used primarily for orthopaedic work, the theatre was not provided with a plaster trap and repeated blockages of the drains ocurred. One of the porters who had to clean out and unstop these scratched his hand and developed an infection with a Gram-negative rod. Cultures of the drain showed that the slow flow had permitted a build-up of the strains of *Pseudomonas aeruginosa*, *Streptococcus faecalis*, *Proteus retgeri* and an atypical coliform, all resistant to the antiseptics then most used—benzalkonium chloride, chlorhexidine and hexachlorophene.

Anaesthetic and similar apparatus

An examination of all apparatus activated by pumps made it clear that in their design no attention had then been paid to the exhaust. We were able to correct this with only the simple addition of filters, made by the instrument curator. This has now become standard commercial practice.

An example of the risk involved in unfiltered aspirators was the frequency of streptococcal infection occurring in other children in a ward when it was necessary to suck out the fossae after a tonsillectomy. Again this was cured by introducing a filter into the exhaust, a method the manufacturers have adopted. Tests of the current commercial filters show that even when they are due to be discarded the external side is still sterile.

The incubators for premature babies received early attention, following a series of infections, usually with pseudomonads. Faults both of design and of management were discovered. The humidifying system had to be topped up with water through a filler with a hinged cap; this was incompletely closed. The instructions

were to fill with distilled water, but it was not specified that this should be sterile and from a sealed bottle and that bottles should be used once only and then discarded. We had long known that distilled water with only atmospheric gases was an adequate culture medium; we found that up to 400,000 organisms per ml. could be supported on this alone. Leifson (1962) has made the same observation.

Since disinfection of these incubators was so difficult, after any infected case the work was entrusted to a commercial firm (Vickers Medical Instruments) who used formalin. Ethylene oxide is also suitable. Tests with bacteria show that the relevant organisms, including *Str. faecalis*, *Ps. aeruginosa*, *Escherichia coli* and *Proteus* spp., are killed by these methods. Between cases the routine cleaning is done with chlorhexidene 0·5 % in 70 % spirit on all accessible surfaces, followed by filling the humidifying system with hypochlorite solution, at the strength used for the feeding bottles, and leaving this for at least 2 hr.

Anaesthetic apparatus itself has always been a focus of anxiety, though proof that it has ever been responsible for cross-infection is lacking. It is always felt that after a patient with tuberculosis or other lung disease has been anaesthetized there is a risk to subsequent patients. In fact, there is a no-return valve in the system and the only parts of machines from which organisms could be returned to the patient are on his side of this. The tubing and masks can be boiled or otherwise disinfected between cases. We were fortunate in finding tubing and connectors which had not been disinfected for 3 months. Swabs from 10 cm. of each end of the elephant tubing were taken, and from the metal joints. Plate counts of up to 2 organisms were found; even these were contaminants since the broth cultures were sterile. This agreed with many previous investigations of apparatus of this sort. The joints were not so satisfactory, up to 10 *S. albus* being found.

The self-disinfecting nature of some rubber is well known, but it was thought that the anaesthetic gases themselves might be sterilizing the apparatus. Nitrous oxide, fluorethane, trilene, penthrane, and cyclopropane were introduced into anaerobic jars in their working concentrations and a full range of upper respiratory organisms exposed to them. They all grew as well as in air alone.

We conclude that the minimum requirements for anaesthetic apparatus is boiling or chemical disinfection of the mask, valve and bag for each case; where special anxiety is felt over any special risk, boiling of all parts on the patient's side of the no-return valve. Although it is usual to send respirators for gas sterilization, this is not strictly necessary where a simple bacterial filter is introduced into the system on the patient's side to protect the apparatus. This alone might be enough to provide a harmless exhaust for the protection of staff and other patients but an exhaust filter is now fitted.

Transmission of organisms

Apart from the spread of microbes by currents of air, there is the part played by individuals in moving them round the hospital. In spite of Semmelweiss, we found the post-mortem room still was a source. It had cracked floors that could not be efficiently cleaned, and teaching and demonstrations were still carried out there irrespective of the nature of the disease or the virulence of the organism

causing death. An examination of the corridors leading from the mortuary to the rest of the hospital showed a much higher count of organisms, particularly from the bowel, than anywhere else. Two groups of students attended a tutorial and their shoes were swabbed. The first group had faithfully attended the P. M. demonstration, then walked a quarter of a mile and climbed six flights of stairs before swabbing; the second group had cut the demonstration. The subject had died with a staphylococcal pneumonia (4 Feb. 1959). Twelve shoes were cultured for each group.

Test group

On MacConky's medium 1–12 Gram-negative rods
Salt mannitol agar 80–440, average 288, *S. aureus*

Controls. No Gram-negative rods, no *S. aureus*. Technicians in the routine laboratory in a different wing of the building, however, gave 0–1 Gram-negative rods but 105 *S. aureus*.

The persistence of contamination of shoes was investigated by painting the sole of a technician's left shoe with *Serratia marcescens*. At intervals areas of 6 cm.^{2} were swabbed and counts made; the results are shown in Table 1. During the time while the shoe was being worn the girl was working, mainly standing, or walking to a canteen.

Table 1. *Survival of* Serratia marcescens *on shoes*

	Number of colonies from areas of 6 cm.^{2}				
	15 min.	30 min.	1½ hr.	2½ hr	4½ hr.
Left (infected)	+ + + +	240	60	75	1
Right (control)	18	12	0	0	0

+ + + + = uncountable.

If this tracer organism is any guide, then bacteria persist long enough to reach any part of the hospital. We felt it was justified to remove the teaching of clinical students from the P.M. room. Repairs to the floors were followed by total counts reduced to 15–70 per 100 cm.^{2} while that for the stairs and doorway was 4000, since here the structure was wooden and not so easily dealt with.

The assumption that only shoes of hospital staff carry pathogens is untrue. We obtained swabs from a shoe repairer's shop not near any hospital. Although the average counts were lower, *S. aureus* 25 per shoe, two gave confluent growth.

ATTEMPTS AT CONTROL

This information, together with the realization that the best way to sample the flora of a ward was to swab the trolly wheels, has permanently biased our barrier-nursing technique. The extent to which the floors represented a reservoir is indicated by the *S. aureus* count of the six main surgical wards at the time—range, 910–2540; average, 1830/100 cm^2. These were wooden floors. Where we have obtained terrazzo, linoleum or plastic covering for them the staphylococcal counts remained lower. Sealing the wood floors with polyurethane has also helped.

Failure to control epidemics without isolation facilities

The control of the spread of staphylococcal infections became almost impossible when a neomycin-resistant staphylococcus, then provisionally grouped as D/77Ad/B5, now 84/85, was introduced into the wards. This occurred in other hospitals that year, and as they have published their experiences we have not previously done so (Quie, Collin & Cardle, 1960; Temple & Blackburn, 1963; Jacobs & Willis, 1963). From 16 August 1962 until December 1963 we maintained an unbroken series of 123 cases, all direct contacts of one another.

Every attempt to stop the spread in a ward by closing it and cleaning it was futile. This was mainly because there were always some cases too ill to be sent elsewhere and because we had no cubicles. For example, in one ward block there were two male and two female wards separated by a central corridor. There were 28 beds in all; the larger had nine beds each, the smaller five. Repeated infections with this staphylococcus persuaded us to close and clean the ward block; all but four patients could be sent home or elsewhere. These remaining cases were confined to one small ward. Among the four, all men, was one with a sinus in the hip infected with the staphylococcus and also a pseudomonas. All other beds were stripped, disinfected and made up with fresh linen and the ward cleaned thoroughly. This was completed by 4 p.m. on one day. At 10 a.m. the following day the beds were sampled by sweep plate. The staphylococcus was already present on 11 of the unoccupied beds, the pseudomonas was on nine. Both were together on seven of these. This patient was a profuse skin shedder.

Attempts at fumigation with formalin vapour were also disappointing. As judged by the usual testing methods, using plates inoculated with a suitable tracer organism and distributed about the room during fumigation, formalin was successful in small wards. A large ward with 27 beds was prepared for fumigation by sealing the windows, a process taking more than a day for two of the maintenance staff. The method of distributing jars containing formalin solution at intervals and then dropping in permanganate of potash was attempted. So violent and unpleasant was the result that they were driven out after only one end had been dosed. The local fire officer sent a team with closed-circuit respirators to repeat the job; this time all pots were activated. Unfortunately plates were not sterilized and 3 days' work was lost. It was clear that only true isolation could solve our problem.

The isolation ward

The only space in the hospital that was free was a floor used as nurses' bedrooms. There appeared to be room for eight cubicles, with the necessary service areas. It was planned to divide the space so that four cubicles could be used for highly infected cases, and the remainder for those needing protective (or reversed) barrier nursing. The latter cases included kidney grafts, aplasias, and similar blood conditions. The committee planned the unit with an air-lock and facilities for changing, between the clean and infected areas. Perhaps fortunately, when the unit opened some months later, one cubicle, the changing area and the air-lock had become a laboratory; it was some time before I realized what had happened.

The sister appointed to take charge of the unit had recently left the Navy, so, when instructed to take both clean and infected cases and to nurse them together, she obeyed orders and did so. At the end of 3 months it was realised just what was happening, but it was also realized that no cross-infection had taken place. The situation was allowed to continue but with the more careful monitoring of the staphylococci of all patients and staff (this has continued until now, 8 years later) The next 6 months' record is given in Table 2.

Table 2. *Mixed barrier nursing: six months' record of seven cubicles*

Infected cases	40
For protective isolation	15
Staphylococcal carrier state	
Uninfected, not a carrier	15
Infected with hospital strain	
Became a carrier	11
Did not become a carrier	10
Carried own strain	2
Infected with own strain, carried own strain	4
Infected with other organisms	
No *S. aureus* carried	8
Carried own strain	3
Carried hospital strain	2

Carriage in the last two patients began outside the unit.

A second major innovation was similarly introduced by accident. It was intended that each cubicle should be entirely self-contained and that nothing should be moved out or in, except food. Everything possible was to be incinerated, and linen sent to the laundry in sealed bags. All was ready for the ward to open when it was found that nothing had been provided for the nurses to wear. As the situation was serious and the beds urgently needed, we sent out and bought housewives' plastic aprons and plastic overshoes, and these formed, and still form, the total protective clothing used, supplemented only with an unlimited supply of sterile gloves. No masks, gowns or head coverings were issued; so that the preparation consists of putting on shoe-covering, or a special pair of shoes or sandals which never leave the unit, and an apron and gloves; similar views have been presented by Hare (1964). After attending a patient, the nurse washes her apron with a sponge from a bowl of 2% benzalkonium chloride, the gloves having been washed on the hands in the cubicle hand basin, then dries off the apron and gloves on a paper towel and goes on with the next case. This modification to the traditional ritual saved at that time £500 p.a. With our present 14-bedded unit, now in the maids' attics, and the grafting unit replacing the previous isolation ward, the saving in laundry alone is about £1,500 p.a. The number of nurses required where there is no robing and scrubbing-up ritual between cases is far less than with more elaborate methods.

Incineration

The reasonable idea that no infected material should be carried through the hospital led to a fiasco. An incinerator was built into the unit next to the sluice and leading from it; it was hoped that all disposable dressings and similar materials would be burnt. The amount of material was underestimated and so the fire blazed night and day. This added greatly to the work of the night staff when no porters were available. The shovel was so heavy that clearing the ashes was a phsyical impossibility for the smaller nurses and maids. The heat generated in a room with limited ventilation stripped the tiles from the walls and split the brickwork. The disposable rubbish is now collected in paper bags which have the closed top turned over and stapled; they can then be taken safely to the main boiler house.

Visitors

The contribution of visitors to the pool of infectious organisms required study. We already had information on shoes; it was necessary to examine the clothes also. Three batches of persons were examined, visitors, porters and house officers; swabs were taken from the trouser turn-ups and cultured. Visitors had no pathogens there; house officers up to 3000 staphylococci per turn-up of the epidemic type; the porters were intermediate. The second sister on the ward had had psychiatric training, and she pointed out the need for unlimited visiting. This is now permitted, the only precautions being that the visitors, like anyone else, wear overshoes. All patients also have television sets, which are self-sterilizing inside and merely need a wipe over with antiseptic on the casing. It was the same sister who insisted on the ward being given a name, as this removed the supposed stigma from isolation, which is connected in the mind of the public with 'pest house'. Once patients were in the ward the special care was appreciated. One patient, with experience of both a London nursing home and our own private beds, on leaving thanked the sister, saying that she would advise all her friends that if they had to be ill they should be sure to be septic.

Barrier mats

The realization that staff and visitors on their shoes and the trolley wheels carried numbers of organisms persuaded us that some sort of antiseptic barrier was required. Overshoes such as those used in the chemical industry were provided, but any plastic or rubber shoe or sandal will serve. These are adequately disinfected every time they pass over an antiseptic mat; The inside flora is unimportant. They are not sterile but their organisms are diluted to a safer number; operating-theatre counts are not required in corridors. We have tried cellular mats filled with liquid antiseptic; these make a mess of the corridors: mats with sticky surfaces which always look dirty; it is possible to step round the edges because of their small size; and plastic fibre carpets with static charges which hold dust. All appear to work, as the count of organisms on the floor beyond is much less than the approaches. Sponge rubber or cellular plastic sheet is useless as it is torn to pieces by trolley wheels. Detergent antiseptics should be used as they destroy

ordinary shoes by detaching the soles, so people soon learn to use the overshoes provided. The mat must block the corridor completely and be not less than two paces long; no one will actually wipe his feet.

The conclusions we have reached are very close to those of Ayliffe *et al.* (1967). Mats are useful if used with overshoes. They also serve to reduce the numbers of persons tramping through the unit and were they chemically useless might still serve a valuable psychological purpose.

Antiseptics

Although the indiscriminate use of antibiotics is probably the most wasteful thing in the hospital budget, the careless use of antiseptics must run it a close second in some places. We ourselves began to bring order into the chaos by limiting the range and concentrations. For example, four preparations of iodine were in use ranging from 0·5 % to 2 %; three were made up in full strength spirit. We reduced the range to one and at the same time saved over £1000 p.a. by diluting all spirit used as an antiseptic or a vehicle to 70 % and so increased its bactericidal power. The total number of antiseptics was reduced to one of each type; this gave economy in buying, storing and dispensing. Care was taken to see that none were incompatible with the detergents used in cleaning. After this our failures were due to misunderstandings.

A hypochlorite preparation was chosen for use in sinks and sluices. While drinking coffee, we were nearly choked by the reek of chlorine from a nearby cubicle. The maid was pouring the neat solution all over the floor. It was found that the consumption had risen to £500 in three months. The reaction of the supply department was to arrange a bulk contract. This brought in an unlimited number of gallon containers with handles and spouts. These being plastic were ideal for filling car radiators, batteries and greenhouse heaters, so the maids were encouraged to empty them. It was clear also that the maids had no idea of the working strength. The supply department had issued instruction in English, saying how many ounces should be used to the gallon; the maids spoke only Italian or Spanish and understood only litres. The amount used by them in three months if properly diluted would, at working strength, have filled a standard swimming bath three times. The dilution and distribution was placed under the pharmacist. In his hands the cost in the next three months fell to £22 10*s*.

A similar situation occurred with our stock solution of benzalkonium chloride. When it was discovered that this was being used undiluted measures were ordered for each ward. This produced no improvement, and when asked, the sisters assured me that no measures had arrived. They were found in the instrument cupboards marked 'For lotions only'. No one knew that lotions were antiseptics so had not used them for anything. We solved the problem by tying a red plastic mug to the handles of the buckets and adjusting the stock so that one mug went to a bucket of water. Field trials showed that the average maid half fills a bucket.

It is assumed that instructions given to domestic staff will be carried out; they, however, introduce their own modifications. There were a series of Gram-negative infections, chiefly with pseudomonads, in the renal graft unit. A phenolic

disinfectant was demanded as a substitute for the benzalkonium chloride previously used. Within a few days complaints were received that the floors were filthy. This was due to the interaction of the rubber soles of overshoes and the antiseptic, the black marks left were almost indelible. Help was sought from the domestic staff of the hospital popularizing this phenolic. Their supervisor explained that they used the antiseptic as instructed and immediately neutralized it with a detergent. The medical staff were satisfied with the smell of phenol, the domestics with the clean appearances of the place. The fact that the phenol was given no time to act had not appeared to them important. The benzalkonium preparation that had been used throughout, although in the laboratory apparently inadequate against pseudomonads, did in fact leave the floors free of them when used.

The infections in this unit led to extensive investigation of the environment. Among the places from which pseudomonads were recovered were the plastic mop-heads—not only those in use but those fresh from the factory. Leigh & Whittaker (1967) showed that the benzalkonium chloride was neutralized by the mop. A full confirmatory study has now been made by Colquitt & Maurer (1969), who compare a number of antiseptics and the effects of mop materials.

RESULTS

Very fortunately soon after the work began a staphylococcus phage-typing unit was established by Professor R. E. O. Williams and it was this that enabled us to record the phage type of every organism isolated from the weekly swabbing of all staff and patients' noses and all wounds.

On the evidence so obtained we can state that there has been no cross-infection of clinical significance since the unit was opened. It should be pointed out that patients with aplasia, leukaemia, burns, radiation accidents and those on immunosuppressive drugs have been nursed by the same nurses at the same time as they were also nursing the most infectious septic cases in the hospital; the two groups were in succession in the same cubicles, or next to one another at the same time.

Up to the end of the experimental period of 9 months, nasal cream containing antiseptics and antibiotics was used. The observation that patients carrying their own strains of staphylococci in the nose did not acquire other strains either from the environment or from their wounds encouraged us to discontinue nasal creams and there is no clinical evidence that this was wrong. Although no clinical cross-infection has occurred there has been a low level of cross-contamination of the environment detected when persistent and exhaustive studies were done as part of an intensive study of the ventilating system (Williams & Harding, 1969).

The most severe test of the adequacy of the method used was in the case of a young woman who had elsewhere been given three times the maximum dose of radioactive gold. The complete aplasia from which she suffered showed no signs of recovery for 7 weeks but recovery was subsequently complete. During her whole illness she developed no infections.

The importance of the technique of barrier nursing and of cleaning demands special teaching efforts. Particularly with the maids, who at the start know little

English, 'in post' teaching is required. The King Edward Hospital Fund, at the Hospital Centre, arranged for teaching films to be made by us with Camera Talks. At least now everyone knows what is supposed to be done daily, weekly and after an infectious case.

The success of the teaching has brought its own troubles. Two luxury hotels have recently opened in our area; the first took 32 of our best girls and the second another 18; this is a tribute to the teaching but not to the pay scales, nor do our staff quarters compare with those in a hotel.

Assessment of a campaign against cross-infection is particularly difficult since the organism at first most important in our hospital, the penicillinase-producing *Staphylococcus aureus*, can now be treated with a range of antibiotics and no longer dominates the picture. Indeed our problems are with organisms of relatively low pathogenicity, which have taken advantage of situations of lowered immunity in the patients on steroids or immuno-suppressive drugs, and which are as likely originally to have formed part of the normal flora of the patient or part of the hospital environment.

We have, however, attempted in two ways to measure our results. The first is to record the staphylococcal coincidences, the occurrence of the same phage type in two cases in the same ward or unit within a month. In the case of infections in the new-born this is ignored since we know that in them all infections are hospital infections.

Table 3. *Staphylococcal cross-infections*

Year	Staphylococcal coincidences	Total staphylococcal infections	
1959	460	—	Incomplete record
1960	240	—	Incomplete record
1961	80	376	—
1962	113	456	50 maternity beds and cots added
1963	133	408	—
1964	130	598	Surgical operations up 1200
1965	74	623	Cubicles up by 14
1966	93	717	—
1967	95	341	—
1968	28	426	—
1969	36	367	—

Table 4. *Post-operative infections for four random weeks in each year*

Year	Type of operation	Total number	Infected on admission	Chest infections	Wound and urine infection
1965	Major	335	0	9	11
	Minor	82	0	0	0
1966	Major	208	14	15	11
	Minor	141	1	0	0
1967	Major	111	11	6	6
	Minor	153	5	0	0
1968	Major	65	4	4	11
	Minor	162	4	0	0

In Table 3 the amount of staphylococcal infection in the wards is given as a measure of the total risk against which the failure rate in the first column must be judged. Certain of the isolates were from carrier sites but the majority were frank infections; the same organism, no matter how many times it was isolated from different sites or lesions in the same patient, was counted once only.

The second check was introduced more recently to silence sceptical colleagues who thought our results too good. Using random number tables, 1 week in each quarter of the year was taken; the notes of every surgical case discharged in these weeks were examined for clinical, laboratory or therapeutic evidence of infection. Table 4 gives the results.

Attempts at assessing the changes over a longer period are not valid because of the changing nature of surgery. In 1959 the commonest operation was for varicose veins, now it is some form of arterial or intra-thoracic surgery.

DISCUSSION

It is difficult to suggest which of many factors was responsible for our satisfactory results. Morale improved as methods became standardized throughout the hospital. Proper cleaning and improved methods of handling laundry were probably useful, but the provision of cubicles where barrier nursing and reversed barrier nursing were possible was more important than all other measures together. The rules must be ruthlessly enforced on paper-boy, priest and physician alike.

The cost of establishing units of our type is one-tenth that of more usual ones. The equipment for a nurse is £1 5*s*. for 9 months, excluding disposable gloves. We were surprised to find that 14 beds were sufficient to meet the needs of a 450-bedded hospital. The success of the cubicle system supports entirely the views of Williams *et al.* (1966) that barrier nursing depends for its success on separate rooms for each patient. Throughout it must be remembered that the private block, in which separate rooms were the only factor differentiating the care from that of general ward patients, had only one staphylococcal coincidence, yet just as much sepsis is admitted here, since a considerable number of cases are flown in because surgery has failed abroad, or the condition is too advanced for local surgeons to take on.

It is impossible to thank all who have helped me. Mr W. A. S. Whittlesea the Instrument Curator, Mr J. W. Perrett the Steward and Mrs D. M. Heyes the Domestic Supervisor were directly involved throughout. My sincere thanks are due to the three sisters, Miss M. Drew, Mrs L. A. Vincent and Miss M. A. Adams, and to Miss Jean Corse, cross-infection technician and in charge of phage-typing of staphylococci.

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J. Hyg., Camb. (1970), **68**, 393
Printed in Great Britain

A survey of infectious mononucleosis in the North-East Regional Hospital Board area of Scotland, 1960–9

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(*Received* 23 *February* 1970)

SUMMARY

This report, based largely on 1258 laboratory proven cases of infectious mononucleosis (IM) detected in the North-Eastern Regional Board area of Scotland during the years 1960–9 inclusive, describes and discusses some of the epidemiological and diagnostic aspects of the disease.

During the period of study, the annual rate of incidence increased fourfold from 11·1 to 44·3 cases per 100,000 population. Evidence is presented to indicate that this does not represent a true increase in prevalence but reflects improvements in disease detection. The annual incidence rates as reported by the C.D.S. for 1967–9 inclusive are regarded as a considerable underestimation of the 'true' incidence of the disease. Over a 3-year period an annual incidence equivalent to 100 per 100,000 population was found in each of two selected group practices. This value was constant and is thought to approximate the 'true' incidence of symptomatic, seropositive IM in the general community.

Of the 1258 seropositive cases, 48·7 % were males and 51·3 % females. The peak age incidence was 15–19 years for both sexes. Twenty-one per cent of the cases were in children under 15 years and only 8·1 % in adults older than 25 years. No significant variation was found in the seasonal or urban/rural incidence. Marked differences were found in the leucocyte patterns of seropositive and 'seronegative' cases.

INTRODUCTION

No long-term study of the incidence and distribution of infectious mononucleosis (IM) in the general community has been previously reported. This communication, based mainly on 1258 seropositive cases detected in the North-East Regional Hospital Board area of Scotland during the years 1960–9 inclusive, describes and discusses some of the epidemiological and diagnostic aspects of the disease and compares the findings with published data mainly derived from short-term studies of special population groups.

MATERIALS AND METHODS

The North-East Regional Hospital Board area of Scotland comprises the City of Aberdeen, the mainland counties of Aberdeen, Kincardine, Banff and Moray and the island counties of Orkney and Shetland. The total regional population at the 1961 census was 479,421 persons. This region is eminently suitable for the epi-

demiological study of IM by reason of its geography and the almost unique role of the laboratory based at the City Hospital, Aberdeen. For almost 50 years this has provided a comprehensive postal laboratory service to the general practitioners of the entire region and, amongst other duties, has also met the laboratory needs of the infectious diseases unit situated at the same hospital.

The major part of this survey is based on the results of 8828 heterophile-antibody tests carried out in the laboratory during the years 1960–9 inclusive. Of the 1258 seropositive results recorded only 9·1 % were derived from hospital in-patients. The data relating to the years 1960–3 inclusive were retrospectively obtained from the laboratory records but thereafter all information was derived prospectively.

The serum or whole blood samples (usually both) were submitted from patients with clinical features suggestive of IM, mainly by general practitioners or the infectious diseases unit. In addition to heterophile-antibody tests, detailed examination of the peripheral blood was undertaken in almost all cases. In many instances more than one blood specimen was received for examination, while repeat examination was routinely requested on those patients who were strongly suspected of having the disease on clinical or haematological grounds but whose serum gave a negative heterophile-antibody test at the initial investigation.

For the purpose of this study, the diagnosis of IM was accepted solely on the basis of the conventionally recognized differential absorption (DA) test. Thus suspects showing the clinical or haematological features of IM were considered to have the disease only if the DA test was positive. Such patients have been termed seropositive. The remaining suspects, i.e. those failing to give a positive DA test on one or more occasions, have, for convenience, been termed 'seronegative'. Further diagnostic differentiation of the latter was impossible because of their vast number, wide source of referral, and the lack of first-hand knowledge of their clinical features. Thus they obviously include a variety of different diseases which may have simulated IM only in their clinical presentation.

Details of the serological methods and the diagnostic criteria applied in this laboratory for the diagnosis of IM have been fully described elsewhere (Davidson, 1967). Briefly, all sera were initially screened by a slide technique, a modification of the sheep-cell test (Moloney & Malzone, 1949) being used until 1966, after which time the method of Hoff & Bauer (1965), using a 4 % saline suspension of formalin-treated horse erythrocytes as antigen, was adopted as the routine procedure. All sera reacting positively with the screening test were then subjected to a full DA test. In this laboratory the lowest titres accepted as being diagnostic are 1/64 before and 1/32 after absorption with guinea-pig kidney. Peripheral blood investigations were carried out according to standard methods and included microscopic examination of a freshly prepared Leishman-stained film.

RESULTS

Annual incidence

The annual number and incidence of the 1258 serologically confirmed cases occurring in the years 1960–9 inclusive and the ratio of seropositive to 'seronegative' cases are shown in Table 1. During the period of study it would appear that the incidence of seropositive cases has increased fourfold. Such an interpretation is seen to be quite fallacious, however, when the 'seronegative' cases recorded during the same period are taken into account. Thus, the apparent and progressive increase in incidence is seen to parallel the rise in the number of heterophile antibody tests performed and the ratio of seropositive to 'seronegative' cases has remained relatively constant throughout. The mean ratio of 1/6 also supports the belief that clinical diagnosis alone is unreliable.

Table 1. *Annual incidence of infectious mononucleosis in the North-East Region of Scotland, and ratio of seropositive to 'seronegative' cases* (1960–9)

Year	Number of cases		Ratio of positive/ 'negative'	Seropositive cases (rate per 100,000 population)
	Sero-positive	'Sero-negative'		
1960	53	300	1:5·7	11·1
1961	81	433	1:5·3	16·9
1962	61	484	1:7·9	12·7
1963	101	665	1:6·7	21·1
1964	101	712	1:7·0	21·1
1965	134	838	1:6·3	27·9
1966	154	872	1:5·7	32·1
1967	165	1042	1:6·3	34·4
1968	196	1014	1:5·2	40·9
1969	212	1210	1:5·7	44·3
	1258	7570	1:6	

The author considers the recorded rates to be a substantial underestimate of the 'true' prevalence of the disease in the community because, during the study, it soon became apparent that a fair number of practitioners in the region were sending few, if any, blood samples to the laboratory from IM suspects.

A detailed study was therefore made of the number of suspected cases referred for laboratory investigation by two group practices selected on the grounds that the doctors were both alert to the disease and laboratory-minded. The seven practitioners involved were not informed that the study was being undertaken. One practice (A) was rural with a list of approximately 5000 patients, while the other (B) was urban with approximately 10,000 patients.

During the 3 years 1966–8 inclusive, practice A submitted specimens from 24, 27 and 29 IM suspects respectively and of these 6, 6 and 7 gave a positive DA test. During the same years, practice B referred samples from 49, 52 and 50 suspected cases of which 10, 9 and 10 gave positive serological results.

Thus for both practices, the annual incidence of seropositive cases appears relatively constant and is equivalent to 100 per 100,000 population, while the ratio

of seropositive/'seronegative' cases also shows little variation but is significantly lower than the mean regional value of 1:6.

Based on this information and an intimate knowledge of the region, the author considers that a reasonably accurate estimate of the annual incidence of seropositive, symptomatic IM occurring in the general community approximates 100 per 100,000 population.

Table 2. *Comparison of reported incidence of seropositive cases in the North-East Region and the rest of Scotland* (1967–9)

	No. of cases		Rate per 100,000 population at risk	
Year	N.E. region	Rest of Scotland	N.E. region	Rest of Scotland
1967	158	528	32·9	11·2
1968	169	545	35·3	11·6
1969	208	644	43·4	13·7

In contrast to these findings, Table 2 records and compares the annual number and incidence of seropositive cases of the disease reported weekly to the Communicable Diseases Scotland (CDS) during 1967–9 inclusive, by this and all other hospital laboratories in Scotland.

Firstly, it is pointed out that although the weekly notifications are conscientiously made by this laboratory, 38 cases were not reported to the CDS during the years 1967–9. Even with this error there is still a threefold difference in the rate recorded for the North-East Region compared with that for the rest of Scotland.

It would be erroneous to infer that the disease is three times more prevalent in the North-East but valid to conclude that the disparity is the result of a multiplicity of factors broadly relating to one or other of two main variables: (*a*) regional variations in the number or proportion of suspected cases referred for laboratory investigation, and (*b*) variations in the accuracy with which these laboratories notify the seropositive cases.

Finally, these results would appear to cast grave doubts on the value and validity of extensive national surveys, a view acknowledged by Newell (1967) after attempting to conduct a similar type of survey in England and Wales.

Age and sex incidence

Of the 1258 seropositive cases reported in this survey, 612 (48·7 %) were males and 646 (51·3 %) were females. The age and sex incidence per 100,000 population at risk is recorded in Table 3 and reveals, as in most other studies, a very low incidence in infancy and in adults over 30 years of age, with the peak incidence occurring in the 15–19 age group for both sexes. Although the peak incidence for both sexes occurs in this age group, a significant difference is seen to exist between the sexes in the rates of incidence within the ages of 15–24 years. Thus within the

15–19 age group, 56·3 % of the cases were female, while in the 20–24 age group only 39·2 % were female.

For comparison, the age incidence of 2056 'seronegative' cases (1022 males and 1034 females) occurring in 1967–8 inclusive is shown in Table 4. In contrast to the above findings, the incidence is seen to be uniformly higher for all age groups and presents a less well defined age distribution.

Table 3. *Age and sex incidence of seropositive cases per* 100,000 *population per year* (1960–9)

	Rate per 100,000 population	
Age group	Males	Females
0–4	2·4	0·5
5–9	26·7	31·0
10–14	26·5	40·1
15–19	148·7	186·4
20–24	132·6	79·0
25–29	20·5	15·7
30 and older	2·1	2·4
All ages	26·8	25·6

Table 4. *Age incidence of 'seronegative' cases per* 100,000 *population* (1967 and 1968)

Age group	No. of 'seronegative' cases	Rate per 100,000 population per year
0–4	63	78·5
5–9	267	350·0
10–14	305	358·3
15–19	385	562·8
20–24	349	582·2
25–29	160	267·5
30 and over	527	99·6
All ages	2056	214·4

Seasonal distribution

Table 5 shows the seasonal distribution of the total 8828 suspected cases investigated during the years 1960–9 inclusive. As can be clearly seen, there is no significant variation in the seasonal incidence of seropositive or 'seronegative' cases.

Rural/urban distribution

No significant difference was found in the rate of incidence of serologically confirmed cases occurring in the rural and urban (City of Aberdeen) communities of the region.

Comparison of leucocyte patterns in seropositive and 'seronegative' cases

Table 6 compares some of the leucocyte changes observed in the initial peripheral blood examination of 330 seropositive and 768 'seronegative' cases. In the

seropositive group the salient features are that almost 50 % of cases had a definite leucocytosis and 90 % had an absolute lymphocytosis. In contrast, no such clearly defined patterns emerged in the 'seronegative' group of patients. Thus, while 16·6 % had an absolute lymphocytosis, a definite neutrophilia was found in 18 % and an eosinophilia in 9·7 % of the cases. These findings would appear to substantiate the view that several quite different disease entities are included within this group because of failure to differentiate them on clinical grounds.

Table 5. *Seasonal incidence of seropositive and 'negative' cases*, 1960–9

	Jan.–Mar.	Apr.–June	July–Sept.	Oct.–Dec.
Seropositive cases	297	361	276	324
% of total	23·6 %	28·7 %	21·0 %	25·8 %
'Seronegative' cases	1945	2013	1726	1886
% of total	25·7 %	26·6 %	22·8 %	24·9 %

Table 6. *Comparison of leucocyte changes in seropositive and 'seronegative' cases*

	Seropositive (330 cases)	'Seronegative' (768 cases)
Leucocytosis (> 10,000/mm.^{3})	48 %	22·3 %
Leucopenia (< 3500/mm.^{3})	0·3 %	3·4 %
Lymphocytosis (> 4500/mm.^{3})	90 %	16·6 %
Neutrophilia (> 7500/mm.^{3})	0·0 %	18 %
Eosinophilia (> 500/mm.^{3})	0·9 %	9·7 %

DISCUSSION

General incidence

The overall incidence of IM remains a matter of dispute. Published estimates, reviewed by Penman (1966), have shown enormous disparity, the extremes of variation being 1·6 and 2000 cases per 100,000 population recorded by Newell (1957) and Yeager (1961) respectively.

Such disconcertingly diverse results can be largely attributed to two major differences in classification relating to whether (i) the diagnosis has been established according to clinical or laboratory criteria and (ii) the prevalence rate has been expressed as a composite of both seropositive and 'seronegative' cases. Thus, extremely high prevalence rates have been a feature of those studies relying solely on clinical diagnosis or where seropositive and 'negative' cases have been combined, whereas low rates have generally been found in those studies based on laboratory diagnosis and including only serologically confirmed cases.

Other important factors contributing to the discrepancies but to a lesser degree have been (iii) the type of population studied, especially if selection has been age-related as in surveys involving students, nurses and servicemen; (iv) duration and manner of case finding including the diligence and care with which the latter has been pursued; and (v) differences in methods and interpretation of serological tests.

Although most of these shortcomings have been avoided in the present survey it is apparent that the method of case finding or collection has exerted a profound influence on the present results. Thus the annual rate appears to have increased in

direct proportion to the increase in the total number of serological tests carried out, the latter presumably being related to the more extensive use being made of the laboratory by general practitioners and their increased awareness of the disease.

The main flaw in a regional survey of this kind is undoubtedly its dependence on a large number of individual doctors, some 300 in this instance, for case finding and referral. When this major variable was reduced by carrying out a study confined to seven doctors in two selected practices a much higher rate of incidence was attained and, moreover, this rate remained relatively constant over a 3-year period.

The evidence presented in this communication therefore appears to indicate that an annual rate of 100 per 100,000 population is a fairly accurate estimate of the number of seropositive cases with symptoms occurring in the general community.

This value probably represents a minimum for the 'true' incidence of the disease, however, as it does not take into consideration (*a*) subclinical cases, (*b*) patients with symptoms who failed to consult their doctor, and (*c*) patients with delayed heterophile-antibody formation who were initially 'seronegative' and had no follow-up tests.

Subclinical forms of the disease would appear to be of infrequent occurrence according to serological testing of healthy blood donors (Barrett, 1941; Hobson, Lawson & Wigfield, 1958; Virtanen, 1962) and careful surveillance of close contacts of the disease (Hoagland, 1955; Evans & Robinton, 1950). Patients in category (*b*) may be more numerous, at least in students (Bender, 1958), although it is probably impossible to assess their number in the general community. Finally, a significant number of cases may be missed owing to delayed heterophile antibody formation, but numerical estimation is again difficult because the onset of clinical symptoms can rarely be dated precisely. In this connexion Hobson *et al.* (1958) found that in 205 cases, where the onset of symptoms could be reasonably defined, a positive heterophile-antibody reaction was obtained in 69 % by the 14th day of the illness, in 90 % by the 21st day and in 94 % by the 28th day. In contrast, Hoagland (1952) recorded seropositive results in 95·3 % of cases within the first 2 weeks of the illness.

Is the incidence of the disease increasing?

Little factual information is available from reliable sources to substantiate the prevailing view that the incidence of the disease is increasing.

Thus an analysis of 1779 cases hospitalized in Stockholm during the years 1940–57 demonstrated a fourfold increase in incidence from 5·3 to 23·2 cases per 100,000 (Ström, 1960). Even allowing for the influence of changes in the social, medical and diagnostic conditions, this was regarded as representing a true increase in disease prevalence.

During the period 1943–8, 79 cases of seropositive IM, equivalent to an annual incidence of 3·25 cases per 100,000, were diagnosed in the North-East area of Scotland (Fullerton & Smith, 1951). In 1969 the annual rate recorded by the same laboratory was 44·3 cases per 100,000, which represents a 13-fold increase over a 25-year period. The question is, does this indicate a real increase in prevalence?

As stated earlier, the gradual increase evidenced over the years may largely be a function of an increasing awareness of the disease on the part of general practitioners, and the submission by them of an increasing number of suspected cases to laboratory investigation.

In addition there is strong supportive evidence that the annual incidence in two selected practices was relatively constant at an equivalent rate of 100 cases per 100,000, which is $2\frac{1}{2}$ times above the current rate for the entire region.

Thus one may reasonably conclude that, during the period of study, no real increase in the prevalence of seropositive glandular fever occurred within the area and the apparent increase merely reflects improvements in disease detection. In some other areas it is probable that higher rates of detection have been achieved since the recent introduction of simplified serological methods which have enabled more laboratories to provide at least a screening service.

Finally, it is suggested that if changes in the incidence of the disease in the general community are to be satisfactorily monitored, their study should be confined to selected group practices.

I am grateful to Dr Audrey Sutherland for statistical help and Dr William Walker for reviewing the manuscript. I also acknowledge the co-operation of general practitioners throughout the Region and the Home and Health Department (Scotland) for permission to use data recorded in the CDS.

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J. Hyg., Camb. (1970), **68**, 401
With 2 plates
Printed in Great Britain

The extracellular antigens of *Micropolyspora faeni:* their significance in farmer's lung disease

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(*Received* 2 *March* 1970)

SUMMARY

A serological analysis of the extracellular antigens of *Micropolyspora faeni* by immunodiffusion with a combination of sera revealed 29 individual antigens. A survey was made of the incidence of precipitins to the antigens in sera from patients with clinical farmer's lung disease (FLD) and other respiratory diseases. Precipitating antibody was found in 75% of farmer's lung cases and in 20% of other cases who had been exposed to the same environment. More precipitin reactions were seen in sera from severe forms of FLD than from milder forms. The distribution of precipitins to individual antigens was not significantly affected by severity of disease.

Most of the patients with precipitins to *M. faeni*, but without the symptoms of FLD, were suffering from mild or moderate symptoms of other respiratory diseases with a history of chronic onset of symptoms. The distribution of precipitins to individual antigens in this group was similar to that in clinical FLD patients but the incidence was considerably lower.

The significance of these results is discussed.

INTRODUCTION

Farmer's lung disease (FLD) is a respiratory hypersensitivity of agricultural workers, associated with the inhalation of dust from mouldy hay (Dickie & Rankin, 1958). The hypersensitivity is of the Arthus type, which is thought to be mediated by precipitating antibody, tissue damage being caused by the formation of antigen-antibody precipitates (Rose & Phills, 1967). The sera of most patients with clinical FLD have been shown to contain precipitating antibody to a thermophilic actinomycete commonly found in mouldy hay, *Micropolyspora faeni* (Pepys *et al.* 1963). A much smaller proportion of workers exposed to mouldy hay who are healthy or have other respiratory disease also show these antibodies. The organism was originally identified as *Thermopolyspora polyspora* but has recently been reclassified (Cross, Maciver & Lacey, 1968). Precipitating antibody to this organism has also been found in sera from many cattle suffering from a wide range of respiratory conditions collectively known as 'fog-fever' which have several features in common with FLD (Jenkins & Pepys, 1965). In addition, sera from farmer's lung patients often contain precipitating antibodies to *Aspergillus*

fumigatus, *Mucor* spp., *Streptomyces* spp. and other thermophilic actinomycetes, notably *Micromonospora vulgaris* (Pepys *et al.* 1963).

For some years laboratories in the Public Health Laboratory Service have aided clinicians in the diagnosis of equivocal cases of respiratory disease by testing sera against extracts of *M. faeni* by immunodiffusion techniques. Interpretation of the results of these tests is difficult as antibody cannot be detected in some cases of clinical FLD and is present in some patients thought to have other respiratory diseases. The present study was undertaken to determine if there is any qualitative difference between the precipitin contents of sera from patients with and without clinical symptoms of FLD.

MATERIALS AND METHODS

Sera

Sera from 40 patients with FLD (group A) and 54 patients with other pulmonary diseases (group B) were obtained from Public Health Laboratories throughout the country. These sera had been sent to this laboratory for serological testing. All patients had a history of exposure to mouldy farm produce. Other clinical details are summarized in Table 1.

Table 1. *Clinical details of patients*

	Group A (total no. 40)		Group B (total no. 54)	
	No.	%	No.	%
Symptoms				
Dyspnoea	33	83	33	65
Fever	12	30	16	30
Rigor	8	20	8	15
Weight loss	13	32	10	19
Severity				
Mild	8	20	14	26
Moderate	22	55	27	50
Severe	10	25	13	24
Mode of onset				
Acute	24	60	35	65
Chronic	16	40	19	35

Group A = farmer's lung disease patients.
Group B = patients with other respiratory diseases.

The two sera used for the serological analysis contained antibodies to more *M. faeni* antigens than any other available; one was from a case of FLD (H 1), the other from a cow with 'fog-fever' (B 1).

Antigen

The organism used as a source of antigen was *M. faeni*, strain 1156, which was isolated by Dr M. Lacey, Rothamsted Experimental Station, Harpenden. This was grown in continuous culture (details to be published) at 55° C. The medium used contained (per litre distilled water): casein hydrolysate (Hopkin & Williams

Ltd), 10·0 g.; dried yeast extract (Difco Ltd), 2·5 g.; sodium glycerophosphate, 2·5 g.; KCl, 0·5 g.; $MgSO_4$ (anhydrous), 0·5 g.; $Na_2HPO_4.2H_2O$, 5·34 g.; KH_2PO_4, 2·72 g. The pH was 7·0.

Culture was centrifuged and the supernatant fluid concentrated by dialysis against 40 % polyethylene glycol (Carbowax 3000, Union Carbide Ltd) in three steps, dialysing against two changes of 0·01 M phosphate buffer, pH 7·0 for 36 hr. after each concentration step. The concentrated material was finally dried from the frozen state and antigen for immunodiffusion tests reconstituted at 25 mg./ml. in 0·9 % saline.

Immunodiffusion tests

The Ouchterlony (1953) immunodiffusion technique was modified as follows. Ten ml. of 1 % Oxoid Agar No. 1 in 0·02 M phosphate buffer, pH 7·0, containing 0·1 % sodium azide, was poured into a plastic Petri dish (90 mm. diam.). Holes, 9·5 mm. diam., were cut in the gel 4·8 mm. apart in the patterns shown in Plate 1. The appropriate holes were filled with antigen and serum and then left for 40 hr. in a saturated atmosphere at room temperature. They were then washed in 0·9 % saline overnight and photographed using dark-ground illumination.

Serological analysis

Two different batches of antigen, A 19 and A 211, used at 25 and 12·5 mg./ml., were tested against two sera, H 1 and B 1, by immunodiffusion. The precipitation pattern between each antigen-antiserum pair and reactions of identity between adjacent patterns were noted; these were interpreted by established criteria (Ouchterlony, 1958).

Serum survey

Sera were tested by immunodiffusion using the arrangement shown in Plate 2. This allowed comparison of their precipitation patterns with the four standard patterns determined by the serological analysis. Precipitins were identified by noting reactions of identity with these four patterns.

RESULTS

Serological analysis

Plate 1 shows the immunodiffusion reactions between two sera and two batches of antigen. An analysis of the precipitation patterns is shown diagrammatically in Fig. 1. Line pattern components (l.p.c.) are numbered from the serum well outwards between A 19 and B 1, and the l.p.c. sequences in the reactions are shown in Table 2. Where two or more l.p.c. appear as one line in the photograph, this is indicated by brackets in Table 2. These results were reproducible provided strict adherence was made to the experimental procedure outlined.

Hereafter 'antigen 1', etc., will refer to the antigen responsible for the l.p.c. which bears that number in Fig. 1.

Small variations were seen in the antigen content of different batches of culture. In most batches antigens 18–29 were not detectable; all had slightly different sequences of l.p.c. in immunodiffusion tests. Such a difference is shown between A 19 and A 211 in Table 2.

Fig. 1. Analysis and numbering system of line pattern components (l.p.c.) of the precipitin reactions in Plate 1.

Serum survey

The incidence of precipitins to *M. faeni* antigens in patients' sera is given in Table 3. No serum, other than the two used for the serological analysis, which were not included in this survey, contained precipitins to antigens 18, 21, 22, 26, 27, 28 or 29. Precipitating antibody to *M. faeni* was detected in 75 % of sera from

Table 2. *Sequence of line pattern components in the precipitation reactions shown in Plate* 1

Reaction			
A19/B1	A19/H1	A211/B1	A211/H1
(1, 2)	(3, 4, 5)	(1, 2)	(3, 4, 5)
(3, 4, 5)	(8, 9, 10)	(3, 4, 5)	(8, 9, 10)
(6, 7)	(1, 2)	(6, 8, 9, 7, 11)	(1, 2)
(8, 9)	23	10	23
(10, 11, 12, 16)	24	13	24
	(6, 7, 25,		6
(13, 14, 15, 17)	26)	12	7
	(11, 12)	14	
(19, 20)			(11, 12)
	(13, 14, 15)	15	
18			25
	16	16	
			(17, 26, 13,
	17	18	14, 15)
		21	16
		22	27
			28

farmer's lung cases and 20 % of sera from other cases. The l.p.c. most frequently given by farmer's lung sera were: 8, 9, 10, 5, 6, 7, 13, 14 and 4, and by sera from cases of other respiratory diseases: 14, 15, 13, 8, 9, 10, 6 and 7.

Most positive sera (75 %) gave either l.p.c. 8 or 9 or 10; 57 % gave all three, together with varying numbers of other l.p.c. Of the remaining positive sera 10 % gave l.p.c. 16 and 17 only, 12·5 % gave l.p.c. 13, 14 and 15 only, 5 % gave l.p.c. 13, 14, 15, 16 and 17 only, and 2·5 % gave l.p.c. 11 and 12 only. There was no significant difference between the two groups of sera in this respect.

In Table 4 the results are shown graded by severity of disease. A considerable difference exists between the numbers of precipitins in sera from mild cases and from moderate and severe cases of both FLD and other respiratory diseases. The difference is statistically significant; $P < 0{\cdot}025$. There is no significant difference, however, between the distribution of precipitins in groups A and B in sera from patients of the same grade of severity.

Sera from patients with chronic or acute modes of onset of symptoms of FLD and other respiratory diseases show very similar distributions of precipitins. This is shown in Table 5. It appears from the results in the table that the presence of

precipitins in sera from non-FLD patients is most frequently associated with a chronic onset of symptoms.

Table 3. *Number and percentage of sera in groups A and B reacting with each antigen*

	Group A (total no. 40)		Group B (total no. 54)		Total (total no. 94)	
Antigen	No.	%	No.	%	No.	%
1	10	25	1	2	11	12
2	11	27	1	2	12	13
3	12	30	2	4	14	15
4	19	47	2	4	21	22
5	20	50	2	4	22	23
6	20	50	4	8	24	25
7	20	50	4	8	24	25
8	23	57	6	11	29	31
9	23	57	6	11	29	31
10	23	57	6	11	29	31
11	6	15	1	2	7	7
12	6	15	1	2	7	7
13	20	50	6	11	26	28
14	20	50	7	13	27	29
15	15	37	7	13	22	23
16	14	35	3	6	17	18
17	14	35	3	6	17	18
19	1	3	—	—	1	1
20	1	3	—	—	1	1
23	14	35	2	4	16	17
24	11	27	2	4	13	14
25	4	10	1	2	5	5
Total no. positive	30	75	11	20	41	43

DISCUSSION

We have investigated the incidence of precipitating antibody to individual *M. faeni* antigens in sera from clinical farmer's lung cases and from cases of other respiratory diseases. Our purpose was to discover if there was any difference between the precipitin content of sera in the two groups.

The results show that the numbers of precipitins present in patients' sera are significantly higher in patients with moderate or severe forms of FLD than in those with milder symptoms, but that the distribution of precipitins to individual *M. faeni* antigens is similar in all grades of the disease. This was also true of the patients with other respiratory diseases. Thus there appears to be a general increase in the incidence of precipitins to all antigens of *M. faeni* with increasing severity of disease. Individual differences in this respect may well be due to variation in the antigenic composition of the strains to which patients are exposed. It is interesting to note that preliminary experiments in this laboratory suggest that antigens 1–5 correspond to the antigenic fraction 'C' of Pepys & Jenkins

(1965), antibody to which was shown to be most frequent in the sera of patients who had suffered several attacks of FLD.

Table 4. *Precipitin reactions in sera from group A and B patients with varying grades of severity of disease*

	Group A			Group B		
Grade of disease ...	Mild	Moderate	Severe	Mild	Moderate	Severe
Total no. of sera ...	8	22	10	14	27	13
No. positive ...	6	16	8	6	4	1
No. reacting with antigen						
1	1	8	1	—	—	1
2	1	8	2	—	—	1
3	1	9	2	—	1	1
4	1	12	6	—	1	1
5	1	12	7	—	1	1
6	1	13	6	—	3	1
7	1	13	6	—	3	1
8	3	15	5	2	3	1
9	3	15	5	2	3	1
10	2	15	6	2	3	1
11	1	2	3	—	—	1
12	1	2	3	—	—	1
13	2	11	7	2	3	1
14	4	9	7	3	3	1
15	1	8	6	3	3	1
16	3	7	4	2	—	1
17	3	7	4	2	—	1
19	1	—	—	—	—	—
20	1	—	—	—	—	—
23	1	10	3	—	1	1
24	—	10	1	—	1	1
25	—	4	—	—	1	—
Average no. of l.p.c./serum	6	12	10	3	8	19

A small number of farmers' lung cases may be attributed to hypersensitivity to other organisms, notably *Micromonospora vulgaris* (Wenzel, Emanuel & Lawton, 1967). However, in many cases failure to detect antibody to *M. faeni* antigens almost certainly reflects a lack of sensitivity in immunoelectrophoresis and, to a lesser extent, in immunodiffusion tests. Jameson (1968), using a more sensitive technique, immunoosmophoresis, detected antibody in many sera from farmer's lung cases and from healthy farmers which were negative by the conventional immunodiffusion test. The numbers of precipitin lines given by these sera were fewer than those given by sera which were positive by the conventional test.

In the chronic stage FLD presents a much greater problem of diagnosis than in the acute stage. Radiological evidence of fibrosis may remain or even increase for years after an attack, without further exposure to mouldy hay, the resulting

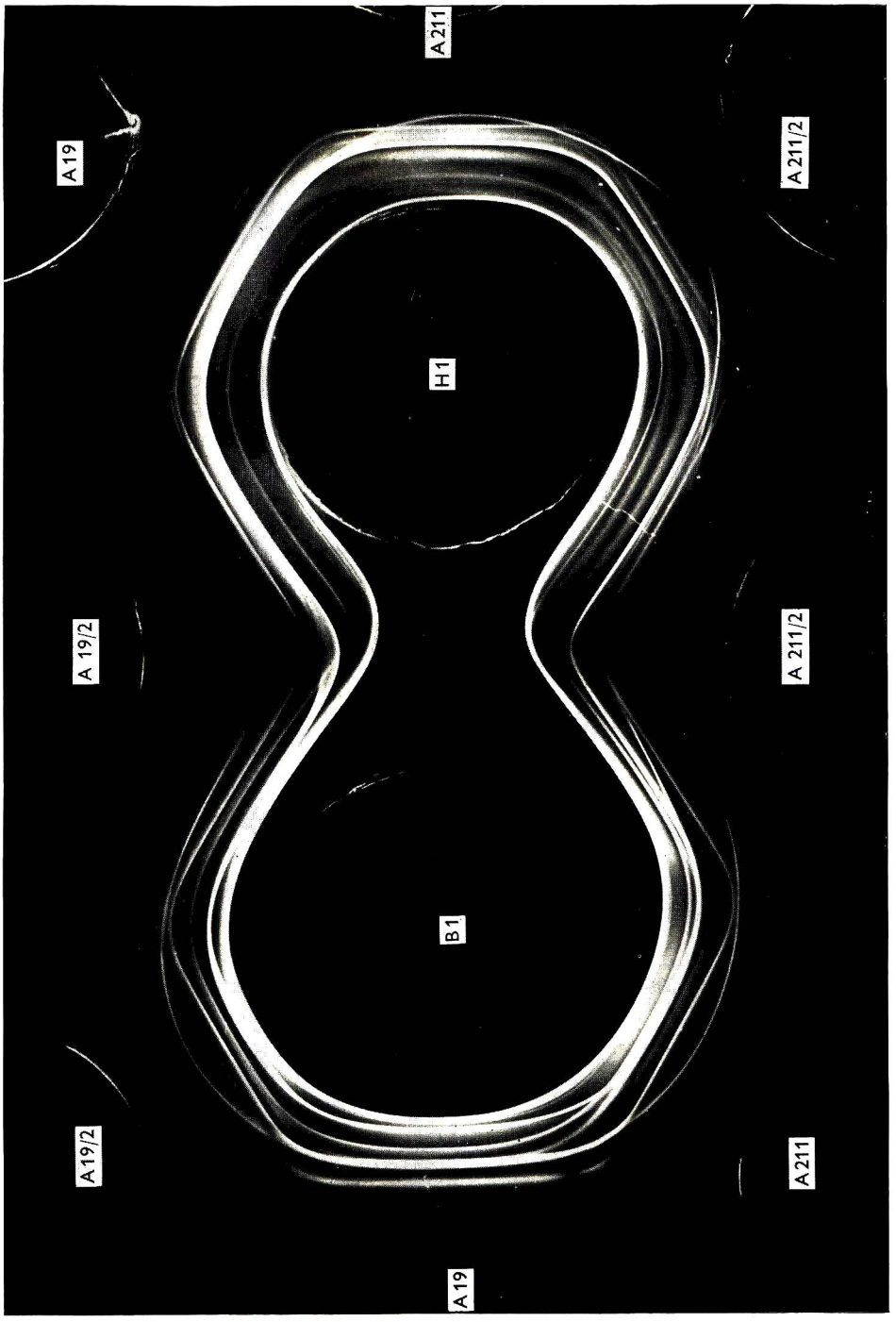
decrease in antibody giving rise to negative serological tests. In addition, the symptoms of FLD in this stage are similar to those of several other diseases, notably chronic 'fibroid' pulmonary tuberculosis, chronic diffuse idiopathic pulmonary fibrosis and other pneumoconioses; careful history-taking is therefore needed to establish a correct diagnosis.

Table 5. *Number of sera from acute and chronic cases in groups A and B reacting with each antigen*

No. of sera reacting with antigen	Acute cases		Chronic cases	
	Group A	Group B	Group A	Group B
1	6	—	4	1
2	7	—	4	1
3	7	—	5	2
4	11	—	8	2
5	12	—	8	2
6	12	—	8	4
7	12	—	8	4
8	14	—	9	6
9	14	—	9	6
10	14	—	9	6
11	5	—	1	1
12	5	—	1	1
13	13	1	7	5
14	13	2	7	5
15	10	—	5	5
16	10	—	4	3
17	10	—	4	3
19	—	—	1	—
20	—	—	1	—
23	7	—	7	2
24	7	—	4	2
25	3	—	1	1
Total sera	19	2	11	9

The occurrence of precipitins in healthy farmers' serum may be due to an attack of FLD in the past which has not been repeated, or to exposure to *M. faeni* to an extent that has not produced obvious symptoms. The predominance of mild and moderate symptoms shown by precipitin-positive patients with other respiratory diseases in this report suggests that in this group the occurrence of antibody may have some clinical significance. It is possible that the patients in question suffer from a mild form of FLD which has escaped diagnosis because of the presence of symptoms of other respiratory diseases.

This work was supported by a grant from the Ministry of Health. The authors are grateful to the many clinicians who supplied sera and clinical details for the investigation. The authors also wish to thank Mr S. Broadhead for expert technical assistance.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A19
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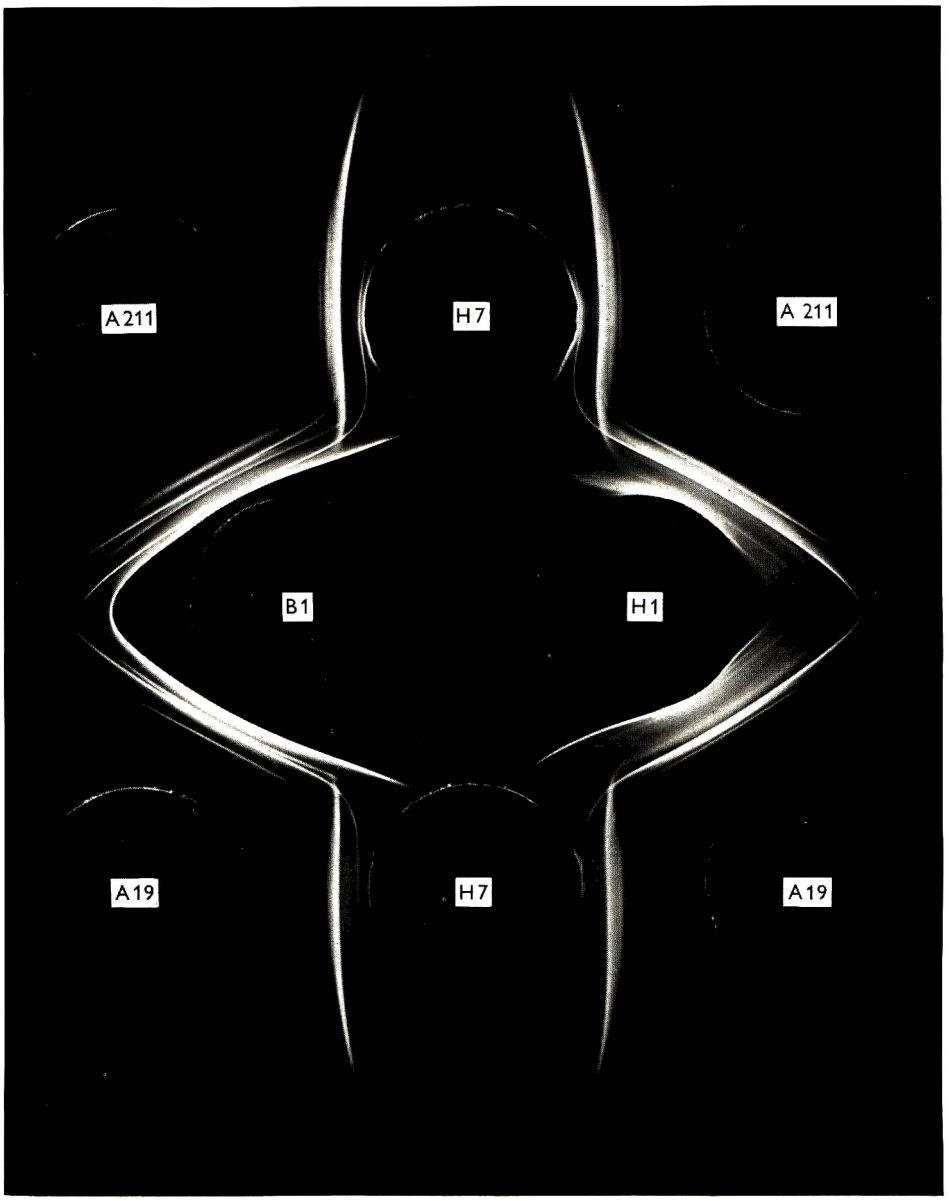
A211
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EXPLANATION OF PLATES

Plate 1

Precipitin patterns given by two batches of antigen at 25 mg./ml. (A 19, A 211) and 12·5 mg./ml. (A 19/2, A 211/2) with a human (H 1) and a bovine (B 1) serum.

Plate 2

The arrangement of sera and antigens used in the analysis of precipitin content of human sera. The precipitation pattern between human serum (in this example H 7) and antigens A 19 and A 211 are compared with those given by four 'standard' reactions (B 1/A 19, B 1/A 211, H 1/A 19 and H 1/A 211).

J. Hyg., Camb. (1970), **68**, 411
Printed in Great Britain

The incidence of complement-fixing antibody to varicella–zoster virus in hospital patients and blood donors

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SUMMARY

The sera of 308 patients, not suffering from varicella or zoster infections, and the sera of 183 blood donors were examined for complement-fixing antibody to varicella-zoster virus. In both groups about 70% of sera from persons aged 11–40 years had antibody titres $\geqslant 1/4$; the incidence was less in the age range 41–60 years and increased in later decades. Antibody titres of 1/16 or 1/32 were noticeably less frequent in those aged 41–60 years than in younger or older groups.

It was concluded that an unchanging titre of 1/16 or 1/32 was of no diagnostic significance. The age distribution of antibody was consistent with the theory that zoster only occurs when antibody has declined.

INTRODUCTION

Although antibody to varicella–zoster virus was demonstrated many years ago (Netter & Urbain, 1926) little is known about the prevalence of antibody in the population.

We have frequently received in the laboratory serum from patients complaining of pain with an apparent nerve root distribution, similar to that of zoster, but with no obvious skin eruption, and if the pain appeared in the chest the provisional diagnosis might be coxsackie virus infection or heart disease. Although no other cause for the pain was found, several of these sera had titres of antibody to varicella–zoster virus of 1/16 to 1/32, but a subsequent rise was rare. It seemed, therefore, that a survey of antibody titres in normal persons and hospital patients would have immediate practical value and might help in elucidating the natural history of varicella–zoster virus infections.

We measured complement-fixing antibody to varicella–zoster virus in all sera sent to the virology laboratory between January and August 1967, and in the sera of 183 blood donors.

MATERIALS AND METHODS

Sera

The routine sera were specimens sent for the diagnosis of common viral infections. Sera from blood donors were kindly supplied by Dr A. E. Preston of the Regional Blood Transfusion Service, Churchill Hospital, Oxford.

On receipt sera were inactivated at 56° C. for 30 min., stored at −20° C., and, if the storage was prolonged, again inactivated before test.

Varicella–zoster antigen

The method of preparing antigen was essentially that of Schmidt, Lennette, Shon & Shinomoto (1964). Human embryo fibroblasts were grown in 6 oz. medical flats, heavily infected with virus and incubated at 36° C. until most of the cells were rounded—usually 4 days. The medium was then discarded, the cells were scraped off with a rubber-tipped glass rod, suspended in 1 ml. veronal buffer for each bottle harvested, frozen, thawed, and disrupted by ultrasonic energy for 3 min. in an H 60 Ultrasonic Cleaner (Headland Engineering Developments Ltd.). After centrifugation at 3000 rev./min. the cell debris was discarded and the fluid stored at −70° C.

Herpes simplex virus antigen

This was prepared by infecting RK 13 cells with a strain of virus isolated from herpes labialis lesions, then harvesting and disrupting as for the V–Z antigen. It was standardized by titration against a serum supplied by the Standards Laboratory, Colindale.

Complement fixation

Tests were done in W.H.O. Perspex trays using 2·5 haemolytic doses of complement and allowing 18 hr. at 4° C. for fixation. The dose of antigen used was determined for each batch by chessboard titration. (See Results.)

RESULTS

Antigen titration

The results of a typical titration of V–Z antigen, as given in Table 1, showed a broad optimum for the dose of antigen reacting with a zoster serum, but no optimum with a varicella serum. For this survey the dose of antigen used was twice the minimum amount giving full fixation at the maximum serum dilution, i.e. a 1/16 dilution of the batch of antigen used in the titration of Table 1.

Specificity of complement fixation

Heterologous antibody rises in varicella–zoster and herpes simplex virus infections have been reported (Kapsenberg, 1964; Ross, Subak Sharpe & Ferry, 1965; Svedmyr, 1965; Schmidt, Lennette & Magoffin, 1969) but Schmidt *et al.* express the opinion that a previous antigenic stimulus with V–Z virus is essential before a herpes simplex infection will elicit a rise in antibody to varicella–zoster virus. There is, at present, no certain way of ensuring the specificity of single determinations, but some check was provided by assembling the results of testing 184 sera for the presence of complement-fixing antibody to both V–Z virus and herpes simplex virus (Table 2). As expected, many sera reacted with both antigens, but 22 % reacted only with herpes simplex antigen and 21 % reacted only with V–Z antigen, showing that different antibodies were detected by the two antigens.

Table 1. *Chess-board titration of varicella–zoster antigen*

Serum dilution	Degree of complement fixation* with: Varicella serum. Antigen dilution 4	8	16	32	Zoster serum. Antigen dilution 4	8	16	32	64	128
8	4	4	3	1	—	—	—	—	—	—
16	4	4	4	2	4	4	4	4	3	1
32	4	4	4	2	4	4	4	4	4	2
64	4	4	4	1	4	4	4	4	4	2
128	4	4	4	1	4	4	4	4	3	1
256	4	4	3	1	4	4	4	4	3	0
512	4	4	3	0	4	4	4	4	3	0
1024	4	4	2	0	3	4	4	4	1	—
2048	4	2	1	0	0	1	1	1	0	—
Control	0	0	0	0	0	0	0	0	0	—

* 4 = Complete fixation; 0 = complete haemolysis; — = not tested.

Table 2. *Sera tested for antibody to V–Z virus and Herpes simplex virus*

		Antibody to H.S.V. Absent	Present
Antibody to V–Z virus	Absent	31	41
	Present	39	73

Total: 184

Table 3. *Titres of antibody to varicella–zoster virus in sera from* 308 *patients*

Age group	No. of patients	Number of sera with titres equal to or greater than 1/4	1/8	1/16	1/32
0–10	19	9 (47)	6 (32)	5 (26)	5 (26)
11–20	59	43 (73)	32 (54)	26 (44)	20 (34)
21–30	98	69 (70)	57 (58)	47 (48)	27 (28)
31–40	35	25 (71)	19 (54)	14 (40)	6 (17)
41–50	23	18 (78)	9 (39)	9 (39)	4 (17)
51–60	26	18 (69)	12 (46)	11 (42)	5 (19)
61–70	19	11 (58)	10 (53)	9 (47)	4 (21)
71–80	20	12 (60)	11 (55)	8 (40)	6 (30)
81–90	9	6 (67)	6 (67)	5 (56)	4 (44)

Figures in parentheses indicate percentages of the total number in each age group.

Antibody in the sera of patients

When sera from patients suspected of having varicella or zoster had been excluded, 308 results remained and these, classified by age group and titre, are shown in Table 3. The lowest percentage of detectable antibody was in the age group 0–10 yr., the period when first infections are being acquired; from 11 to 50 yr., over 70 % of patients had antibody and in the older groups there was a

slight fall in the incidence. The percentages with titres equal to or greater than 1/8, or 1/16, were not particularly remarkable but did show a similar trend to those with titres of 1/4. The incidence of titres of 1/32, or higher, showed a broad minimum in the group 31–60 yr., with higher percentages in younger and older groups.

Antibody in the sera of blood donors

The results on the sera of 183 blood donors are set out in Table 4 and they show an unequivocal fall in the percentage with antibody, at each of the four levels in the group 41–60 yr. As there were only five donors over 60 yr. the increased percentage in the last group has little significance.

Table 4. *Titres of antibody to varicella–zoster virus in sera from* 183 *blood donors*

Age group	No. of donors	Number of sera with titres equal to or greater than 1/4	1/8	1/16	1/32
11–20	17	12 (70)	10 (59)	8 (47)	1 (6)
21–30	64	39 (61)	24 (37)	14 (22)	4 (6)
31–40	37	26 (70)	15 (41)	7 (19)	0
41–50	33	17 (52)	9 (27)	2 (6)	1 (3)
51–60	27	12 (44)	6 (22)	3 (11)	1 (4)
61–70	5	4 (80)	4 (80)	1 (20)	0

Figures in parentheses indicate percentages of the total number in each age group.

DISCUSSION

The results from the two sets of sera differ in that the patients showed higher percentages with antibody than did the blood donors, presumed to be healthy, and no satisfactory explanation for this observation can be offered. On the other hand, the two series concur in showing a fall in the proportion of individuals with antibody in the group 41–60 yr. Figures presented by Schmidt *et al.* (1969), in a study of the immunological relationship between herpes simplex and varicella–zoster viruses, provide information on 75 sera from San Francisco which show a similar trend, though they do not comment on it. They give, by age group, the number of patients with herpes simplex virus infections who had complement-fixing antibody to V–Z virus in the acute-phase serum: 1–10 yr., 6%; 11–20 yr., 59%; 21–30 yr., 75%; and 31 yr. and over, 31%.

All these results contrast sharply with the reported incidence of antibody to herpes simplex virus as illustrated by a recent survey by Smith, Peutherer & MacCallum (1967), who also give references to 18 similar surveys, all of which show that the percentage of individuals with antibody to herpes simplex virus increases steadily with increasing age.

It is now generally accepted that herpes–zoster is a localized resurgence of virus which has remained latent from a previous attack of varicella and Hope-Simpson (1965) suggested that this reactivation can only occur when antibody has fallen

below some critical level. As varicella usually occurs in the first 20 years of life and zoster mostly in those over 50 yr. old, the Hope-Simpson theory postulates a decrease in antibody level with advancing years and, as a corollary, one might expect in the higher age groups a lower percentage of the population to have detectable antibody. This, in fact, is what we have found.

In his review of 192 cases of herpes zoster, seen in general practice, Hope-Simpson records the incidence per 1000 population, per year, as 2·58–2·92 between the ages of 20 and 49 years, rising to 5·09 in the sixth decade and progressing to 10·1 in the ninth decade. The incidence of zoster began to increase in the age groups in which we found the minimum incidence of antibody.

Whether or not the onset of an attack of zoster is determined by the pre-existing antibody titre is difficult to decide, because by the time the first serum is collected a secondary rise of antibody may have begun. There are reports in the literature of zoster patients who did not have detectable antibody in the acute-phase serum and in our records of 74 cases of zoster (of which 71 had convalescent titres $\geqslant 1/64$) 15 had a titre of less than 1/4 in the first serum.

Although this type of complement fixation test is satisfactory for demonstrating changes in antibody titre in sequential samples of serum, it is not a good technique for single determinations. Neutralization tests with V–Z virus would be very laborious to apply to large numbers of sera (Caunt & Shaw, 1969) and the immunofluorescent technique (Schmidt, Lennette, Woodie & Ho, 1965), which we used on 60 sera, gives no more precision than the complement fixation test and is more time-consuming. However, when applied to many sera, a total of 491, it is unlikely that the imprecision of a single CF test has obscured the pattern of incidence.

We conclude that, with the technique used, unchanging titres of 1/16 or 1/32 have no diagnostic significance. Further, the age distribution of antibody to V–Z virus is consistent with the theory that latent virus only becomes active when antibody has declined.

We thank Dr A. E. Preston of the Regional Blood Transfusion Service, for the supply of sera from donors and we are very grateful to Mrs P. Huish, Mr G. D. W. Curtis and Mr D. Day for their able technical assistance.

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J. Hyg., Camb. (1970), **68**, 417
Printed in Great Britain

Nasal acquisition of *Staphylococcus aureus* in a subdivided and mechanically ventilated ward: endemic prevalence of a single staphylococcal strain

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(*Received* 20 *March* 1970)

SUMMARY

An investigation was made of nasal acquisition of *Staphylococcus aureus* and of staphylococcal wound sepsis in a hospital ward divided into two sections and provided with mechanical ventilation, so that there was no transfer of air from one of the sections to the other. Although the strains of *S. aureus* found in the air, and those colonizing the noses of patients, in the protected section could seldom be related to patients nursed elsewhere in the ward, the mechanical ventilation did not lead to any significant reduction in the degree of contamination of the air or in the rate of nasal acquisition of *S. aureus*.

Even in the protected section, nearly 20% of the strains of *S. aureus* recovered from the air could not be related to known nasal carriers. Since this proportion was nearly as great as that found in the absence of directed air-flow, it seems probable that these strains were derived either from undetected sources within the section or were dispersed from the clothes of persons who entered it.

Nearly one-third of the nasal acquisitions in the ward could not be related to known nasal carriers, but about one-half of these (16%) were probably 'spurious' and half of the remainder (8%) could be related to strains recovered from patients' lesions or drawsheets, leaving no more than 8% unaccounted for. A short investigation in which both drawsheet and perineal samples were examined showed that drawsheet samples did not give a reliable indication of perineal carriage unassociated with nasal carriage.

During the period of the investigation, a single strain of *S. aureus* that was resistant to a wide range of antibiotics established itself in the ward. The most notable character of this strain was the profuse dispersion of it by carriers. As a consequence, staphylococcal wound sepsis increased, with nearly three-quarters of

the infections attributable to this strain, and nasal carrier rates increased with length of stay in the ward, over 20 % of patients who stayed 5–6 weeks acquiring the strain.

INTRODUCTION

In an attempt to reduce the incidence of wound sepsis, part of which we thought might be due to pre-operative acquisition of hospital strains of *Staphylococcus aureus*, a male surgical ward was divided into two parts by a wood and glass

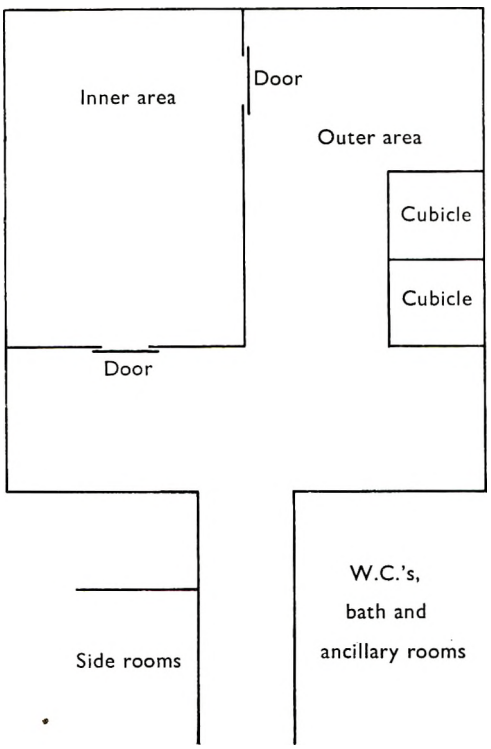

Fig. 1. Schematic plan of the ward.

partition reaching from floor to ceiling (Shooter *et al.* 1963). As far as possible, patients were admitted to the inner part before operation and transferred to the outer area after operation (see Fig. 1). Nasal acquisition of tetracycline resistant strains occurred twice as often among patients in the 'post-operative' (outer) area as among those in the 'pre-operative' (inner) area. However, the natural air movements in the ward resulted in substantial exposure of patients in the inner area to airborne strains dispersed by patients in the outer area and, in fact, about half of the nasal acquisitions by patients in the inner area that could be referred to particular patient sources appeared to be derived from patients in the outer area.

Later, we tried to prevent airborne transfer of staphylococci between the two parts of the ward by means of mechanical ventilation, first of the inner area only and later of both sections of the ward. We now report the results of the following experiments.

(1) To prevent the airborne transfer of staphylococci from the outer area to the inner, mechanical ventilation of the inner area was installed with an input of filtered fresh air, equivalent to about 10 air changes per hour, introduced through ceiling diffusers. The excess air escaped into the outer area of the ward around the edges of the floors and through grilles beside them. Even when the doors between the two parts of the ward were opened the direction of air flow was consistently outwards from the inner ventilated section. Observations were carried out with this arrangement from March 1963 until December 1964, and during this time the plant was out of action only for four periods each of less than 24 hr.

(2) Any apparent effects of this combination of ventilation and subdivision might, however, be attributable to the provision of a positive ventilation system in the inner area rather than to the separation of the two parts of the ward. The ventilation plant was therefore extended so that a separate supply of filtered fresh air was provided at approximately ten air changes per hour to each part of the ward. An air outlet, via a disused chimney, carried part of the input to the inner area directly out of the building but a substantial fraction escaped, as previously, into the outer area of the ward. Observations were made from February 1966 until June 1967, but during this period the ward was closed for cleaning for 4 weeks and part or all of the ventilating plant was out of action on eight occasions for periods of between 1 hr. and 1 day.

During both periods of observation we studied the carriage of *S. aureus* by patients and staff and the occurrence of staphylococcal sepsis. In 1962, several different strains of *S. aureus* had spread among the patients but no one of them was prevalent in the ward for long. Early in the present experiments, however, a new strain of *S. aureus* was introduced into the ward and became established in both parts. It was lysed by phages 84 and 85, or occasionally only by one of these two phages, and was resistent to penicillin, tetracycline, erythromycin and neomycin. The widespread dissemination of this strain among patients in both parts of the ward, especially during 1966–7, so confused the identification of sources for nasal acquisition as to make evaluation of the effects of the ventilation arrangements of doubtful significance.

METHODS

Patients

Patients were admitted to both parts of the ward but those admitted with sepsis, known to have been hospital in-patients within the previous 12 months or not expected to undergo operations, were placed in the outer area. As far as possible patients infected with or carrying tetracycline-resistant strains of *S. aureus* were transferred to the isolation cubicles or to the side rooms off the entrance corridor. The same staff attended all patients, and patients in isolation were generally not barrier-nursed. Staff found to be carrying tetracycline-resistant strains were treated with Soframycin nasal spray, given hexachlorophane soap to use and kept off duty until clear. Dusting powder, containing 0·33% hexachlorophane, was used extensively on the skin of patients.

Patients nursed in the inner area had to pass through the outer area in order to

use their own separate bathroom and W.C. but they were instructed not to linger or talk with patients in the outer area.

Bacteriology

A nasal swab was taken from each patient as soon as possible after admission, usually within 24 hr., and thereafter weekly on a fixed day of the week. Swabs were taken from operation wounds at first dressing and subsequently on any sign of sepsis, and also from other septic lesions not arising in operation wounds. Weekly nasal swabs were taken from the ward nurses and from as many of the medical and auxillary staff as possible. One representative of each colonially distinguishable type of *S. aureus* was selected for further examination. During 1966–7, impression plates were taken from patients' under bed-sheet (drawsheet) in the area in contact with the perineum, and perineal swabs were obtained during limited periods totalling about 9 weeks. Up to ten colonies of *S. aureus* from each plate were examined.

Air samples were taken weekly on the fixed swabbing day by impingement onto phenolphthalein phosphate serum agar using a slit sampler. The duration of sampling was 1 hr. and the volume of air sampled was 108 ft.^{3} (about 3 m.^{3}). Up to ten colonies of *S. aureus* from each plate were examined.

All cultures of coagulase-positive staphylococci were classified as *S. aureus* and were phage typed and tested for resistance to penicillin and tetracycline.

Sepsis

Wounds that on examination showed visible pus were recorded as septic. Wounds from which pathogenic organisms were cultured but where there was no visible pus were recorded as colonized. Sepsis was also suspected when pus cells were seen in the Gram film and a pathogenic organism was found on culture; the wound was then re-examined for signs of visible pus.

Method of analysis

Measurements of the nasal carrier rate and the rate of acquisition of new strains of *S. aureus*, and determinations of the probable source of infection or colonization, were in general made by methods described previously (Lidwell *et al.* 1966). In this investigation, however, the collection of weekly nasal swabs and air samples on the same day reduced the number of assumptions that had to be made about the carriage state in the intervening periods.

RESULTS

Airborne Staphylococcus aureus

Table 1 shows the results of the air sampling in the two periods of investigation (1963–4 and 1966–7). Possible sources for the strains isolated were looked for among patients and staff carriers detected on the same day or, if no swab had been obtained from one of the staff on that day, in the previous or subsequent week. Untypable strains were always recorded as derived from unknown sources even if

a carrier of an untypable strain was present in the ward. Untypable strains comprised about 40 % of those strains without identifiable source.

Although the total number of airborne *S. aureus*, varying from 56–106/1000 ft.^{3} (28 m.^{3}) for the two parts of the ward during the two periods of observation, were substantially lower than those observed in open wards in the hospital in previous

Table 1. *Source and numbers of* Staphylococcus aureus *isolated from the air*

Sampling position	Strain	Source of strain	No. of colonies of coagulase-positive staphylococci/1000 ft.^{3} of air sampled: In 1963–4	In 1966–7
Inner	Any other than 84/85	Inner	13·2 (8·7)	7·5 (5·0)
		Outer	1·3 (0·5)	2·6 (1·6)
		Outer (+cubicles and side)	3·0 (0·9)	2·8 (1·3)
		Staff	5·6 (0·3)	2·8 (0·2)
		Ambiguous	6·2	3·8
		Not known	8·8	6·5
		Total (all origins)	36·8	23·4
	84/85	All origins	19·9	39·4
	All	Overall total	56·7	62·8
Outer	Any other than 84/85	Inner	5·1 (3·4)	2·2 (1·5)
		Outer	21·5 (7·9)	4·1 (2·6)
		Outer (+cubicles and side)	26·1 (7·7)	5·7 (2·6)
		Staff	9·6 (0·6)	2·4 (0·2)
		Ambiguous	23·8	2·8
		Not known	23·7	6·9
		Total (all origins)	88·3	20·0
	84/85	All origins	17·3	46·2
	All	Overall total	105·6	66·2
Any	84/85	All origins	18·5 (16·8)	42·5 (14·7)
Any	Tetracycline resistant *not* 84/85	*All origins	2·4 (2·4)	2·4 (1·4)
Any	Any other than 84/85	*All origins	52·7 (2·2)	17·6 (1·0)
Vol. sampled in each position			29,800 ft.^{3}	6800 ft.^{3}

'Not known' includes untypable strains.

Figures in parentheses give the count derived from a single source carrier (i.e. the number of colonies/1000 ft.^{3} of air divided by the average number of carriers present in the position and of the type specified: see Table 6).

* Excluding 'no possible source identifiable' but including untypable strains. (As the 1963–4 records do not differentiate between these two categories of strains, the number of untypable strains for the period was estimated as 40 % of the aggregate of untypables and strains without identifiable source—the proportion observed during the period 1966–7.)

years, i.e. about 200/1000 ft.^{3} (Noble, 1962), they differed little from the counts observed during the period after the partition had been erected but before any mechanical ventilation was introduced, i.e. 70–80/1000 ft.^{3} (Shooter *et al.* 1963).

The 84/85 strain accounted for about 26 % of airborne *S. aureus* in 1963–4 and by 1966–7 this had risen to 71 %. Owing to the widespread dissemination of the strain there was usually more than one carrier present at any given time so that the

sources from which the air strains were derived could often not be identified. In allotting the airborne staphylococci to probable sources in various parts of the ward, we therefore excluded this strain from consideration.

During 1963–4 the distribution of other strains from positively identified patient-sources conformed with the air-flow pattern. Staphylococci found in the air were much more often referable to sources in the same area than to sources elsewhere in the ward. But whereas less than one-tenth (ratio $1{\cdot}3/(13{\cdot}2+1{\cdot}3)$) of such strains recovered in the inner area appeared to be derived from sources in the outer area, nearly one-fifth (ratio $5{\cdot}1/(21{\cdot}5+5{\cdot}1)$) of those recovered in the outer area were related to sources in the inner area. The origin of the small number of strains found in the air of the inner area for which no sources other than patients in the outer area could be discovered is not known. It seems unlikely that they were due to reverse air flow. Carriers of these strains in the inner ward or among the staff may have escaped detection, especially if they carried the organisms only in small numbers or on sites that were not examined. Alternatively, the strains may have been brought into the area on the clothes of the nurses and then dispersed into the air (Speers *et al.* 1969).

The distribution of airborne staphylococci attributable to single source carriers (shown in parentheses in Table 1) was in general similar to that for staphylococci attributable to all known sources.

After the installation of mechanical ventilation in both parts of the ward in 1966 the sources of the air strains recovered in the two parts of the ward were more evenly distributed, in each area between one-quarter and one-third apparently originating from sources in the other area. The total count of airborne staphylococci was also nearly equal in the two areas. During 1963–4 the count in the unventilated outer section had been nearly twice that in the inner area.

There was a considerable difference between the number of airborne staphylococci dispersed by single carriers of the 84/85 strain and of other strains of staphylococci. In 1963–4, single carriers of the 84/85 strain contributed on average 16·8 staphylococci/1000 ft.^{3} to the air of the ward, but single carriers of all other staphylococci contributed only 2·2/1000 ft.^{3} of air and single carriers of tetracycline-resistant staphylococci other than the 84/85 strain contributed only 2·4/1000 ft.^{3} of air. A similar difference was observed in 1966–7.

Excluding the 84/85 strains, no source could be identified for 24 % of the air-strains recovered in 1963–4 and 33 % of those recovered in 1966–7. These figures may be compared with 23 % of airborne strains without identifiable sources in the unventilated divided ward (Shooter *et al.* 1963). Since the introduction of positive ventilation in place of the previous general inflow of air from outside the ward led to no reduction in the proportion of airborne strains without an identifiable source it seems unlikely that any significant proportion of these were brought into the ward by air currents from other parts of the hospital.

As there was more than one possible source for 20 % of the air strains other than 84/85 only about 50 % of these strains could be related to probable sources of dispersion. In 1966–7 when 67 % of the strains were 84/85 types this resulted in identified unique sources for little more than 16 % of all air strains.

Nasal acquisition of Staphylococcus aureus

The overall rates for apparent nasal acquisition, lying between 36 and 71/1000 patient weeks (Table 2), were appreciably lower than those reported for the ward divided but unventilated (100 and 120/1000 patient weeks in the inner and

Table 2. *Sources and rates of nasal acquisitions of* Staphylococcus aureus *by patients*

Position at time of acquisition	Strain	Position of source	No. of strains acquired/1000 patient weeks in the ward: In 1963–4	In 1966–7
Inner	Any other than 84/85	Inner	3·5 (2·3)	—
		Outer	1·2 (0·4)	—
		Staff	4·7 (0·3)	—
		Ambiguous	5·9	—
		Not known	12·9	—
		Total (all origins)	28·2	44
	84/85	All origins	8·2	27
	All	Overall total	36·4	71
Outer	Any other than 84/85	Inner	0·9 (0·6)	—
		Outer	7·7 (2·8)	—
		Staff	10·5 (0·7)	—
		Ambiguous	5·7	—
		Not known	25·9	—
		Total (all origins)	50·7	25
	84/85	All origins	6·7	33
	All	Overall total	57·4	58
Any	84/85	All origins	8·4 (7·6)	33 (11·4)
Any	Tetracycline resistant *not* 84/85	*All origins	2·7 (2·7)	5 (3·0)
Any	Any other than 84/85	*All origins	29·3 (1·2)	21 (1·1)
Patient weeks recorded			2375	1134

'Outer' does *not* include cubicles or side rooms.
Figures in parentheses give the rate of acquisition from a single source carrier (see Table 1).
* Excluding 'no source identifiable' but including untypable (see Table 1).

outer areas respectively; Shooter *et al.* 1963) and in 1963–4 they were lower in the inner ventilated area than in the outer area. Even including acquisition of the 84/85 strain, acquisition of tetracycline resistant strains was also lower in 1963–4 than during the preceding study; in 1966–7, however, when over half of all apparent acquisitions were of the 84/85 strain, the rate was essentially similar to that observed in the outer area of the unventilated divided ward.

The widespread dissemination of the 84/85 strain during 1966–7 and the preponderance of acquisitions of this strain had the result that only six of the 75 apparent nasal acquisitions observed during this year could be related to particular carriers in identified situations during the week preceding the apparent nasal

acquisition. It was therefore not profitable to attempt any analysis of the location of sources of nasal acquisition during this period.

Examination of the results for the period 1963–4 shows that when the source was identifiable it was usually in the same area of the ward as the recipient, and that staff carriers were generally individually weak sources, although since they were numerous an appreciable proportion of all nasal acquisitions appeared to be derived from them.

Drawsheet samples and perineal swabs

As in previous analyses of this type, a substantial proportion of apparent acquisitions could not be related to any known source in the ward. Some of these acquisitions were certainly spurious, since examination of repeated nasal swabbings of the same individual shows that intermittent recovery is not uncommon (Parker, John, Emond & Machacek, 1965). There are also more antibiotic sensitive strains among these 'acquisitions' without apparent source than among acquisitions from known sources, a fact which correlates with the greater frequency of sensitive strains in nasal swabs taken from patients on admission compared with those from patients who have been in hospital for some time. Even when allowance is made for this (see Lidwell *et al.* 1966), there are still appreciable numbers of apparent nasal acquisitions including some of antibiotic resistant strains without any known source. Some people carry *S. aureus* on the skin of the perineum but not in the nose, and it has been suggested that perineal carriage may dispose towards greater dispersal of the organisms carried, so that these individuals might be more potent sources of cross-infection than plain nasal carriers (Hare & Ridley, 1958). It seemed possible that the widespread dissemination of the 84/85 strain observed in the present investigation was associated with perineal carriage. Regular perineal swabbing of patients was possible only during a short period of the investigation, but the drawsheet of each patient was sampled weekly during 1966 and 1967.

Table 3 shows an analysis of the 23 apparent acquisitions during 1966 and 1967, excluding seven acquisitions of untypable staphylococci, for which there was no known source among nasal carriers in the ward. These acquisitions were divided into two fractions (see Lidwell *et al.* 1966) with a distribution of antibiotic sensitivities similar to that found in staphylococci in admission swabs (line 2) and that for acquisitions from known sources (line 3). This suggested that 11·6 of the 23 acquisitions were 'real'. However, the inclusion of staphylococci isolated from drawsheets and lesions as possible sources produced sources for nine of the 23 acquisitions (line 4). The antibiotic sensitivity distribution of the remaining 14 (lines 5, 6) was such as to suggest that eight of them were possibly spurious in the sense described above, leaving six or no more than 8% of the original total as probably genuine acquisitions without any identified source (line 7). The contribution of the drawsheet impression samples to this was, however, small since in only two instances did a drawsheet sample identify a possible source that was not already indicated by a lesion swab.

The relationship between nasal carriage and drawsheet samples is shown in Table 4. Of 173 nasal carriers, excluding carriers of 84/85, only 22% yielded

Table 3. *Acquisitions without identifiable sources* (1966–7)

Acquisitions	No. of acquisitions with the following antibiotic sensitivity pattern: S	P	T	All sensitivities
1. Without nasal source	8	5	10	23 (31 %)
2. By admission swab sensitivity distribution	6·6	4·0	0·8	23 | 11·4
3. Leaving 'probably real' (known source, sensty. distribution)	0·5	2·3	8·8	11·6 (16 %)
4. Acquisition related to drawsheet or lesion sources	3	1	5	23 | 9*
5. Leaving without any identifiable source	5	4	5	14 (19 %)
6. Without identifiable source, by admission sensitivity	4·6	2·8	0·6	14 | 8·0
7. Leaving 'probably real' without any identifiable source	0·2	1·2	4·6	6·0 (8 %)
8.† Acquisitions related to drawsheet or lesion sources, by admission sensitivity	1·9	1·2	0·2	9 | 3·3
9.† Leaving 'probably real', from drawsheet or lesion source	0·2	1·1	4·3	5·7

S = Sensitive to penicillin and tetracycline; P = resistant to penicillin, sensitive to tetracycline; T = resistant to tetracycline.

* Of these nine, seven could be related to lesions and eight to drawsheets, six were related to drawsheet and lesion simultaneously. A part of these drawsheet/lesion acquisitions may also be spurious and due to missed nasal admission positives (see lines 8, 9).

† Applying the method of distribution according to antibiotic sensitivities, it is probable that no more than six of the nine acquisitions (line 4) were 'real'.

Note: lines 6 and 8 add to give line 2; lines 7 and 9 add to give line 3.

Table 4. *Isolation of* S. aureus *from drawsheets of patients and its relation to nasal carriage by the patient in the bed*

S. aureus in nasal swab	Examined	Drawsheets yielding *S. aureus*: 84/85 strain	Not 84/85 strain: Same as nasal strain	Not 84/85 strain: Different from nasal strain	Without *S. aureus*
Present: 84/85 strain	72	42 (58)*	—	0	30
Present: other strain	173	8 (5)	38 (22)	3 (2)	124
Absent	843	57 (7)	—	27 (3)	759

* In parentheses, percentage in nasal carriage class with positive drawsheet culture.

drawsheet samples positive for the same strain and 6 % were positive for a strain that differed from that carried in the nose. In conformity, however, with the greater dissemination of the 84/85 strain, this strain was recovered from 58 % of drawsheet samples from nasal carriers of the strain. Nearly 7 % of drawsheet samples from non-nasal carriers also yielded colonies of the 84/85 strain while only 3 % showed colonies of other strains.

The results obtained on the limited number of occasions when nasal, perineal and drawsheet samples were obtained at the same time are analysed in Table 5. Although the strains isolated from two or more sites were usually identical, the drawsheet samples gave a poor indication of perineal carriage. There were 30 occasions on which *S. aureus* was isolated from the drawsheet, but the same strain was not isolated either from the nose or the perineum on 12 of these occasions; the same strain was isolated from perineum and drawsheet on nine occasions, but on four of them the patient was a nasal carrier, so that unsuspected carriage was detected on only five occasions. The drawsheet culture was negative for the strain carried in the perineum eight of 17 times, and six of 11 times in patients who were not nasal carriers. Thus, nearly half of the positive drawsheet cultures appear to have been 'false', and half of the perineal carriers were missed.

Table 5. *Simultaneous culture for* Staphylococcus aureus *of samples from drawsheet, perineum and nose*

(No isolation was made from any site from 122 of the 185 sets of samples.)

	Number of occasions						
	Drawsheet +				Drawsheet −		
	Perineum +		Perineum −		Perineum +		Perineum −
Strain isolated*	N +	N −	N +	N −	N +	N −	N −
84/85	2	4	7	7	0	2	6
Other	2	2†	3‡	3	2	3	20
Total	4	6	10	10	2	5	26

\+ = *S. aureus* isolated; − = *S. aureus* not isolated; N = nasal swab.
* Except where indicated, strains isolated from two or more sites were identical.
† One pair: drawsheet, strain 84/85; perineum, other strain.
‡ One pair: drawsheet, other strain; N, strain 84/85.

Nasal carriage and length of stay in hospital

The changes in nasal carriage of coagulase-positive staphylococci with the length of time the patients have been in the hospital show interesting variations from place to place and at different times, and it seems that they may be a good index of the epidemiological situation in a ward (Lidwell *et al.* 1966). It is much easier to measure these changes than to carry out an analysis of individual sources of cross-infection such as had been given in the earlier sections of this paper. Only coagulase and antibiotic sensitivity testing is required and much less detailed patient records are needed. In Fig. 2 we present the results obtained in the ward under study both before and after the introduction of mechanical ventilation and show also, for comparison, the situation in an undivided unventilated ward in another part of the same hospital. The intrusion of the 84/85 strain is clearly seen. In the earliest years the overall carriage rate in both wards increased with length of stay. Carriage of strains sensitive to all antiobiotics or resistant to penicillin only showed little change with duration of stay while carriage of strains resistant to

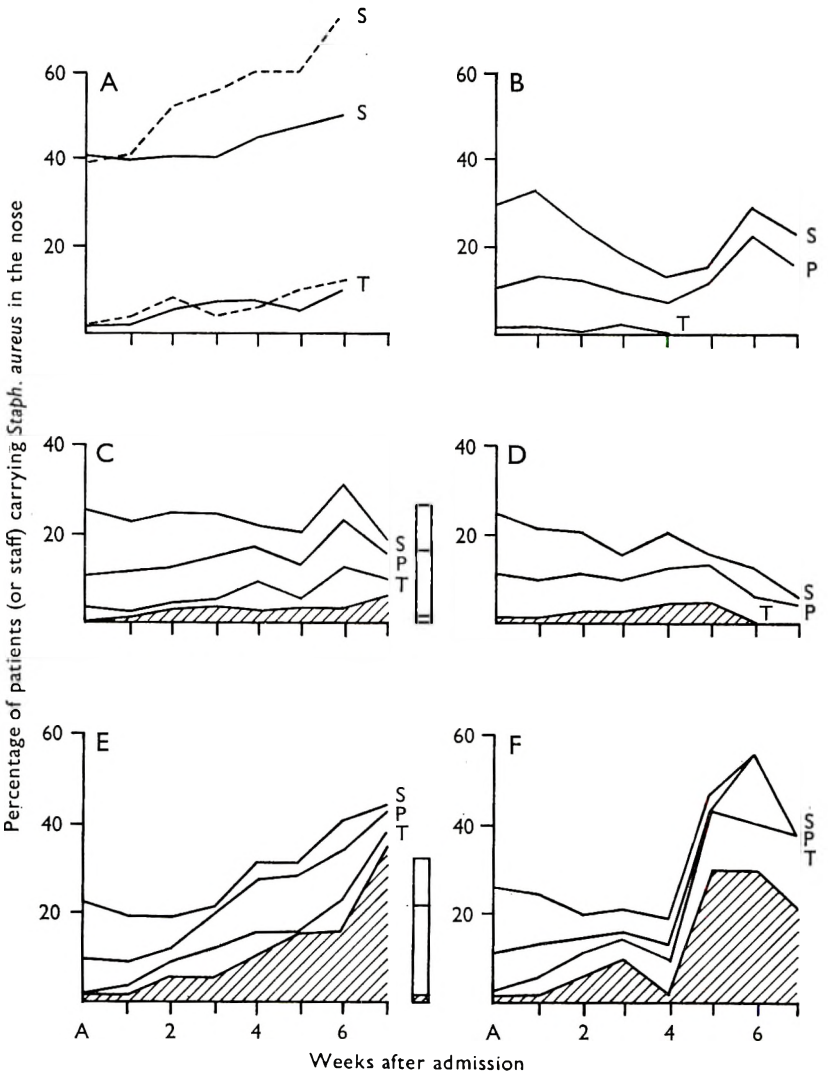

Fig. 2. Changes in nasal carriage of *S. aureus* during hospital stay. The top line, labelled S in each section, shows the percentage of patients carrying *S. aureus* in the nose after varying length of stay in the ward. The line P shows the percentage carrying strains resistant to penicillin or tetracycline or both and the lower line T the percentage carrying tetracycline resistant strains, the hatched areas indicating the part due to carriage of the 84/85 strain. (A) Year 1961. Full lines refer to the divided unventilated ward, broken lines to an undivided unventilated ward elsewhere in the hospital. (B) Year 1966. Patients in the same undivided unventilated ward. (C) Year 1963–4. Patients in the outer area of the divided ward. The inner area only was mechanically ventilated. (D) Year 1963–4. Patients in the ventilated inner area of the divided ward. (E) Year 1966–7. Patients in the outer area of the divided ward. Both areas were mechanically ventilated. (F) Year 1966–7. Patients in the inner area of the divided ward. The histograms between (C) and (D) and between (E) and (F) show the average rate of nasal carriage by members of the staff working in the divided ward.

tetracycline and other antibiotics increased. This has been a general experience in open hospital wards (Lidwell *et al.* 1966). However, experience in some subdivided wards in recent years has shown situations in which carriage of sensitive staphylococci was reduced during hospital stay and there was little countervailing increase in resistant strains. It seems likely that this change is due partly to a more effective use of antibiotics but it may also reflect a reduction in the opportunities for cross-infection in the situations described or a change in the disseminating abilities of the prevalent strains of staphylococci.

The results given in Fig. 2 show that in this hospital, in the undivided unventilated ward as well as in the ward under study, there has been during recent years a loss of sensitive strains during stay in hospital without any significant increase in the carriage of multiple resistant strains. In 1966 and 1967, however, this was true in the divided ward only for strains other than 84/85; this strain spread so effectively that there was an overall increase in the nasal carriage rate with length of stay in the ward entirely attributable to acquisition of this strain. There is some slight evidence that acquisition was delayed in the inner area compared with the experience of the outer section.

Table 6. *Average numbers of carriers of coagulase-positive staphylococci present*

	1963–4	1966–7
Unambiguously located (strains other than 84/85)		
In inner area	1·51	(1·5)
In outer area	2·72	(1·6)
All patients		
Strains other than 84/85	7·24	3·88
84/85	0·93	2·28
Tetracycline resistant strains other than 84/85	0·55	1·08
Staff		
Strains other than 84/85	16·95	14·70
84/85	0·17	0·60
Tetracycline resistant strains other than 84/85	0·44	0·60

Nasal acquisition and the numbers of airborne staphylococci

The potential value of ventilation systems designed to reduce the exposure to airborne bacteria depends not only on the proportion of cross-infection attributable, directly or indirectly, to airborne micro-organisms but also on the relationship between the numbers of airborne organisms to which the patient is exposed and the risk of infection which this represents. Nasal carriage of *S. aureus* is not in itself a disease but is the principal reservoir of the organism. The exchange of different strains between patient and patient and between patient and staff is then the mechanism by which particular strains, especially those resistant to antibiotics that are less often used in the normal population, maintain themselves endemically in the hospital environment. Examination of the data presented in Tables 2 and 3 together with the figure for the numbers of possible source carriers given in Table 6 shows that the chance and rate of nasal acquisition is related to both the airborne counts and to the number of possible sources. Neither relationship is, however, very close and, as in the two other situations in which we have

made a similar analysis (Lidwell *et al.* 1966, and unpublished data from a partially subdivided modern ward at the Queen Elizabeth II Hospital, Welwyn), there is a better correspondence between the rate of acquisition per possible source carrier, and the exposure to airborne staphylococci per source carrier in the same relationship to the recipient. The exposure per source carrier and the rate of acquisition per source carrier are given in Tables 2 and 3, in brackets, and the relationship between them is exhibited on a log. scale in Fig. 3. As observed previously the relationship, for the figures from the 1963–4 observations on strains other than 84/85, can be represented by a straight line with a slope substantially less than unity. For the present observations this slope, about 0·6, is appreciably greater

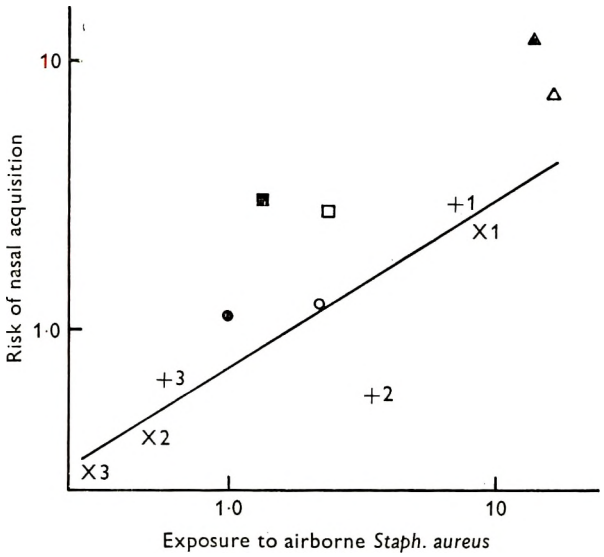

Fig. 3. Relation between the risk of nasal acquisition and the exposure of airborne *S. aureus*. Logarithmic scales for both co-ordinates. Risk of nasal acquisition: per potential source (carrier) per 1000 patient weeks. Exposure to airborne *S. aureus*: colony count/carrier/1000 ft.^{3} (28 m.^{3}) of air sampled. Line drawn with slope of 0·60. ×, Acquisitions by patients in the inner area, all strains except 84/85. +, Acquisitions by patients in the outer area, all strains except 84/85. (1) Acquisitions from patients in the same area. (2) Acquisitions from patients in the other area. (3) Acquisitions from staff carriers. ○, Average rate of acquisition and exposure to *S. aureus* for all strains other than 84/85 by all patients from all sources. □, Average rate of acquisition and exposure for tetracycline resistant strains other than 84/85 by all patients from all sources. △, Average rate of acquisition of and exposure to the 84/85 strain for all patients from all sources. The symbols ●, ■, ▲ give the comparable figures for the period 1966–7 when detailed source breakdown was impracticable.

than in the other two situations, 0·20 and 0·25 respectively, but the accuracy of these estimates is not high, especially in the present situation. A relatively consistent relationship of this kind lends force to the contention that the airborne route is the most important one with respect to nasal acquisition. The low values of the slope imply that reduction in the airborne transport of staphylococci, e.g. by ventilation systems, will produce a much less than proportionate reduction in the

rate of nasal acquisition. A tenfold reduction would, in the evidence presented here, lead to a reduction of the order of about threefold, or rather over $1\frac{1}{2}$-fold and rather less than twofold in the other two situations referred to.

Points are also shown on the figure for the average acquisition rates for both observation periods, for tetracycline resistant strains other than 84/85, and for 84/85 strains. The average figures for 1966–7 are between 40 and 50 % higher than those for 1963–4 but no detailed breakdown by sources was possible, as has been explained earlier. We have no explanation, other than the inherent variability of this kind of observational data, to account for this. In each year the tetracycline resistant strains were acquired about twice as frequently as the average of all strains for a given level of exposure. The 84/85 strain was similar to other tetracycline resistant strains in this respect, i.e. the great capacity this strain showed for spreading did not appear to be due to any greater colonizing potential but entirely to the much greater extent to which carriers dispersed it into their environment.

It must be pointed out that the limited effect of reduction in airborne staphylococci on nasal acquisition consequent on the below unity slope of the relationship as shown in Fig. 3 applies to each epidemiologically distinguishable strain separately, the effects of different strains would appear to be additive, i.e. any environmental measure which reduced the airborne levels by reducing the number of possible sources would be expected in this respect to produce a proportionate reduction in the rate of nasal acquisition.

Wound colonization and sepsis

Table 7 shows the figures for the two periods of observation. The rapid rise in the proportion of sepsis due to the 84/85 strain is at once apparent although the overall difference between the two periods is negligible. The rates during both periods (7·6 and 7·8 % respectively) were, however, substantially higher than in the divided unventilated ward (5·4 %; Shooter *et al.* 1963), or in the undivided ward the year previously (3·8 %; Williams *et al.* 1962).

Table 7. *Wound colonization and sepsis due to* Staphylococcus aureus

	1963–4	1966–7
Number of wounds examined	485	475
Percentage colonized with *S. aureus*	11·4	13·0
Percentage septic with *S. aureus* (all strains)	7·6	7·8
Percentage septic with 84/85 strain	1·6	5·7
Percentage septic without *S. aureus*	2·3	3·2
Total wound sepsis (%)	9·9	11·0

There have been some changes in the character of the surgery performed in this ward over the years but this had not been great, and application of the rates given in the second of the above references to the operation list for 1966–7 leads to an expected sepsis rate of only 4·7 %. Examination of the distribution of sepsis due to the 84/85 strain over the different types of operation does not suggest any associa-

tion with any particular types of operation. The widespread dissemination of this strain has, therefore, been accompanied by a significant overall rise in wound sepsis. There was also no significant difference in the incidence of sepsis between patients nursed in the two different ward areas.

DISCUSSION

The changes that take place in the nasal carriage of *S. aureus* by patients during successive weeks of their stay in hospital are now somewhat different from those observed over 7 years ago. At that time, it was the general experience that the total carrier rate of patients increased progressively during their stay in hospital. This was due to the acquisition by many patients of multiple-antibiotic resistant 'hospital' staphylococci with little corresponding loss of sensitive strains or of strains resistant only to penicillin. In a number of hospitals the more sensitive strains are now lost more quickly than formerly, and this is probably attributable to changes in the pattern of antibiotic usage, and particularly to giving penicillin in large dosage. The fact that in some hospital wards the rate of acquisition of multi-antibiotic resistant staphylococci has decreased, so that the total effect is a falling carrier rate during stay in hospital, may be attributable to hygienic improvements and to avoiding giving ineffective antibiotics to patients infected or colonized with resistant strains.

The introduction of the 84/85 strain into our ward interrupted this comfortable progress. The great potential of this strain for spreading seems to depend on its being dispersed by carriers some ten times more profusely than any other strain encountered. In addition to having a rather wide range of antibiotic resistance, it may also possess some other undetected character that enables it to multiply to higher levels in colonized sites, thereby increasing the number of organisms dispersed into the environment from them. Some part of the wider dispersal of the strain might also arise from better survival in the environment, including the air, but we have no evidence of this either. Clinically the strain does not seem to present any special characteristics, except that like most 'hospital' strains belonging to phage group III it seldom causes boils in patients or staff. By the end of the second period of observation, however, nearly 75% of all cases of staphylococcal sepsis were due to it, and the total staphylococcal wound sepsis rate in the ward had probably risen by 50%. In this changing epidemiological situation it was clearly very difficult to assess any effects due to sequential changes in the environment such as ventilation. In spite of the advent of the 84/85 strain the total count of airborne coagulase-positive staphylococci in the air of the ward did not rise significantly above the level to which it had fallen in the immediately preceding period, and nasal acquisition of new strains also remained low, except for substantial acquisition of the 84/85 strain following several weeks in the ward. It is clear, however, that mechanical ventilation with fresh air at as high a rate as ten air changes/hr. in the ward was ineffective in controlling the spread of this epidemic strain and led to no reduction in the incidence of sepsis in surgical wounds. A similar conclusion was reached by Whyte, Howie & Eakin (1969) who found no

difference between the rates of nasal acquisition and sepsis in a 'race-track' type ward (internal concentric corridor) mechanically ventilated at 7–8 air changes/hr. and those experienced in two open Nightingale type wards.

The widespread dispersion of the 84/85 strain also complicated attempts to investigate in greater detail the possible origins of new strains appearing in the noses of patients that could not be traced to other patients or staff carriers. It was thought that impression plates from the drawsheets might reveal undetected perineal carriers as sources of this acqusition. In fact, very few additional sources were recognized by this procedure, but the systematic study of swabs from all lesions enabled all but 8% of the nasal acquisitions to be plausibly accounted for. A smaller study in which perineal swabs as well as drawsheet impressions and nasal swabs were taken at the same time showed, however, that the drawsheet cultures were a very unreliable indicator of perineal carriage.

Finally it was possible from the data obtained during the first part of the study, before the 84/85 strain became too widespread, to examine the relationship between exposure to airborne *S. aureus* and the chance of nasal acquisition of the strain. In conformity with observations reported elsewhere, the risk of acquisition of a single strain increased less than any increase in airborne exposure to it. This effect was somewhat less pronounced than in two other situations in which similar studies had been made, but it is possible that the differences reflect no more than the substantial error inherent in the estimates. Considered on an absolute scale, the risk of acquisition for an equivalent dose was similar to that found in another divided surgical ward (Lidwell *et al.* 1966) and of the order of half that in a general medical ward (unpublished). In all the three situations, the risk of acquisition of a resistant strain, including the 84/85 strain, was of the order of twice that found for all strains, predominantly those sensitive to all antibiotics or resistant to penicillin only.

Our thanks are due to the nursing staff of the hospital, to Miss Horgan for work in the laboratory, to the Board of Governors of the Hospital for a grant towards the expenses of the work and to the then Ministry of Health who provided and installed the ventilating plant.

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J. Hyg., Camb. (1970), **68**, 435
Printed in Great Britain

Experimental encephalitis following peripheral inoculation of West Nile virus in mice of different ages

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(*Received* 23 *March* 1970)

SUMMARY

Experimental arbovirus infections of mice provide a convenient model to study factors which determine the occurrence or severity of encephalitis following extraneural infection with certain neurotropic viruses. Varying doses of West Nile or Powassan viruses were inoculated by intraperitoneal or intramuscular routes into mice of varing ages; individual variables were manipulated to influence the outcome of infection. Three patterns of pathogenesis were delineated: (1) Fatal encephalitis, preceded by early viraemia, and invasion of the central nervous system. (2) Inapparent infection, with no detectable viraemia and no evidence of central nervous system invasion. (3) Subclinical encephalitis, usually preceded by trace viraemia, with minimal transient levels of virus in the brain. In this latter type of subclinical infection with a potentially lethal virus, the immune response probably plays an important role in recovery.

INTRODUCTION

Human infections with neurotropic viruses are known to range in their manifestations from fatal encephalitis through aseptic meningitis to inapparent extraneural infection. It is well established that experimental arbovirus infections in mice also range from inapparent to lethal, following peripheral virus inoculation. Furthermore, the outcome of infection is influenced by a number of readily manipulated variables, including virulence and dose of virus, route of inoculation, and age of host (Albrecht, 1968; Cole & Wisseman, 1969; ElDadah, Nathanson & Sarsitis, 1967; Grossberg & Scherer, 1966; Johnson & Mims, 1968; Lennette & Koprowski, 1944). Thus experimental arbovirus infections can be used to delineate some of the factors which may determine the occurrence of encephalitis following peripheral virus infection.

The present investigation was designed as a series of pair comparisons, in each of which a single experimental variable was altered to convert inapparent into lethal infection. For each of these pairs, the comparative pathogenesis was studied, in an attempt to define factors critical to the outcome of infections.

MATERIALS AND METHODS

Animals

MBR/ICR albino Swiss outbred mice were obtained from a commercial breeder (Hazelton-Carbia, Laurel, Maryland). Pregnant mice were followed to determine exact age of young. The term 'newborn' is used throughout to refer to animals 1–3 days of age.

Virus

West Nile virus strain Egypt 101 (E101) had had approximately four egg followed by six newborn mouse brain passages (ElDadah *et al.* 1967). Powassan virus had had about eight newborn mouse brain passages (Thind & Price, 1969*b*). Viruses were inoculated intracerebrally (0·02 ml., without anaesthesia), intraperitoneally (0·05 ml., left lower quadrant), or intramuscularly (0·05 ml., gastrocnemius). All virus doses are expressed as newborn mouse intracerebral LD50.

Titrations

For determination of 50% lethal (LD50) and infectious (ID50) end-points, eight animals were inoculated with each decimal dilution of virus. After 4 weeks observation, survivors were bled from the orbital plexus for serum antibody and challenged by intracerebral inoculation of 100 LD50. Animals with antibody or resisting virus challenge were considered to have undergone inapparent infection (ElDadah *et al.* 1967).

Pathogenesis experiments

To follow the course of infection, four mice in each experimental group were killed daily. Heparinized bloods or sera were pooled for viraemia or antibody determinations, respectively. Following perfusion with saline, tissues from all animals were removed for immunofluorescent examination. For histological preparations 2–4 additional mice were killed at 2 and at 3 weeks after infection; in the case of lethal infections, animals were killed when moribund.

Heparinized bloods were tested for viraemia by intracerebral inoculation of newborn mice with decimal dilutions prepared in 0·75% bovine plasma albumin in phosphate-buffered saline. In those instances where no viral antigen was detected by immunofluorescence (Figs. 4B, 6B, 7B), tissues were also homogenized, centrifuged, and supernates inoculated into newborn mice (Cole & Wisseman, 1969; ElDadah & Nathanson, 1967).

Histological methods

For immunofluorescent (IF) staining, animals were perfused with sterile saline, organs removed, embedded in 10% gelatin, and 8 μ sections cut at $-20°$C. The direct fluorescent antibody method was used with Evans blue counterstain added (Cole, Nathanson & Rivet, 1970; ElDadah & Nathanson, 1967). Tissues routinely examined were: brain (parasagittal section), cross-section of thigh (muscle and peripheral nerve), liver, spleen and kidney. Immunofluorescence was graded sep-

arately for each tissue, according to a semiquantative scale: 0 = none seen; 1 = a few scattered cells; 2 = 1 to 25% cells stained; 3 = 25 to 75% cells stained; 4 = over 75% cells stained. Within each experimental group, observations were usually similar for different mice killed on the same day, and a median of four animals is recorded in the figures.

For light microscopy, mice were perfused with 10% formalin containing 1% acetic acid, paraffin sections were cut at 10 μ, and stained with haematoxylin and eosin (ElDadah & Nathanson, 1967).

Haemagglutination-inhibition (HI) antibody

Sera were treated with acetone and tested in microtitre plates with six units of antigen prepared by the sucrose acetone method (Clarke & Casals, 1958; ElDadah *et al.* 1967). Inhibition at a serum dilution of 1/20 or greater was considered to represent specific antibody (recorded as + in Figs. 2–7).

RESULTS

Age-specific titrations of West Nile and Powassan viruses

The upper half of Fig. 1 shows that West Nile virus had a high intracerebral titre in mice of all ages ($10^{8\cdot3}$ LD50/0·02 g. in newborn and 10^{7} LD50 in adult animals). Following intramuscular or intraperitoneal inoculation, the titre was high in newborns ($10^{8\cdot3}$ LD50/0·05 g.), but decreased with increasing host age. By the intramuscular route, no deaths occurred in mice age 4 weeks, and by the intraperitoneal route few deaths occurred in mice age 6 weeks or over.

Infectious titres (ID50) were determined by testing survivors of titrations for haemagglutination-inhibition (HI) antibody, followed by intracerebral virus challenge with 100 LD50. In newborn mice inoculated by any route, LD50 and ID50 were identical; that is, there was no evidence of sublethal infection. A few older mice surviving intracerebral inoculation had undergone sublethal infection, but the ID50 was no more than $10^{0\cdot5}$ greater than the LD50. Following intraperitoneal or intramuscular injection, the age-specific ID50 was about $10^{6\cdot5}$ at 4 weeks of age and $10^{5\cdot5}$ at 12 weeks of age. Thus, peripheral inoculation of West Nile virus regularly produced inapparent infections in older mice.

Powassan virus was included in the study, because it was known to possess high neuro-invasiveness following peripheral inoculation in older mice, in contrast to the low peripheral virulence of West Nile virus. The lower half of Fig. 1 shows that, following intraperitoneal or intramuscular inoculation, the LD50 titre of Powassan virus in newborn mice was $10^{9\cdot3}$, falling to 10^{8} and to 10^{6} in mice 4 and 12 weeks of age, respectively. By any route, the ID50 of Powassan virus was only slightly greater than the LD50, even in mice age 12 weeks.

Influence of experimental variables on pathogenesis

A systematic study of the course of infection was made for different inoculation routes in mice of varying ages, using both West Nile and Powassan viruses. The different combinations studied are listed in Table 1. Since this study was designed

to contrast pathogenesis under circumstances where a single experimental variable markedly influenced the outcome of infection, the list in Table 1 has been arranged to delineate the comparisons which were made. Figs. 2–7 follow the same scheme.

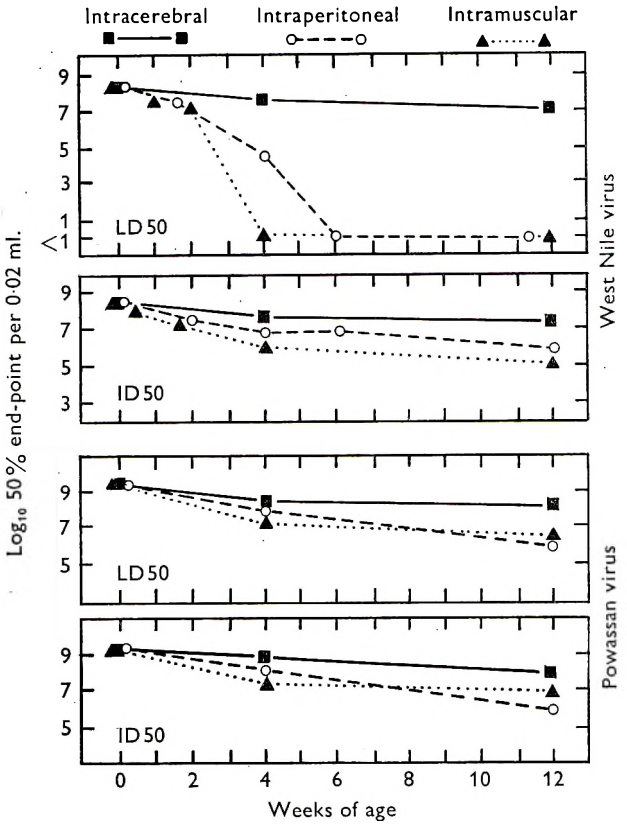

Fig. 1. West Nile and Powassan virus age-specific lethal (LD 50) and infectious (ID 50) titres in mice following intracerebral, intraperitoneal, and intramuscular inoculation. ID 50 based on HI serum antibody in survivors bled 4 weeks after infection, and on intracerebral challenge at same time with 100 newborn mouse i.c. LD 50 of homologous virus.

Effect of age

The pathogenesis of West Nile virus infection following intracerebral inoculation of 1000 LD 50 in mice age 1–3 days and age 4 weeks are presented in Fig. 2. At both ages a regularly fatal encephalitis occurred, and the data are shown for comparison with infection following peripheral inoculation, presented in Figs. 3–7.

Fig. 3 contrasts intraperitoneal inoculation of 1,000,000 LD 50 of West Nile virus in mice age 1–3 days and in mice 12 weeks of age. In newborn mice, virus appears in the blood by day 2 and in the brain by day 3, with most animals dying 5–7 days after inoculation. In animals age 12 weeks there was no mortality or signs of illness and, although viraemia was not detected, virus did invade the central nervous system. Viral antigen was detected in scattered individual neurones in brains examined 8 days after infection.

Table 1. *Summary of pathogenesis studies of experimental arbovirus infections in mice; pair comparisons of variables which influence the occurrence of lethal encephalitis**

Key variable in each pair comparison	Virus	Route of inoculation	Dose $\log_{10}$ LD50	Age	Outcome	CNS invasion	Pathogenesis summarized in fig.
Age	West Nile	i.c.	3·0	1-3 days	Died	+	2
	West Nile	i.c.	3·0	4 weeks	Died	+	
Age	West Nile	i.p.	6·0	1-3 days	Died	+	3
	West Nile	i.p.	6·0	12 weeks	Survived	+	
Age	West Nile	i.m.	3·0	1 week	Died	+	4
	West Nile	i.m.	3·0	4 weeks	Survived	−	
Route	West Nile	i.p.	6·0	4 weeks	Died	+	5
	West Nile	i.m.	6·0	4 weeks	Survived	+	
Dose	West Nile	i.p.	6·0	4 weeks	Died	+	6
	West Nile	i.p.	3·0	4 weeks	Survived	−	
Virus	Powassan	i.m.	3·0	4 weeks	Died	+	7
	West Nile	i.m.	3·0	4 weeks	Survived	−	

* Virus dose in newborn mouse i.c. LD50/inoculum.

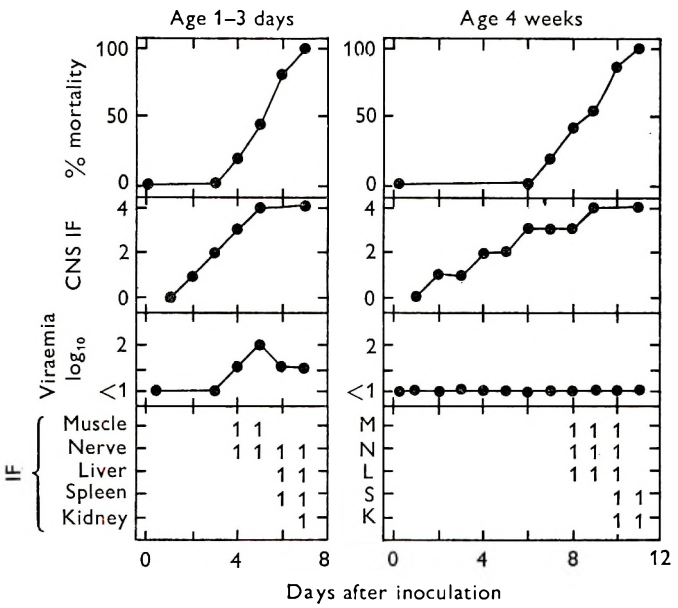

Fig. 2. Course of West Nile virus infection in mice, following intracerebral inoculation of 1000 newborn mouse i.c. LD50. Comparison of lethal infections in animals age 1–3 days and age 4 weeks. See methods for immunofluorescence (IF), grading scale. Viraemia: $\log_{10}$ newborn mouse i.c. LD50/0·02 ml. whole blood. Haemagglutination-inhibiting antibody (HIAB): +, titre of 1/20 or greater.

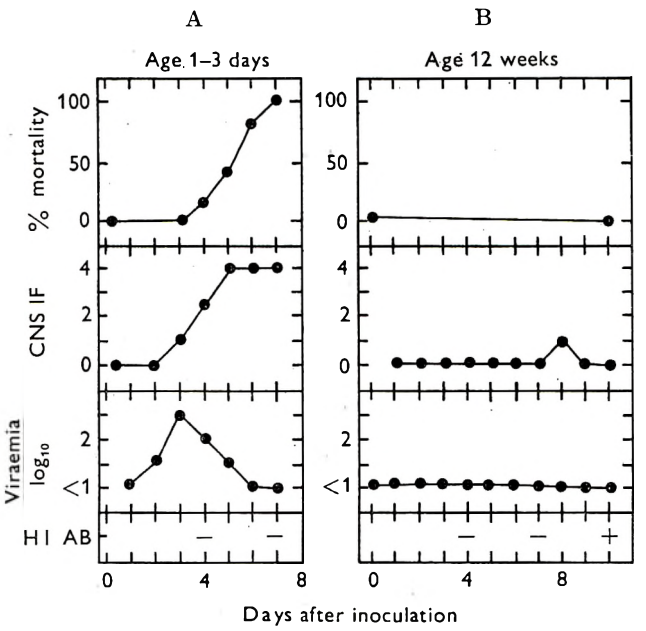

Fig. 3. Course of West Nile virus infection in mice, following intraperitoneal inoculation of 1,000,000 newborn mouse i.c. LD 50. Comparison of lethal infections in animals age 1–3 days and inapparent infection in animals age 12 weeks. See caption for Fig. 2.

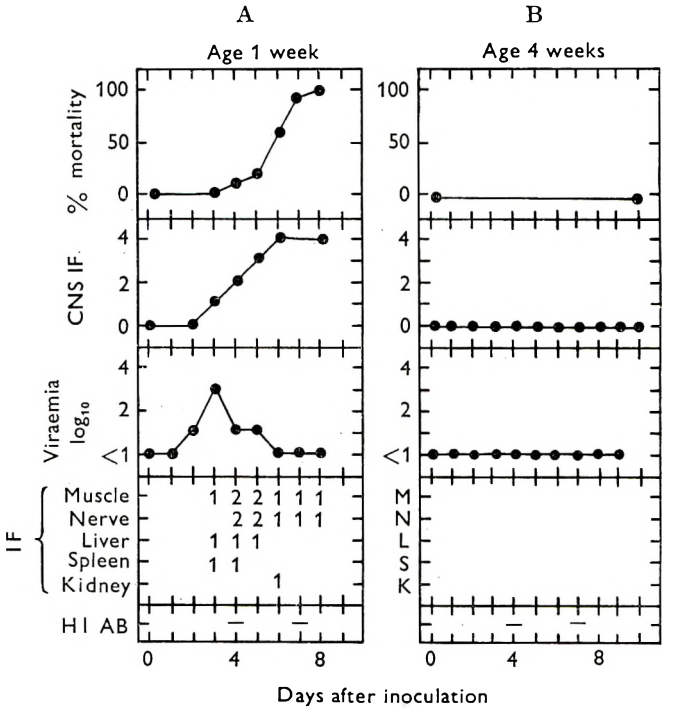

Fig. 4. Course of West Nile virus infection in mice, following intramuscular inoculation of 1000 newborn mouse i.c. LD 50. Comparison of lethal infection in animals age 1 week and inapparent infection in animals age 4 weeks. Peripheral tissues of animals age 4 weeks were also negative on subinoculation. See caption for Fig. 2.

Fig. 4 presents another age comparison. Death regularly occurred 5–8 days after inoculation in 1-week-old mice injected intramuscularly with 1000 LD50 of West Nile virus. Viraemia was detected on days 2–5, and immunofluorescent staining revealed widespread infection in brain and other tissues, beginning about 3 days after infection. By contrast, mice age 4 weeks had inapparent infections, following intramuscular inoculation of 100 LD50. Furthermore, direct titrations and immunofluorescent observations failed to reveal virus either in blood, or in a variety of extraneural tissues including the inoculated muscle.

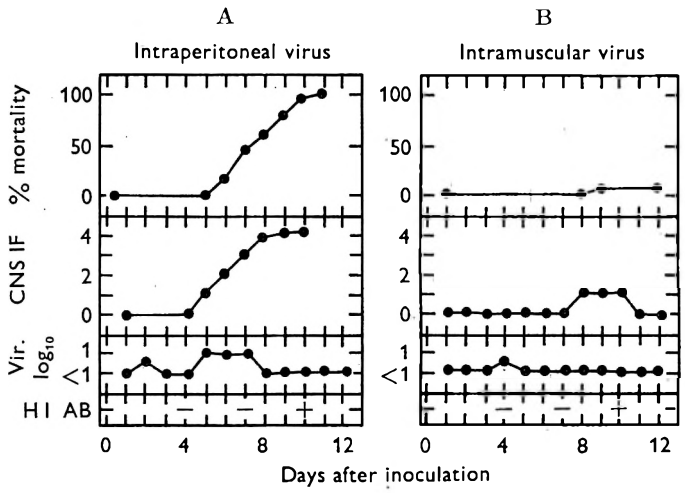

Fig. 5. Course of West Nile virus infection in mice age 4 weeks, following inoculation of 1,000,000 newborn mouse i.c. LD50. Comparison of lethal intraperitoneal infection and inapparent infection following intramuscular inoculation. See caption for Fig. 2.

Route of inoculation

Fig. 1 indicates that mice 4 weeks of age were not killed by intramuscular injection of any dose of West Nile virus, but did succumb to intraperitoneal injection of large virus doses. Fig. 5 shows a comparison of fatal intraperitoneal and subclinical intramuscular inoculation of 1,000,000 LD50. The critical difference appears to be the occurrence of viraemia, which was irregularly detected on days 2–7 following intraperitoneal injection, with invasion of brain about day 5, followed by fatal encephalitis. By contrast, intramuscular injection was followed by minimal viraemia detectable only on day 4; invasion of the central nervous system occurred late (minimal amounts of viral antigen seen on days 8–10), and only an occasional mouse (3 of 42) developed clinically apparent encephalitis.

Virus dose

A comparison of lethal and sublethal infection, under conditions where the critical variable was virus dose, is shown in Fig. 6. Mice age 4 weeks, injected by the intraperitoneal route, all died after a dose of 1,000,000 LD50, while those given 1/100 LD50 survived. The larger dose produced viraemia, with subsequent invasion of the brain; the smaller dose failed to produce detectable viraemia or evidence of

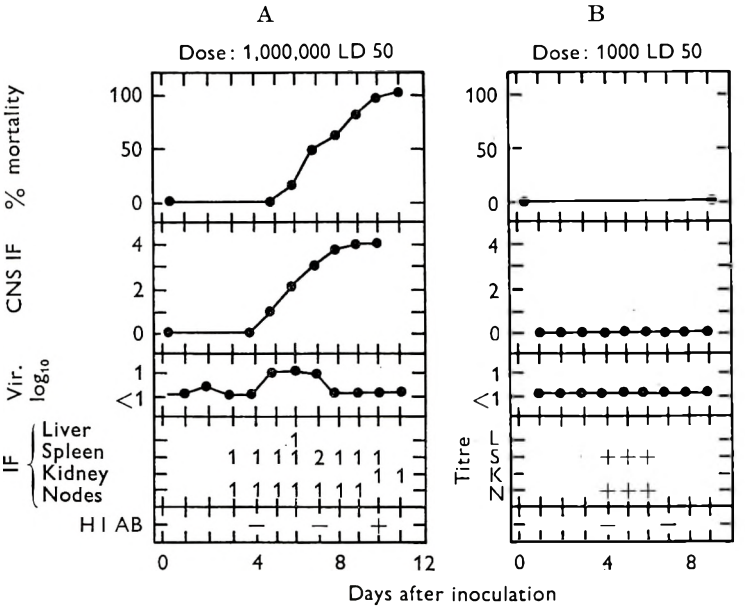

Fig. 6. Course of West Nile virus infection following intraperitoneal inoculation in mice age 4 weeks. Comparison of lethal infection produced by large inoculum (1,000,000 newborn mouse i.c. LD 50) and inapparent infection produced by smaller inoculum (1000 LD 50). For animals given 1000 LD 50 peripheral tissues (including peritoneal lymph nodes) were tested by subinoculation since they were negative for immunofluorescent viral antigen. +, titre ≧ 10 LD 50/0·02 g. See caption for Fig. 2.

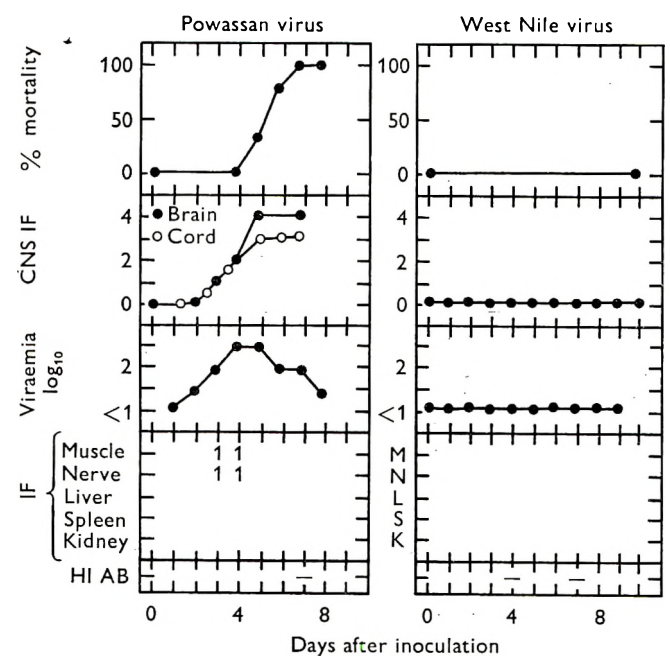

Fig. 7. Course of West Nile and Powassan virus infections in mice age 4 weeks, following intramuscular inoculation of 1000 newborn mouse i.c. LD 50. Comparison of lethal Powassan virus infection and inapparent West Nile virus infection. Peripheral tissues of animals given West Nile virus were also negative on subinoculation. See caption for Fig. 2.

central nervous system invasion, but virus was found (by subinoculation) in spleen and peritoneal lymph nodes on days 4–6.

Viral virulence

To delineate the influence of viral virulence, West Nile was compared with Powassan virus, as shown in Fig. 7. Mice 4 weeks of age were inoculated intramuscularly with 1000 LD50 of each virus. Under these conditions West Nile virus produced neither detectable viraemia nor infection of the central nervous system, and virus was not detected in a variety of tissues. By contrast, Powassan virus produced a marked viraemia, present from days 2–8, with invasion of brain, spinal cord, and other tissues, first detected on day 2 or 3, followed by death of all animals on days 5–7.

Histological evidence of encephalitis

Each of the groups of animals in Figs. 2–7 were examined for evidence of encephalitis. Lethal infections (Figs. 2, 3A, 4A, 5A, 6A, 7A) were accompanied by readily apparent histological changes, including leptomeningitis, perivascular cuffs, and focal infiltrates; neuronal necrosis or outfall were patchy and most frequent in cerebral cortex and hippocampus. In instances where there was no evidence of viral invasion of the CNS (Figs. 4B, 6B, 7B), there was no histological evidence of encephalitis. In circumstances where a subclinical CNS infection occurred (Figs. 3B, 5B) a mild meningo-encephalitis was seen in some brains, with little detectable neuronal outfall; failure to see changes in some animals may have been due to transient nature of inflammation (animals were killed at 14 and 21 days after infection), to the limited number of sections examined, or to absence of neuroinvasion.

DISCUSSION

This study distinguishes three categories of infection following peripheral arbovirus inoculation: (1) widespread infection with subsequent overwhelming involvement of the brain; (2) minimal immunizing infection without involvement of the CNS; and (3) an intermediate category where virus reaches the CNS, but undergoes only limited replication. These markedly different courses of infection pose two obvious questions which require further consideration: first, what are the differences in the extraneural phases of infection which determine whether CNS invasion occurs; and second, why, in some instances, does a potentially lethal neurotropic virus regularly invade the CNS and yet give rise to only a limited subclinical infection?

Extraneural phase of infection

Recent reviews by Johnson & Mims (1968) and by Albrecht (1968) describe the sequence of events in arbovirus encephalitis. There is early multiplication in extraneural tissues, followed by viraemia, with subsequent spread into the brain. Albrecht (1968) summarized a number of studies suggesting that variations in the extraneural phase of infection could be an important determinant in the occurrence of CNS invasion. The present study is consistent with this view. Thus, *age* comparisons (Fig. 3, 4) show a much greater viraemia in young than old mice, which

correlates with the distribution of immunofluorescence in tissue and indicates that extraneural virus replication was much more widespread and intense in young animals (Fig. 4). Other variables being constant, *route* of inoculation can determine outcome; Fig. 5 indicates that intraperitoneal injection produces an earlier and more prolonged viraemia than intramuscular injection, perhaps reflecting differences in the relative efficiency with which the virus inoculum reaches susceptible cells (cf. Figs. 4B, 6B). Under conditions where there is a delicate balance between parasite and host, virus *dose* can be critical. Fig. 6 shows that a large dose can initiate viraemia; a small dose fails to produce detectable viraemia (minimal levels of virus were detected in peritoneal lymph nodes and spleen), and there is no subsequent CNS invasion.

The high peripheral virulence of Powassan virus in older mice (in contrast to West Nile virus) correlates with much higher viraemia titres (Fig. 7). This may solely reflect the greater ability of Powassan virus to replicate in those extraneural sites which seed virus into the circulation. However, arboviruses can also vary markedly in the rates with which they are cleared from the circulation, which in turn could influence viraemia potential (Postic, Schleupner, Armstrong & Ho, 1969).

Virus replication in the central nervous system

Of the three types of infection seen in this study, two appear relatively readily understood. Lethal encephalitis is characterized by cytolytic infection of a high proportion of neurones and glial cells; death may occur so rapidly that histological changes as seen by conventional light microscopy are not fully developed (Albrecht, 1968; ElDadah & Nathanson, 1967). At the other end of the range, infections in which virus does not become established in the brain necessarily produce no changes in the CNS.

Of most interest is the intermediate category of infection in which virus reaches the CNS, but undergoes an abortive subclinical cycle of replication and then disappears. Under appropriate experimental conditions, this pattern occurs with regularity (Figs. 3B, 5B). Since West Nile virus injected intracerebrally, even in small inocula, is potentially capable of producing lethal encephalitis in mice of any age (Fig. 1), the occurrence of sublethal encephalitis requires explanation.

It is clear from this and prior studies (Albrecht, 1968; ElDadah & Nathanson, 1967) that in an abortive CNS infection a small number of widely scattered neurones (and possibly glia) are infected; these cells are destroyed, usually with concomitant production of mild inflammatory changes. Thus, infection is clearly established throughout the CNS, but fails to progress even though a potentially susceptible substrate is available.

The occurrence of subclinical CNS infection following extraneural inoculation with highly virulent arboviruses is probably a relatively common laboratory phenomenon, since findings similar to ours have been reported for a number of arboviruses: louping-ill (Doherty, 1969), Langat (Thind & Price, 1969*a*; Webb *et al.* 1968), Japanese encephalitis (Huang & Wong, 1963) and Venezuelan equine encephalitis (Gleiser, Gochenour, Berge & Tigertt, 1961). These descriptive studies of pathogenesis indicate that virus invasion of the CNS occurs later during abortive

infection than it does during lethal infection. In the present study, virus was first detected in the CNS on day 8 in abortive infections (Figs. 3B, 5B) and on days 3–5 (Figs. 3A, 4A, 5A, 6A, 7A) in lethal infections. This suggests that, if CNS invasion is sufficiently delayed, host defence mechanisms can outrace the virus and abort the infectious process (Mims, 1964).

One of the defence mechanisms which plays an important role in abortive encephalitis is the immune response. The use of immunosuppressive techniques to convert abortive arbovirus infection into lethal encephalitis (Cole & Nathanson, 1968; Nathanson & Cole, 1970; Weiner, Cole & Nathanson, 1969) provides ample evidence for this view.

Since a number of group B arboviruses are both efficient inducers of interferon as well as sensitive to its action (Finter, 1967), the interferon response could also be of importance in the outcome of infection. However, several relevant studies of arbovirus infections in mice indicate that interferon production appears to be directly related to virus replication, more interferon being detected in tissues of animals in which virus titres are highest (Cole & Nathanson, 1968; Cole & Wisseman, 1969; Subrahmanyan & Mims, 1966; Vainio, Gwatkin & Koprowski, 1961). In our view, such findings fail to suggest a significant role for interferon in this experimental model. A more important role has been proposed for interferon in studies of genetically determined differences in the susceptibility of mice to West Nile virus (Baron, 1970; Hanson, Koprowski, Baron & Buckler, 1969).

The expert technical assistance of John Hodous and Huguette Rivet is gratefully acknowledged. Powassan virus was received from W. H. Price and I. S. Thind.

This research was supported in part by Public Health Service training grant NB 05627 and research grant NB 05363, as well as by a special research fellowship NB 01791 (L. P. W.) and research career development award NB 21945 (N. N.), from the National Institute of Neurological Diseases and Stroke.

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Hyg., Camb. (1970), **68**, 447
Printed in Great Britain

Temperate bacteriophages of *Escherichia coli* O119:B14

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(*Received* 23 *March* 1970)

SUMMARY

Lysogeny was detected in 98·8% of the 343 *Escherichia coli* O119:B14 strains. A suitable indicator strain *E. coli* KS was selected to demonstrate the presence of temperate phages in this serotype. A great diversity in the temperate population was observed based on their lytic patterns and neutralization studies. No definite relationship could be established between the biochemical reactions and the flagellar antigens of the lysogenic strain and its temperate phage though some temperate phages released by *E. coli* O119:B14 strains with certain flagellar antigens did give specific lytic patterns and were serologically identical. Lysogenic strains, which did not release temperate phages spontaneously, were u.v. inductible. Cross-reactions with lysogenized colonies which were immune to corresponding phages also confirmed diversity of temperate phages in *E. coli* O119:B14.

INTRODUCTION

Escherichia coli serotype O119:B14 was first isolated from a case of calf diarrhoea and was reported as 034W by Wramby (1948). Smith (1953) was the first to demonstrate its importance in cases of infantile gastro-enteritis. Since then many authors have isolated it in different countries. Chambon (1955); Coetzee & Pretorius (1955); Banerjee, Chatterji & Praminik (1956); Quilligan *et al.* (1957); Cooper, Walters & Keller (1956); Gamble & Rowson (1957); Sacrez, Levy, Minck & Poirier (1958); Lutz, Grad & Bass (1959); Ayoma (1960); McCallum, Le Minor, Le Minor & Chabbert (1961) and Hiroki (1961) isolated these strains from epidemic or sporadic cases of infantile gastro-enteritis. This serotype has been studied in detail by many authors from the point of view of antigenic structure, fermentation reactions and susceptibility to antibiotics. The present study was undertaken first to demonstrate the presence of temperate phages in *E. coli* serotypes O119:B14 in order to establish a phage typing system useful for epidemiology and secondly to see the relationship between the biochemical characters and antigenic structures of *E. coli* O119:B14 and the temperate phages derived from them.

MATERIAL AND METHODS

Bacterial strains

In total 343 strains of *E. coli* O119:B14 from the collection of Institut Pasteur, Paris, were studied. These strains were isolated from epidemic or sporadic cases of gastro-enteritis in Germany, France, Belgium, United States, Great Britain and

Mexico. Biochemical, serological and antibiogram studies of these strains were done by McCallum *et al.* (1961) who divided the strains into flagellar types H_4, H_5, H_6, H_8, H_{27}, H_{32} and H_{40}. Non-motile strains were designated as H-.

Bacteriophages

The lysogenic property of *E. coli* strains was demonstrated by classical techniques on an agar plate inoculated with an indicator strain, using either a drop of broth culture, or a drop of supernatant fluid of a broth culture to which a few drops of chloroform had been added, followed by centrifugation at 6000 rev./min. for 10 min. *Shigella paradysenteriae* Y 6R, *E. coli* Bordet, *E. coli* B, *E. coli* 36 and *E. coli* K (S and R used as suffix for smooth and rough variants respectively) were used as indicator strains. Mostly *E. coli* KS was used as the indicator strain of choice for detection of temperate phages in cultures of *E. coli* O119:B14. In cases where the above two techniques failed, the presence of temperate phages was demonstrated either by 'mixed culture' technique or by u.v. induction. In the former case *ca.* 10^4 bacteria/ml. of the suspected lysogenic strain were mixed with an equal number of cells of the indicator strain. The mixed culture was incubated at 37° C. for 4–5 hr. in a water bath agitator and then filtered through a Chamberland L3 filter. The filtrate was tested for the presence of bacteriophages.

For u.v. induction experiments (Lwoff, Siminovich & Kjeldgaard, 1950) an ultra-violet lamp developing an energy of 4000 ergs/mm.^{2}/min. at a distance of 50 cm. was used. Irradiation of diluted log. phase liquid culture (about 10^3 cells/ml.) in thin layers was done in Petri dishes. Immediately after irradiation, the culture was incubated for 3 hr. at 37° C. in a water bath, at the end of which a few drops of chloroform were added and the culture centrifuged at 6000 rev./min. for 10 min. The supernatant fluid was titrated on the indicator strain for the presence of phage.

Purification of bacteriophages

Cultures were sometimes polylysogenic. Purification of phage was done by cutting out a piece of agar showing a well-isolated plaque in the centre with a scalpel and transferring it to a flask containing 30 ml. of nutrient broth. After incubation at 37° C. for 3–8 hr., the broth was filtered and spotted in serial dilutions so as to get isolated plaques on nutrient agar plates seeded with the propagating strain. Single plaque propagation was repeated at least three times before obtaining pure bacteriophage suspension. Different plaques showing different morphology from the same polylysogenic strain were propagated separately.

Preparation of high-titred bacteriophages and titration

Preparation of high-titred bacteriophages and titration were usually done according to Adams (1959). In some cases a high titre was obtained by incubating phage–host suspension at 37° C. for a few hours and then leaving it at laboratory temperature or even at 4° C. until lysis occurred. The lysate was treated with a few drops of chloroform to lyse the bacterial cells and centrifuged to get rid of

suspended bacteria and debris. The supernatant fluid was filtered through a Chamberland L3 filter and stored at 4° C.

Serology of bacteriophages

For purposes of identification and antigenic classification, phage antisera were prepared in rabbits. Phage suspensions in broth containing *ca.* 10^9 particles/ml. were diluted 1/5 in peptone water and injected two or three times into rabbits intraperitoneally in doses of 50 ml. each at weekly intervals. The rabbits were bled 15 days after the third injection and the serum was tested against the corresponding bacteriophage by neutralization tests (Adams, 1959). Usually high-titred antisera were obtained except in a few cases where even the addition of adjuvants like Freund, ascorbic acid, pantothenic acid and nicotinic acid did not help. In neutralization experiments 0·5 ml. of 1×10^7 p.f.u./ml. of a bacteriophage in nutrient broth was mixed with 0·5 ml. of antiphage serum in varying dilution. The mixture, after incubation at 37° C. for 30 min., was diluted 1/10 in broth and titrated on the indicator strain to get the p.f.u./ml. at each serum dilution. The maximum dilution of the serum was recorded where phage was neutralized completely.

Phage resistance by lysogenization

The resistant colonies appearing in a lytic area were picked and subcultured two or three times on agar slants to remove any carry over of the original bacteriophage. These lysogenized colonies were then verified as resistant to superinfection by the corresponding bacteriophages and able to release the lysogenizing phage on the appropriate indicator strains. The lysogenized colonies were designated by the name or number of the *E. coli* indicator strain followed by the number of the phage in parentheses, e.g. KS (6719).

Designation

A temperate phage was either designated by the strain number of *E. coli* O119:B14 from which it was derived or, in case this phage was propagated to get a high titre, it was designated by the number of the *E. coli* strain from which the phage was derived with the name of the propagating strain as denominator, e.g. phage 6719/KS isolated from *E. coli* O119:B14 number 6719 and propagated on *E. coli* KS.

RESULTS

Demonstration of lysogeny by homologous indicator strains

Temperate phages of *E. coli* O119:B14 very rarely show lysis on strains of the same serotype. In cross-culture technique only seven out of 343 strains gave lysis on seven other indicator strains of *E. coli* O119:B14. These seven temperate phages could be divided into four groups based on their lytic pattern (Table 1). The first group consisted of phage 6719 derived from the strain *E. coli* number 6719 with flagellar antigen H_4. Further studies proved that serologically this phage was different from other temperate phages. The second group which included phages

13485, 967 and 16180 derived from *E. coli* strains with flagellar antigens H_6, H_6 and H_{32} respectively showed same lytic pattern. The third group consisting of phages 16114 and 16115 derived from lysogenic *E. coli* with H_8 and H_{40} respectively gave similar lytic pattern. They were found to be related in their antigenic properties (Table 6). The fourth group was represented by two phages derived from the same strain 15755 with flagellar antigen H_5, one giving clear plaques (C) and the other turbid (T). They also gave more or less similar lytic pattern. In neutralization studies they were observed to be identical. The clear plaque-producing phage was derived as virulent mutant of the wild-type turbid plaque-producing phage.

Table 1. *Lysogeny within strains of* Escherichia coli O119:B14 *employing homologous indicator strains*

Indicator strains			Phages from lysogenic strains with flagellar antigens							
									15755 H_5	
Nos.	Flagellar antigen	Bio-type	6719 H_4	13485 H_6	967 H_6	16180 H_{32}	16114 H_8	16115 H_{40}	T	C
15766	H_6	2	4 pl	–	–	–	–	–	6 pl	+ +
14001	H_8	1	–	–	–	–	+	CL	–	–
16167	H_8	2	–	–	–	–	+	CL	–	–
18867	H_{32}	1	–	SCL	+ + +	+ + +	–	–	+ + +	SCL
15664	H-	2	–	–	–	–	–	–	–	SCL
968	H_6	2	+	+ +	1 pl	8 pl	–	–	+ + +	+ + +
15379	H_6	2	–	–	–	–	–	5 pl	–	–

T, turbid plaques; C, clear plaques; CL, confluent lysis; SCL, semi-confluent lysis; pl, actual number of plaques.

Table 2. *Frequency of lysogeny in* Escherichia coli O119:B14

Technique	No. of strains tested	Phage detection by indicator strains				
		E. coli K			Y 6R	
		Colony S	Colony R	*E. coli* B	Colony S	Colony R
Chloroform and centrifugation	343	297* (86·6 %)	198 (57·7 %)	131 (38·2 %)	188 (54·8 %)	236 (68·8 %)
U.v. irradiation	–	34 (9·9 %)	6 (1·7 %)	–	19 (6·4 %)	22 (5·5 %)
Mixed culture with *E. coli* KS	–	8 (2·3 %)	–	–	–	2 (0·6 %)
Total	343	339 (98·8 %)	–	–	–	–

* Represents the number of strains found lysogenic.

Demonstration of lysogeny by heterologous indicator strains (frequency of lysogeny)

Forty-eight strains representing different species in the family *Enterobacteriaceae* were tested as indicator strains to temperate phages of *E. coli* O119:B14. Amongst them, *Shigella paradysenteriae* Y 6R (F. M. Burnet), *E. coli* K (R. Legroux), *E. coli* B (Demerec and Fano) and *E. coli* 36 (F. M. Burnet) were found to be very

sensitive. In total 297 out of 343 strains, i.e. 86·6 % were found to be spontaneously lysogenic as demonstrated by the ordinary method of chloroformed culture filtrate (Table 2). Out of the remaining 46 strains, 42 strains were found to be lysogenic either by u.v. induction or by mixed culture technique or by both methods. Only four strains were found to be non-lysogenic or were defective lysogenic.

Table 3. *Optimum time for u.v. induction of* Escherichia coli O119:B14 *strain* 15755

U.v. irradiation time (sec.)	Phage titre assayed on *E. coli* KS—p.f.u./ml.	Observation
0	0	
15	$1{\cdot}6 \times 10^5$	1⁰ With increase of u.v. irradiation dose, plaques diminish in size
30	$4{\cdot}4 \times 10^5$	
60	$4{\cdot}4 \times 10^6$	
90	5×10^6	2⁰ The number of plaque-forming units increase up to 90 sec. of u.v. irradiation and then a u.v. killing effect is obtained
120	$1{\cdot}6 \times 10^6$	
180	$2{\cdot}8 \times 10^5$	
240	$2{\cdot}8 \times 10^5$	

U.v. induction of lysogenic Escherichia coli

Table 3 shows the optimum time of u.v. irradiation to induce lysogeny in a strain 15755 which did not show any lysogeny either by the ordinary classical method of chloroformed culture filtrate or by mixed culture technique. The optimal time of irradiation to get the maximum plaque formers from a lysogenic strain was found to be 90 sec.

Lytic pattern

Forty-five lysogenic strains were selected on the basis of their differences in flagellar antigen, biotype and geographical origin; 45 phages isolated from these strains were purified, propagated and titrated on *E. coli* KS. These temperate phages employed in routine test dilution gave nine lytic patterns when tested on seven different indicator strains. As shown in Table 4, lytic patterns produced by temperate phages of H_4, H_5 and H- *E. coli* strains were all different from each other and from other groups. The phages derived from different strains of *E. coli* O119:B14:H6 gave different lytic images. A number of temperate phages derived from *E. coli* strains with H6 gave similar lytic patterns. Different lytic patterns were obtained with the phages derived from *E. coli* strains in the same geographical site, e.g. *E. coli* strains isolated in lower Alsace gave phages that produced four different lytic patterns on a set of indicator strains.

Cross-immunity between lysogenized colonies produced by different phages

A lytic pattern for the lysogenized colonies was obtained employing corresponding lysogenizing phages. As shown in Table 5, each lysogenized strain was immune to lysis by the corresponding phage but was lysed by other phages. Most of the lysogenized strains showed a specific lytic pattern which in certain cases was related to the flagellar antigen of the temperate phage-producing strain, e.g.

lysogenizing phage derived from *E. coli* H_4 gave specific pattern for K (6719). On the contrary, phages derived from *E. coli* strains with same flagellar antigen H_6 formed lysogenized colonies which gave different lytic patterns, e.g. phages 7629, 13076, 966, 21003 and 17575.

Table 4. *Lytic pattern obtained by* 45 *phages derived from* Escherichia coli *strains with different flagellar strains*

	Lysogenic strains			Phages giving	Lytic pattern of indicator strains						
Lytic pattern	Flagellar antigen	Biotype H_6	Origin	similar pattern	*E. coli* KS	*E. coli* KR	*E. coli* Y	*E. coli* 6 R Bordet	*E. coli* B	*E. coli* K 125	*E. coli* Lisbonne
1	H_4	—	Pennsylvania	1	+	+	+	+	+	+	+
2	H_5	—	Lower Alsace	1	+	+	+	+	+	+	−
3	H_6	1 (3)	Berlin (3)	9	+	+	+	+	+	−	−
		2 (5)	Lower Alsace (4)								
			Aberdeen (2)								
4	H_6	2	Strasbourg	2	+	+	+	−	+	+	+
			Aberdeen								
5	H_6 (26)	1 (25)	Lower Alsace (13)	27	+	−	+	−	−	+	+
	H_{32} (1)	3 (1)	Liege (4)								
			Lyon (1)								
			Berlin (9)								
6	H_6	2	Lower Alsace	1	+	+	+	−	−	−	+
7	H_6	2	Berlin	1	+	+	−	−	−	−	−
8	H_6	2	Berlin	2	+	+	+	−	+	+	−
			Lyon								
9	H-	2	Lower Alsace	1	+	+	+	−	−	−	−

( ) Indicates the number of strains.

Table 5. *Cross-reactions between the lysogenized* Escherichia coli *KS colonies and the corresponding phages*

	Phages with flagellar antigens of the lysogenic strains											
Lysogenized colonies resistant to lysis	6719 H_4	15755 H_5 C	15755 H_5 T	7629 H_6	13076 H_6	11235, 974, 15379 H_6	966 H_6	21003 H_6	17575 H_6	16443, 18807 H_{32}	16180 H_{32}	15764 H-
KS (6719)	−	CL	CL	CL	CL	CL	CL	CL	CL	+ + +	CL	CL
KS (15755)	CL	CL	−	CL	SCL	CL	+ + +	CL	CL	+ + +	CL	CL
KS (7629)	CL	CL	CL	−	CL	CL	CL	CL	SCL	+ + +	CL	CL
KS (13076)	SCL	CL	CL	CL	−	CL	+ + +	CL	SCL	−	SCL	CL
KS (11235-974-15379)	CL	CL	CL	−	−	—	+ + +	CL	SCL	−	SCL	CL
KS (966)	CL	CL	CL	CL	−	CL	−	CL	−	−	SCL	CL
KS (21003)	CL	CL	CL	SCL	CL	CL	CL	−	SCL	−	+ + +	CL
KS (17575)	CL	CL	CL	CL	CL	CL	CL	CL	−	SCL	CL	CL
KS (16443)	CL	CL	CL	CL	CL	CL	SCL	CL	SCL	−	CL	CL
KS (18807)	CL	CL	CL	CL	CL	CL	SCL	CL	SCL	−	CL	CL
KS (16180)	CL	CL	CL	CL	SCL	CL	SCL	CL	SCL	SCL	−	CL
KS (15764)	−	CL	−	SCL	SCL	CL	CL	CL	SCL	+ + +	SCL	−

CL, confluent lysis; SCL, semiconfluent lysis; + + +, above 100 plaques;
T, turbid plaques; C, clear plaques.

Serology

The great diversity amongst lysogenic phages of *E. coli* O119:B14 was confirmed by neutralization tests. Antisera against 11 phages isolated from lysogenic strains with different flagellar antigens were prepared. Antisera against phages derived from the clear and the turbid plaques of *E. coli* strain 15755 were also prepared. Certain phages (6719, 7629, 15106, 15755, 15764, 16114 and 16115) were excellent antigens and produced high titred neutralizing antisera. On the other hand phages 9623, 13076 and 16180 gave very poor antisera in different lots of rabbits even in the presence of various adjuvants. The antisera produced against the first two phages were removed from Table 6 because of the complete lack of

Table 6. *Cross-neutralization of* 27 *temperate phages by antiphage sera*

	Phages														
Anti-phage serum	6719 H_4*	15106 H_6	16114 H_8	16115 H_{40}	15755 H_5 C	15755 H_5 T	15764 H-	7629 H_6	16180 H_8	14178 H_6	77251 H_6	20850 H_4	9623 H_6	13076 H_6	13 phages selected at random
6719	**8000**†	—	—	—	—	—	100	—	—	8000	1200	8000	—	—	—
15106	—	**3000**	—	100	—	—	—	—	—	—	—	—	—	—	—
16114	—	—	**3000**	3000	—	—	—	—	—	—	—	—	—	—	—
16115	—	—	4000	**4000**	—	—	—	—	—	10	10	10	—	—	—
15755 C	10	—	—	20	**8000**	8000	1000	—	—	10	10	10	—	—	—
15755 T	10	—	—	10	8000	**8000**	1000	—	—	10	10	10	—	—	—
15764	—	—	—	—	100	100	**1000**	—	—	10	10	10	—	—	—
7629	—	—	—	—	—	—	—	**2000**	—	—	—	—	—	—	—
16180	—	—	—	—	—	—	—	—	**10**	—	—	—	—	—	—

* Flagellar antigens of *E. coli* strain that produced the corresponding phages. T, turbid plaque-forming phage.
† Highest dilution of serum which neutralized the phage completely. C, clear plaque-forming phage.

specific phage antibodies. With the help of nine antiphage sera, 27 different temperate phages were divided into six groups by neutralization experiments. The first group consisted of temperate phages 6719, 14178 and 20850 which were neutralized completely at a serum dilution of 1/8000. Phage 7251 was neutralized partially up to 1/1200 which showed its partial antigenic relationship with the first three phages. The second group was represented by a single phage 15106 which was neutralized by its homologous antiserum at a dilution of 1/3000. The third group consisted of phages 16114 and 16115 which cross-neutralized each other completely by their homologous antisera. The fourth group was represented by two phages derived from *E. coli* strain 15755. There was a complete cross-neutralization of these phages by their homologous antisera. The antisera produced against these phages also neutralized phage 15764 at a dilution of 1/1000 which indicated that phage 15764 was partially related to phage 15755. The fifth group consisted of one phage 7629; phages 16180, 9623, 13076 and 13 other phages which were not neutralized by the antisera available were grouped together temporarily in the sixth group.

DISCUSSION

Most bacteria carry certain temperate bacteriophages but frequently it is impossible to demonstrate the presence of these phages because of the lack of the suitable indicator strain. The data represented in Tables 1 and 2 indicate that the frequency of lysogeny (2 %) was poor in *E. coli* O119:B14 when different strains of the same serotype were used as indicator strains; on the contrary, after having selected 45 different heterologous indicator strains, spontaneous lysogeny in the same serotype could be detected in 86·6 % of the strains. All the remaining strains, except four, were also detected as lysogenic by u.v. induction or mixed culture technique.

It has been extremely difficult to establish a relationship between the nature of the temperate phage carried by a lysogenic strain and its biochemical or antigenic character. The phages 6719 and 15755 derived from *E. coli* strains with flagellar antigens H_4 and H_5 respectively gave specific lytic patterns with homologous *E. coli* O119:B14 indicator strains (Table 1), heterologous *E. coli* indicator strains (Table 4) and a set of immune lysogenized colonies (Table 5). This led to the conclusion that the phages 6719 and 15755 were different from each other and from other temperate phages. It was further confirmed by neutralization studies that the antigenic structure of these phages was different. Similar lytic patterns (Table 1) were obtained for phages 16114 and 16115, with similar antigens associated in neutralization tests (Table 6), though derived from lysogenic *E. coli* with two different flagellar antigens H_8 and H_{40} respectively. Owing to the lack of more *E. coli* H_4, H_5, H_8 and H_{40} strains, no definite relationship could be established between their flagellar antigens, the specific lytic pattern obtained by their temperate phages and the phage antigens involved in neutralization test.

Studies with lysogenized colonies (Table 5) proved that the temperate phages of *E. coli* O119:B14 offered specific self-immunity to lysogenized strains and that these lysogenizing phages, though very diversified, could still be grouped, e.g. phages 11235, 974, 15379 and 16443 and 18807 based on the specific lytic pattern of the lysogenized strains. Here it was interesting to observe the similarity in the lytic patterns of *B. coli* KS lysogenized by phages derived from *E. coli* with the same flagellar antigens. The above first three phages were derived from *E. coli* H_6 and the last two from *E. coli* H_{32}.

In spite of the fact that almost all of the *E. coli* O119:B14 strains were lysogenic and that the temperate phages released by these strains were very diversified, the strains of *E. coli* O119:B14 were rarely sensitive to the temperate phages of other strains of the same serotype. This may be explained by the fact that probably these strains were polylysogenic and that immunity to lysis exists as an interaction between the phages or may be due to the presence of few common prophages within the cell. Because of this lack of sensitivity of the serotype towards its temperate phages, it was not possible to establish a rational phage typing scheme based on the temperate phages of the same serotype as was possible in *Salmonella typhi* and *S. paratyphi B*. However, an empirical phage-typing system was established with the help of the virulent phages isolated from sewage (Kasatiya, 1963).

Phage serological studies also supported the diversity of temperate phages in this serotype. In one case antigenic similarity between the temperate phages was related with the flagellar antigen of the corresponding lysogenic *E. coli* strain, e.g. phages 6719 and 20850, both derived from strains with flagellar antigens H_4, were serologically identical. On the other hand temperate phages of strains with the same flagellar antigens did show different antigenic structure in neutralization experiments (phages 16180, 7629, 15106 and 14178). The data proved that the temperate phages of *E. coli* O119:B14 were serologically independent, except in few cases, of the flagellar antigens of the corresponding lysogenic strain.

The author greatly appreciates the guidance of Doctor André Lwoff of Cancer Institute, Villejuif and Doctor Pierre Nicolle of Pasteur Institute, Paris during this work.

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J. Hyg., Camb. (1970), 68, 457
With 1 *plate*
Printed in Great Britain

Recoveries of bacteria after drying and heating in glutamate foams

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(*Received* 24 *March* 1970)

SUMMARY

A method of drying bacteria is described, in which the bacterial suspension was made in 40% sodium glutamate, and 0·1 ml. volumes of this in 8 ml. ampoules were dried *in vacuo* while being held in a water bath at 25° C. After 1 hr. with the pump still running, the ampoules were immersed in water at 100° C. The partly dried suspension expanded rapidly into a homogeneous white foam. After 30 min. the ampoules were taken off the manifold; small tubes containing dry P_2O_5 were inserted in the ampoules which were then sealed in air. Preliminary results with three organisms, *Salmonella ndolo*, *Staphylococcus aureus* and *Serratia marcescens* showed high survivals immediately after the 'foaming' period, and good stability after 1 or 2 days at 100° C.

INTRODUCTION

In a previous study (Annear, 1966) it was shown that bacteria suspended in sodium glutamate could be recovered in appreciable numbers after the suspensions were dried *in vacuo* while immersed in a bath at 100° C. Under these conditions vigorous spluttering occurred and the suspensions dried rapidly in foamy flakes patchily distributed on the walls of the ampoules.

Subsequent studies have shown that a more controlled foaming resulted if the suspension was initially dried from the liquid state at a lower temperature (*ca.* 25° C.) before being subjected to a higher one. Under these conditions the suspension dried initially as a scaly bubbly residue in the bottom of the ampoule (Plate 1 A). When subjected to a high temperature (100° C.), and while still being evacuated, the desiccate expanded and set within a second or two into a fairly homogeneous white foam. The density and volume of the foam was modified by the volume and/or glutamate concentration of the solution dried (Plate 1 B, C). The appearance of the foam did not seem to be affected by the interval between the initial drying and the heating, desiccates left on the pump for several days responding in a similar manner to those left for 1 hr. Water loss from the desiccates during foaming is undoubtedly accelerated both because of the increased temperature and because of the enormous increase in surface area which occurs in the dried material.

The results of several experiments are published here to illustrate the method of drying micro-organisms in glutamate foams and the relative stability of the

desiccates to high temperatures when held in air and in the presence of dry P_2O_5. Previous studies by Annear & Bottomley (1965) have shown that bacteria sealed under such conditions but dried by means other than those described here were remarkably stable to heat.

METHODS

The organisms chosen are shown in Table 1. They were grown on nutrient agar plates for 18 hr. at 30° C. and heavy suspensions made in 40 % glutamate. Ampoules of 8 ml. capacity were inoculated with 0·1 ml. volumes of these suspensions which were then dried *in vacuo* on a horizontal manifold using P_2O_5 as a desiccant and while held in a water bath at 25° C. (Annear, 1961). Drying was carried out under these conditions for 1 hr., most of the water being removed within the first few minutes to leave behind scaly residues. While still being pumped, the ampoules were then immersed in a boiling water bath to produce foaming and were left at this temperature for a period of 30 min. to effect further dehydration of the foams.

Table 1. *Recoveries (log_{10}) of bacteria after drying, foaming and heating in sodium glutamate*

		Recovery				
		Immediately after drying	After foaming	After exposure to 100° C. for		
Organism	Inoculum			1 day	2 days	3 days
Salmonella ndolo (NCTC 8700)	9·0	8·9	8·8	7·2	5·1	2·1
Staphylococcus aureus (NCTC 6571)	8·9	8·9	8·7	6·1	3·4	< 1
Serratia marcescens (NCTC 1377)	8·8	8·8	8·7	7·1	5·3	3·0

After removal from the manifold, small tubes of dry P_2O_5 were introduced into the ampoules which were then sealed in air. The ampoules were transferred to a boiling water bath and viable counts made on the desiccates at daily intervals. Counts were also performed immediately after the first stage of drying at 25° C. and after the initial exposure to 100° C. used to produce the foams. All counts were made on blood agar plates after slow rehydration of the desiccates (Annear, 1965).

RESULTS AND DISCUSSION

The results in Table 1 show that very little loss occurred with any of the three organisms tested during either the initial drying from the liquid state at 25° C. or during the high temperature treatment used to produce the foams. The survival of the organisms during subsequent exposure to 100° C. indicates reasonable stability and permits reliable prediction of high survival in such desiccates during storage at ambient temperatures. However, a more systematic study of the various factors involved would be necessary for the understanding of survival in this type of desiccate.

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(*Facing p.* 459)

It is not known at present what application if any this particular method of drying has. It does offer a means of drying a small volume of suspension from the liquid state efficiently because of the enormously increased surface area the desiccates present when expanded into foams. In this respect the desiccates resemble the peptone foams made from freeze-dried peptone plugs (Annear, 1956). Foaming in those preparations was shown to be dependent upon entrapped air in the matrix of the freeze-dried peptone.

While entrapped air would seem also to be involved in the production of the glutamate foams, it is of interest that failure has resulted in attempts to produce foams with a number of other compounds commonly used in preservation of micro-organisms. Compounds tested included glucose, sucrose, sorbitol, mannitol and higher molecular weight compounds such as dextrins, starch, dextrans and polyethylene glycols. None foamed as glutamate did and it would be interesting to find an explanation for this behaviour which at present seems fortuitously associated with a compound so useful in the preservation of micro-organisms.

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EXPLANATION OF PLATE

Glutamate desiccates before and after foaming. Organisms dried in 40 % sodium glutamate.

(A) Desiccate resulting from drying 0·1 ml. of suspension *in vacuo* at a bath temperature of 25 °C.

(B) Appearance of (A) after subjecting it to 100 °C. *in vacuo*.

(C) As for (B) but resulting from an initial suspension of 0·5 ml. volume.

J. Hyg., Camb. (1970), **68**, 461
Printed in Great Britain

Postnatal decline of maternally acquired rubella antibodies

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SUMMARY

The postnatal decline of maternally acquired rubella antibody was studied in a large group of infants. A high degree of variability was found in the rate of antibody decline (half-life). Ninety-two babies had rubella antibody half-lives lying between 14 and 70 days and three had values considerably higher. There was no significant difference between the rubella antibody half-lives of the sexes. The antibody titre at birth was weakly correlated with both birth weight and gestational age. There was a highly significant positive correlation between the baby's antibody titre at birth and that of its mother. There was a positive relationship between the half-life and the persistence of rubella antibody. Some babies had no detectable antibody by 2 months whereas others still possessed antibody at 9 months. It was found that the relationship between the half-life and the rubella antibody titre at or near birth could be described by a rectangular hyperbola.

INTRODUCTION

A number of studies have shown that maternally acquired antibody in the newborn child decreases at an exponential rate after birth (Allansmith, McClellan, Butterworth & Maloney, 1968; Schultze & Heremans, 1966). Most of these studies have been related to total IgG immunoglobulin and few have been concerned with the rate of decline of specific antibodies.

An opportunity to study the decline of specific maternal antibody in a large group of newborn babies was recently given to us by an intensive longitudinal perinatal survey. A study was made of the decline of rubella antibody, in the first instance, for a number of reasons. First, a previous (unpublished) study by us had indicated that 79% of infants could be expected to have rubella antibody of maternal origin in their sera at birth. Secondly, a sensitive quantitative test (haemagglutination-inhibition test) had recently been developed for rubella antibody and this test was readily adaptable to the micro-system needed for the small volumes of sera available. A third and practical reason for studying the decline of rubella antibody was related to the diagnosis of congenital rubella infections. A positive serological diagnosis of congenital rubella is indicated by the failure of the affected infant's antibody titre to decline in a 'normal' manner following birth. However, to our knowledge, no detailed studies of the 'normal' decline of rubella antibody had been reported.

This paper reports not only the decline of maternally acquired rubella antibodies in a large group of babies, but also the relationship between the rates of antibody decline and persistence of antibody and initial antibody titre.

MATERIALS AND METHODS

Study group

The babies in this study were born between April 1967 and June 1968 at the Royal Hospital for Women, Paddington, N.S.W. Of the 120 babies in the original group, 95 (79%) possessed rubella antibody; these composed the study group. The birth weights and gestational ages of the babies are summarized in Tables 1 and 2.

Table 1. *Classification of the study group by birth weight*

Birth weight (g.)	No. of babies in group	Birth weight (g.)	No. of babies in group
1100–1500	12	3001–3500	5
1501–2000	28	3501–4000	5
2001–2500	27	Total	95
2501–3000	18		

Table 2. *Classification of the study group by gestational age*

Gestational age (weeks)	No. of babies in group	Gestational age (weeks)	No. of babies in group
28–30	6	39–42	31
31–34	23	43–44	5
35–38	30	Total	95

Sera for the study

Blood was taken from the mothers at parturition, from the babies at birth or within 10 days following birth ('initial serum') and also at regular intervals for a period of 2–12 months. Generally four to six sera were collected from each infant. Serum was removed from the clotted blood and was stored at $-30°$ C.

Serological techniques

Rubella antibody was determined by the haemagglutination-inhibition (HI) test (Stewart *et al.* 1967), modified in that non-specific inhibitors were removed from the sera by manganese chloride and heparin (Cooper, Matters, Rosenblum & Krugman, 1969), that the sera were treated with pigeon red blood cells to remove naturally occurring agglutinins and that 0·2% pigeon cells were used in the test.

In order to minimize bias, the babies whose sera were to be tested on a given day were selected by an appropriate ranking procedure and all the sera from a given baby were tested at the same time. Ten samples of a standard positive serum were included in each test as a check on the within-test and between-test variability.

Each baby in the study group was found to possess detectable antibody in at least three but usually four to six sequential sera. The antibody titres were punched onto data cards and were analysed in an IBM 360 Model 50 computer.

Using these antibody titres, a linear regression of log. titre against time (days from birth) was computed, and the print-out included the rate of rubella antibody decline (half-life) for each baby.

RESULTS

Rate of antibody decline (half-life)

A frequency distribution of the half-life values obtained is shown in Fig. 1. Half-lives ranged from 14 to 259 days with a mean of 43 days and a mode of 36 days. In most cases the straight line obtained from the regression equation was a good fit to the observed values. With six babies, including the baby with a rubella antibody half-life of 259 days, a twofold or greater rise or fall in rubella antibody titre, as compared to the titre at birth, took place during the first month of life prior to the commencement of exponential antibody decline. In addition, four babies had a stationary antibody titre for between 1 and 2 months of life, after which antibody declined in an exponential manner.

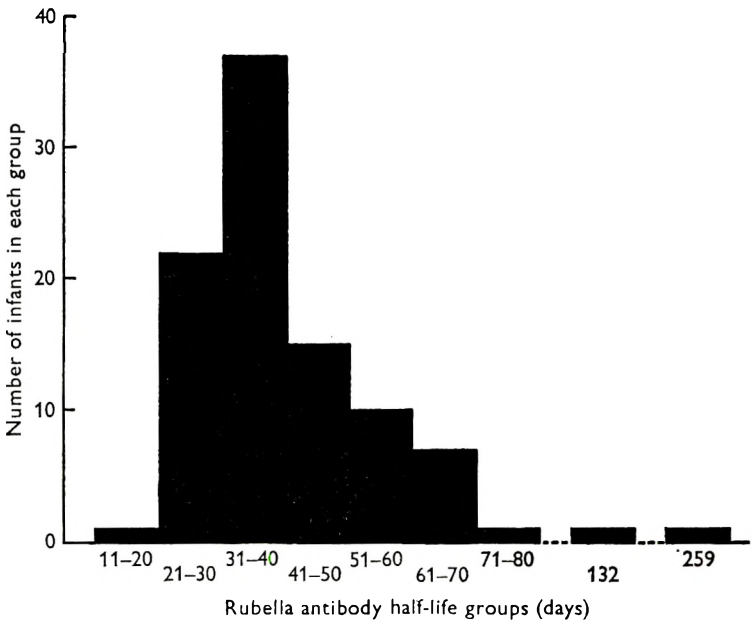

Fig. 1. Frequency distribution of rubella half-life values.

Antibody status in twins

There were 10 pairs of twins in the original group. The antibody status (i.e. presence or absence of antibody) of both twins in a pair was always identical. Seven pairs were antibody positive. In these pairs, not only was the titre of rubella antibody at birth similar for both twins of a pair, but the half-lives of rubella antibody in both twins were also similar.

Sex of the babies

There was no statistically significant difference between the mean rates of antibody decline of the sexes. The frequency distributions of half-lives in the two sexes were almost identical.

Relationships between initial antibody titre, birth weight and gestational age

There was a positive correlation between initial antibody titre and birth weight and between initial antibody titre and gestational age. In both cases there was a considerable scatter of points and the relationships were only weakly significant ($P = 0{\cdot}05$).

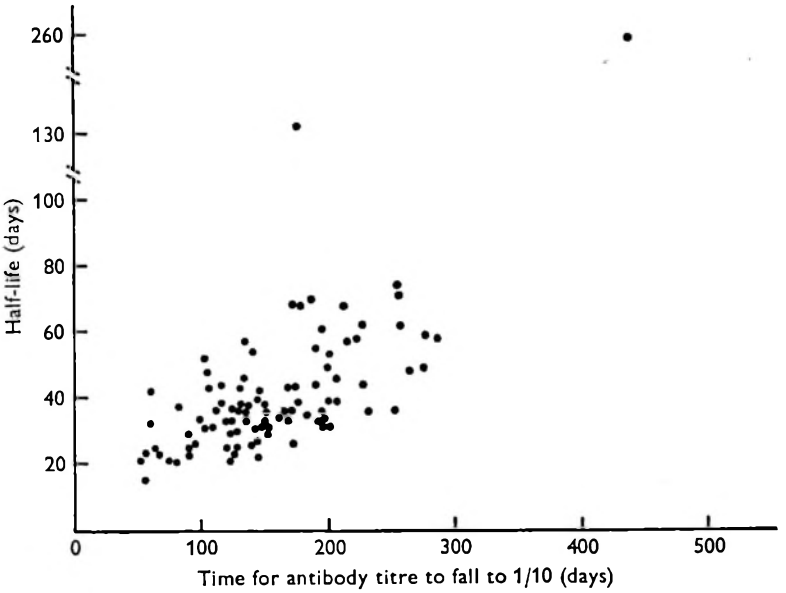

Fig. 2. Relationship between half-life and time calculated for rubella antibody to decline to a titre of 1/10.

Relationship between antibody titres of infants at birth and those of their mothers

There was a highly significant linear correlation between the baby's initial rubella antibody titre and that of its mother ($r = 0{\cdot}99$, $P < 0{\cdot}001$). Only 8% of the babies had a titre twofold greater or lower than that of their mothers, and these differences were never greater than sixfold.

Duration of detectable maternally acquired rubella antibody

The lowest titre of rubella antibody detectable by our test system was 1/10. In order to obtain some idea of the persistence of rubella antibody, the number of days from birth for the titre to fall to 1/10 was calculated from the regression equation for each baby. The relationship between half-life and the number of days for the titre to reach a level of 1/10 for each baby is shown in Fig. 2. It can be seen that there is a positive relationship between half-life and the duration of antibody. In addition, it was observed that 17 babies (18%) had detectable antibody at 6 months of age and seven (5%) had antibody at 9 months.

Relationship between half-life and initial antibody titre

The decline of maternally acquired antibodies in the infant is a reflexion of the catabolism of IgG. It has been shown that IgG catabolism is related to the IgG concentration in the serum such that increasing concentrations of IgG lead to an increase in its catabolism and hence a decrease in its half-life (Brambell, Hemmings & Morris, 1964; Brambell, 1966). It has been postulated that this relationship between half-life and concentration of IgG takes the form of a rectangular hyperbola.

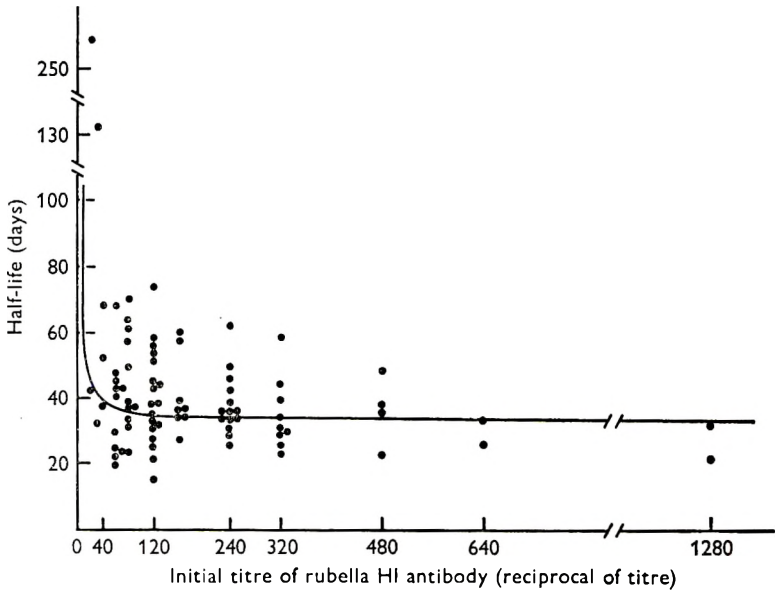

Fig. 3. Relationship between half-life and initial titre of rubella antibody in infants. The curve (Brambell *et al.* 1964) is

$$\text{half-life} = \frac{\text{initial titre ln } 2}{0{\cdot}02\ (\text{initial titre} - 3{\cdot}25)}$$

In this study the relationship between the rate of decline of an IgG antibody of a given specificity and its titre in the infants' serum at birth was determined. The relationship between the half-life of rubella antibody in these babies and the titre of antibody at birth could be described by a rectangular hyperbola (Fig. 3). Few of the babies had an initial titre of 1/40 or less and consequently few points were obtained at the lower end of the antibody axis. The variability between babies over the range of initial antibody titres was not constant and increased at the lower end of the antibody axis. For this reason, the linear equivalent of the model proposed by Brambell *et al.* (1964) (see Fig. 3) was used in this study. It was found that there was a statistically significant relationship ($P = 0{\cdot}01$) of the type described. Although the measurement errors of initial titre and half-life are correlated, it was found that this correlation had a negligible effect on the significance of the relationship between half-life and initial titre.

DISCUSSION

The relationship noted here between the antibody titres in the mother and infant at birth has been previously reported both for total IgG and also for other specific antibodies (Schultze & Heremans, 1966). Similarly, a relationship between IgG levels and both birth weight and gestational age has been demonstrated (Hobbs & Davis, 1967; Jones, 1969). This study simply serves to extend these relationships to rubella antibody in a large series of children.

On the other hand, there are two aspects of this study which are of considerable importance. First, it is noteworthy that there has been a high degree of variability between babies in the rate of decline of maternally acquired rubella antibody. To our knowledge, this has not been previously reported. The half-life of maternally acquired antibody in infants has usually been considered as lying between 20 and 40 days. This has applied to total IgG as well as to a wide range of viral antibodies (Schultze & Heremans, 1966) including polio antibody (Perkins, Yetts & Gaisford, 1958), measles antibody (Strauss & Zeman, 1967), and rubella antibody (Vesikari, Vaheri, Pettay & Kunnas, 1969).

The observations recorded above with this large group of babies indicate that the upper limit of the half-life range must now be extended to at least 70 days and possibly even higher (Fig. 1). Within this group of children there were babies who had lost all detectable antibody by 2 months of age and others who still possessed detectable antibody at 9 months. As rubella antibody undoubtedly declines to titres below the level of detectability of the HI test, it is probable that in many babies such antibody persists beyond 9 months. It has been suggested that the time for the complete disappearance of herpes simplex antibody of maternal origin was from 3 to 15 months (Anderson & Hamilton, 1949), and for group-B arbovirus antibody, 6 months or longer (Carey, Myers, Wilson & Manoharan, 1968).

The wide range of rubella half-lives obtained in this study indicates that caution must be exercised in the interpretation of antibody titres in the serological diagnosis of congenital rubella infections. It is doubtful whether laboratory confirmation of the diagnosis of congenital rubella can be made solely on the presence of rubella antibody at 6 or 9 months of age. This criterion has occasionally been used in the confirmation of a diagnosis (Hardy, McCracken, Gilkeson & Sever, 1969; McCracken *et al.* 1969).

It was not considered likely that babies in this series with long half-lives had a congenital rubella infection, since none of the babies had any of the known congenital abnormalities of *in utero* rubella infection. In addition, in all 12 babies having a half-life of 60 days or greater, the antibody level eventually reached a titre of less than 1/10. In congenital rubella the antibody titre does not decline during the first 6 to 12 months of life (Michaels, 1969; Vesikari *et al.* 1969), although it may start to decline at 15 to 18 months of age (Hardy *et al.* 1969). Similarly, it was not considered that the long half-lives encountered were due to the presence of non-specific inhibitors in some of the sera. All the results obtained, including those from the negative control sera, indicated that all of the inhibitors had been removed from the sera. Little is known about the host factors which affect the

rate of decline of passively acquired antibodies, although it has been suggested that the basal metabolic rate may influence their catabolism (Solomon, Waldmann & Fahey, 1963; Schultze & Heremans, 1966).

The second finding of considerable importance is the relationship between half-life and initial titre. There have been a number of studies which have suggested that the catabolism of passively acquired IgG is related to its concentration in the serum in such a way that as the IgG concentration is increased the rate of catabolism also increases (Brambell *et al.* 1964; Brambell, 1966). Such a phenomenon has been reported for a number of species including the mouse (Fahey & Robinson, 1963; Fahey & Sell, 1965), the rabbit (Andersen & Bjørneboe, 1964) and man (Solomon *et al.* 1963; Waldmann & Schwab, 1965; Waldmann, 1969). Although most of these studies have been carried out in adults, it has been found that the half-life of passively administered gammaglobulin in children with agammaglobulinaemia was 50–60 days whereas, in normal children, it was 20–40 days (Gitlin, Janeway, Apt & Craig, 1959).

Although this study has been with antibody of one specificity, as distinct from total IgG, these results are in reasonable agreement with the model postulated by Brambell *et al.* (1964). To our knowledge, these results represent the first detailed study in infants of the relationship between the postnatal decline of maternally acquired antibody of a given specificity and the titre of such antibodies at birth. The results suggest that with a specific antiviral antibody, namely rubella antibody, babies having high titres at birth lose their antibody rapidly, whereas in babies with low initial titres, antibody declines over a longer period of time.

Precise information such as this on the postnatal decline of specific maternally acquired antibodies is important for several reasons. Maternal antibody in the infant may provide protection against infection (Anderson & Hamilton, 1949), it may affect the efficacy of vaccination in infants (Perkins *et al.* 1958) and it has recently been suggested by Chanock *et al.* (1968) that it may even be a factor in the pathogenesis of certain virus infections. Future studies will be carried out using different antigens with the same sera in an attempt to determine whether other antiviral antibodies, especially viral respiratory ones, decline in the same manner as rubella antibody. An attempt will also be made to determine whether the half-life of antibody of a given specificity depends entirely on its own concentration in the serum or whether it is influenced by the level of other serum antibodies.

We thank Dr C. McGilchrist, Department of Mathematics, University of New South Wales, for advice and assistance in the statistical analysis of the results; Mr R. W. Thomas, Department of Mechanical Engineering, University of New South Wales, for writing the computer programs and for organizing the computing; Mr K. Deason, Department of Medical Illustration, University of New South Wales, for preparing the illustrations; and Dr C. J. Lewis and the premature nursery laboratory staff of the Royal Hospital for Women, Paddington, for their assistance in the collection of sera. This work was assisted by a grant from the National Health and Medical Research Council of Australia.

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J. Hyg., Camb. (1970), 68, 469
With 2 plates
Printed in Great Britain

Antigenic differences within the species *Mycoplasma hominis*

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(*Received* 6 *April* 1970)

SUMMARY

Membrane and soluble fractions of one genital and two oral strains of *Mycoplasma hominis* were compared by immunodiffusion and polyacrylamide gel electrophoresis. Differences were demonstrated between the membrane antigens of the three strains by immunodiffusion, and the membrane proteins also gave dissimilar patterns in polyacrylamide gel electrophoresis. The soluble fractions gave identical lines in immunodiffusion tests and similar patterns in polyacrylamide gel electrophoresis.

When the strains were cross-titrated in metabolic inhibition (MI) and indirect haemagglutination (IHA) tests, statistical analysis of the results revealed significant differences between the strains. Previously, growth-inhibition, MI and IHA activity was shown to be associated with the membrane antigens of *M. hominis*, so the intraspecies differences revealed by MI and IHA correlate with the differences in the membrane antigens demonstrated by immunodiffusion. Growth-inhibition tests, which might also have been expected to show intraspecies differences, did not do so, probably because of the insensitivity of the test. In contrast to MI and IHA, complement-fixation (CF) tests revealed a high degree of relatedness between the strains. This is consistent with the observation that the soluble antigens of *M. hominis* participate in the CF reaction, and that the soluble antigens of different strains are identical in immunodiffusion tests.

INTRODUCTION

Lemcke & Hollingdale (1968) and Hollingdale & Lemcke (1969) showed that the membrane antigens of a genital strain of *Mycoplasma hominis* gave rise to antiserum active in growth-inhibition (GI), metabolic inhibition (MI) and indirect haemagglutination (IHA). Complement-fixing (CF) activity, on the other hand, was associated with antigens in the soluble cell fraction as well as the membrane. It was also possible, by immunodiffusion and immunoelectrophoresis, to distinguish the membrane antigens from those in the soluble fraction.

Since intraspecies differences between strains of *M. hominis* have been demonstrated by several serological techniques (Nicol & Edward, 1953; Taylor-Robinson, Ludwig *et al.* 1965; Purcell *et al.* 1967), the membrane and soluble fractions of three strains of *M. hominis* were compared by immunodiffusion to see if any

differences could be detected between the antigens of the three. The strains were also compared by MI, IHA and CF, and the results analysed statistically to determine the degree of relatedness.

MATERIALS AND METHODS

Cultures

The three *M. hominis* strains examined most fully were a genital strain, SC4 (Hollingdale & Lemcke, 1969), and two oral strains, DC63 and V2785 (Taylor-Robinson, Somerson, Turner & Chanock, 1963). Six other genital strains were included in growth-inhibition tests: H34 and 4387P (Lemcke, 1964) and four strains isolated by Nicol & Edward (1953)—H23 (PG25), H26 (PG23), H50 (PG21) and D419 (PG26).

Fractionation

To obtain membrane and soluble fractions, SC4, DC63 and V2785 were grown, disrupted by sonic treatment and fractionated as described by Hollingdale & Lemcke (1969). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystallized bovine serum albumin (Puriss. grade, Koch–Light Laboratories, Colnbrook, Bucks.) as a standard.

Antisera

The rabbit antisera to whole cells of SC4, H34 and 4387P were those prepared by Hollingdale & Lemcke (1969) and Lemcke (1964, 1965). Antisera to whole cells of DC63 and V2785 were prepared by a regime of inoculation similar to that used for SC4, after the strains had been adapted to grow in liquid medium containing rabbit serum. Pre-immunization sera were obtained from all the rabbits and included as controls in every serological test.

Polyacrylamide gel electrophoresis

For membrane fractions, the method of Rottem & Razin (1967) was used except in the preparation of the membrane extract. Membranes washed six times and resuspended in dilute sodium chloride/tris buffer (0·0075 M sodium chloride, 0·0025 M tris) at pH 7·4 were mixed with phenol–acetic acid–water (2 : 1 : 0·5, w/v/v) to give a final protein concentration of 2 mg. per ml. After standing at room temperature for 30 min. and at 4° C. overnight, the mixture was centrifuged at 9500 *g* for 30 min. at 4° C. Two volumes of the clear supernatant was mixed with 1 volume of sucrose (40 %, w/v) in acetic acid (35 %, v/v) and 0·15 ml. of the mixture layered on the gel.

Soluble fractions were compared by a modification of the method of Davis (1964), in which solutions for analysis are run through a 7·5 % polyacrylamide gel in a tris (3·0 M) buffer, pH 8·9, and the electrode buffer consists of tris/glycine (0·01 M tris, 0·077 M glycine) at pH 8·3. Soluble fractions in dilute sodium chloride/tris buffer were diluted with electrode buffer to a final concentration of 2·0 mg. protein/ml. This was then mixed with an equal volume of 60 % (w/v) sucrose

solution in electrode buffer and 0·075 ml. layered on the gel. Electrophoresis was carried out for 90 min. at a constant current of 2 mA. per tube.

Serological tests

The methods for immunodiffusion, metabolic inhibition (MI) and indirect haemagglutination (IHA) were as described by Hollingdale & Lemcke (1969). For IHA, tanned erythrocytes were sensitized with sonicated suspensions of the mycoplasmas at 0·125 mg. protein per ml.

Growth-inhibition (GI) tests were carried out as described by Clyde (1964). For each strain the inoculum was a 10^{-1} dilution of an 18 hr. culture (0·02 ml. per 5 cm. plate). The 5 mm. disks (Whatman AA) applied to the agar surface contained 0·02 ml. of undiluted serum, and all the sera were tested simultaneously against the same strain.

For complement fixation (CF) tests, antigens were harvested from 6-day broth cultures, washed twice in saline (0·85%, w/v), resuspended in saline containing thiomersalate (0·0001 %, v/v) and stored at 4° C. The method for CF was that of Bradstreet & Taylor (1962) except that the microtitre system with unit volumes of 0·025 ml. was used and complement was guinea-pig serum stored without preservative at −30° C. The optimal concentration of antigen, i.e. the dilution giving the maximum serum titre with its homologous antiserum, was the same for all three strains, namely 0·033 mg. cell protein per ml. All cross-titrations were carried out with antigens at this concentration.

Measurement of serological relationships

In metabolic inhibition, indirect haemagglutination and complement fixation, SC4, DC63 and V2785 were cross-titrated against the corresponding antisera in three replicate tests. The resulting mean titres were then analysed according to Alling (1967) and Purcell *et al.* (1967), so as to derive a numerical estimate, with confidence limits, for the degree of relatedness.

When two antigens are tested against the homologous and one heterologous antiserum to give four titres, T_{hom1}, T_{hom2}, T_{het1}, T_{het2}, each titre can be expressed in the form cb^y, where c is the reciprocal of the dilution of the least dilute serum used and b is the dilution factor for successive dilutions (e.g. for twofold dilutions, $b = 2$). Thus, for a titre of 1280 obtained from an initial dilution of 1/10 and a dilution factor of 1/2, $1280 = 10 \times 2^y$ and $y = 7$. The degree of serological relationship, R, is expressed in $\log_b$, here $\log_2$.

$$R = \frac{T_{\text{het1}}\, T_{\text{het2}}}{T_{\text{hom1}}\, T_{\text{hom2}}}$$

Hence

$$\log_b R = \frac{y_{\text{het1}}\, y_{\text{het2}}}{y_{\text{hom1}}\, y_{\text{hom2}}}$$

When the antigens are identical, therefore, $R = 1$ and $\log_b R = 0$, whereas when they are completely unrelated, $R = 0$ and $\log_b R = -\infty$. Thus, the more $\log_b R$ deviates from 0, the greater the differences between the strains.

RESULTS

Immunodiffusion

The soluble fractions of the three strains, SC4, DC63 and V2785, gave very similar patterns with antisera to any of the three (Plate 1, *a–c*). In contrast, there were pronounced differences between the membrane antigens (Plate 1, *d–f*, and Table 1). The homologous membrane gave the most complex series of lines, the heterologous membranes sharing some but not all of the components. With all three sera, DC63 and V2785 membranes shared a line which was not present in SC 4.

Table 1. *Immunodiffusion tests with detergent-lysed membranes and antisera to whole cells of three strains of* Mycoplasma hominis

Plate no.	Anti-serum well	Precipitin lines*						Antigen well†
1*d*	SC4	−	+	(+ +)	+ +	+	.	SC4
		+	+	+ +	− −	−	.	DC63
		+	−	+ +	+ −	−	.	V2785
1*e*	DC63	+ +	+	+	−	+ +	.	DC63
		(+ +)	−	−	+	+ +	.	SC4
		+ +	+	−	−	+ −	.	V2785
1*f*	V2785	+	+	?	+	+	+	V2785
		−	+	+ +	−	−	−	SC4
		+	+	+ +	−	−	−	DC63

* Scoring from left to right denotes order of lines from centre (antiserum) well to outer (antigen) well.

† Membranes lysed with 5 mg. Triton X-100 per mg. membrane protein; final concentration in wells, 2 mg. protein per ml.

+, Single line; + +, two lines, very close together, which may fuse into a single line (+ +); + −, one of two lines missing; −, − −, one or two lines missing; ?, obscured by other precipitin lines.

Polyacrylamide gel electrophoresis

There were minor differences between the proteins of the soluble fractions but the general pattern was the same (Plate 2*a*). The membrane fractions gave more protein bands than the soluble fractions, but there were pronounced dissimilarities, especially among the slower-moving components in the upper part of the gels (Plate 2*b*).

Metabolic inhibition

The results of the titrations are shown in Table 2. All the relatedness (R) values fell outside the 95 % confidence interval. This suggests that the strains were not identical with respect to the antigens reacting in this test, and confirms the results of Purcell *et al.* (1967).

Indirect haemagglutination

The results are shown in Table 3. As with MI tests, the relatedness values for the three strains were outside the 95 % confidence interval.

Complement fixation

The results are shown in Table 4. All titres were within 25 % of the homologous reaction. All the relatedness values fell within the 95 % confidence interval. By this test, therefore, the three strains seem to be closely related.

Table 2. *Relationships among three strains of* Mycoplasma hominis *as shown by metabolic inhibition tests*

	Reciprocal of titre with antiserum		
Antigen	SC4	DC63	V2785
SC4	**1280–2560**	80	160–320
DC63	160–320	**1280**	160
V2785	160–320	80	**1280**

Homologous titres in bold type.

Relatedness ($\log_2 R$) from three replicate tests

	SC4	DC63
DC63	−7·67	
V2785	−6·33	−8·00

Ninety-five per cent confidence interval, −1·98 to +1·98.

Table 3. *Relationships among three strains of* Mycoplasma hominis *as shown by indirect haemagglutination tests*

	Reciprocal of titre with antiserum		
Antigen*	SC4	DC63	V2785
SC4	**2560**	640	320–640
DC63	160	**1280**	80–160
V2785	80–160	80	**640–1280**

Homologous titres in bold type.

* Tanned erythrocytes sensitized with sonicated suspensions of organisms at 0·125 mg. cell protein per ml.

Relatedness ($\log_2 R$) from three replicate tests

	SC4	DC63
DC63	−5·0	
V2785	−5·33	−6·66

Ninety-five per cent confidence interval, −1·62 to +1·62.

Growth inhibition

The results obtained with nine strains of *M. hominis* against antisera to five strains is shown in Table 5. All the strains were inhibited by the antisera. However, measurements of the zones of inhibition were within too small a range to permit differentiation of the strains on this basis.

Table 4. *Relationships among three strains of* Mycoplasma hominis *as shown by complement fixation*

	Reciprocal of titre with antiserum		
Antigen	SC4	DC63	V2785
SC4	**640–1280**	640–1280	640–1280
DC63	640	**1280**	640–2560
V2785	640–1280	640–1280	**1280–2560**

Homologous titres in bold type.

Relatedness ($\log_2 R$), from three replicate tests

	SC4	DC63
DC63	−1·0	
V2785	−0·66	−1·33

Ninety-five per cent confidence interval, −1·98 to +1·98.

Table 5. *Results of growth-inhibition tests on* Mycoplasma hominis

	Zone of growth-inhibition (mm.) with antisera				
Strain	SC4*	DC63	V2785	H34†	4387P
SC4	**5**	3	1–2	5	2
DC63	3	**2–3**	3	2	NR
V2785	3	3	**5**	4	3
H34	4	1–2	4	**5**	4–5
4387P	1–2	1–2	2–3	2	**3**
H23 (PG25)	2–3	2–3	2–3	3	2–3
H26 (PG23)	3	2–3	NR	3	2–3
H50 (PG21)	2	2–3	4	4–5	2
D419 (PG26)	1	2–3	3–4	1–2	1

Homologous reaction in bold type
NR = no result.
*, † Representative of results with four (*) or three (†) other antisera against these strains.

DISCUSSION

Diversity within the species *M. hominis* has been demonstrated in various ways. Somerson *et al.* (1966), using a nucleic acid homology test, showed that eight strains were genetically heterogeneous. The cell proteins of five strains examined by Razin (1968) were also heterogeneous, in that they gave differing patterns in polyacrylamide gel electrophoresis. Serological differences between strains were noted by Nicol & Edward (1953) in agglutination tests and by Purcell *et al.* (1967) in metabolic inhibition tests. Taylor-Robinson, Ludwig *et al.* (1965) demonstrated differences between two strains in indirect haemagglutination tests with human sera, but not with hyperimmune rabbit sera.

We have shown that the serological intraspecies differences observed are attributable to differences in the membrane antigens. These differences were also

apparent in the electrophoretic patterns of the membrane proteins, which showed greater dissimilarities than the proteins of the soluble fractions from the same strains. It is noteworthy that the differences were demonstrable even with fractions prepared by sonic treatment, which often releases some membrane antigen into the soluble fraction (Hollingdale & Lemcke, 1969). Still clearer results might be expected with fractions prepared by alternate cycles of freezing and thawing.

Our results also showed that tests such as metabolic inhibition and indirect haemagglutination, which are dependent on membrane antigens, reveal the greatest intraspecies differences. In contrast, complement fixation tests, in which other antigens besides those in the membrane participate, tended to mask the differences. These results are consistent with those previously obtained by Card (1959) and Lemcke (1964) in which 73 genital strains of *M. hominis* were found to be closely related by CF.

If there is a similar heterogeneity within other *Mycoplasma* species, the choice of tests for identification and differentiation will influence any conclusions about the relatedness of the strains under test. Complement fixation tests will emphasize similarities between strains of the same species, and tests such as MI and IHA and other agglutination tests will tend to reveal intraspecies differences. Fluorescent antibody techniques, which are probably dependent on surface antigens, may also belong to the second category, although sensitivity may be a limiting factor in detecting strain differences, as it is in GI tests.

In our hands, GI, although previously shown, like MI, to be dependent on membrane antigens, was not sufficiently sensitive to detect intraspecies differences. Even D419 (PG26), which was found by Razin (1968) to be resistant to inhibition by three heterologous *M. hominis* antisera, was inhibited to some extent by all the antisera tested. There were differences between the sizes of the inhibition zones produced by different strains with any one antiserum, but in such an insensitive test it was impossible to evaluate their significance. Moreover, the size of the inhibition zone being related to the concentration of the colonies, the results obtained with any one antiserum are comparable only when the inoculum is the same for all strains, and this is difficult to ensure when a number are being tested simultaneously.

With immunodiffusion tests, the results are dependent on the mode of preparation of the antigen used. Thus, in gel-diffusion experiments, where mycoplasma suspensions disrupted by sonic treatment or alternate freezing and thawing were used, seven genital strains of *M. hominis* gave identical precipitin patterns (Lemcke, 1965). However, membranes must be lysed with detergents before the main precipitating components are released (Hollingdale & Lemcke, 1969), so that Lemcke's (1965) precipitating antigens were derived mainly from the soluble fraction and any dissimilarity due to membrane antigens would not have been evident. It is noteworthy that most gel-diffusion studies on mycoplasmas have been made with either frozen–thawed or sonic-treated antigens (Taylor-Robinson *et al.* 1963; Dinter, Danielsson & Bakos, 1965; Taylor-Robinson, Soběslavský & Chanock, 1965; Kenny, 1969). It is highly probable, therefore, that the antigens demonstrated in these studies were mainly soluble and not membrane antigens.

Both membrane and soluble antigens are present in mycoplasmas lysed with Triton X-100 (Hollingdale & Lemcke, 1969), but the precipitin patterns given by such lysates are rather complex.

Since MI and IHA tests reveal the greatest intraspecies differences in *M. hominis*, the use of any one strain to detect MI and IHA antibody in man may not give fully representative results. For example, Taylor-Robinson, Ludwig *et al.* (1965) found that DC63 was a more sensitive detector of IHA antibody than PG21. It is advisable, therefore, where the infecting strain is not available, to use more than one strain in screening human sera for MI and IHA antibody to *M. hominis*. The CF test should also be included, since it is dependent on antigens common to different strains of *M. hominis*. Moreover, CF antibody appears earlier in the infection than MI antibody (Jones & Tobin, 1969).

We do not consider it is justifiable to separate *M. hominis* strains into more than one species as suggested by Razin (1968). The strain differences we have demonstrated in membrane proteins and antigens are comparable with those noted by Razin in the cell proteins, but the results of GI and CF tests indicate a basic relationship between the strains which does not obtain between distinct species of *Mycoplasma* (Lemcke, 1964; Clyde, 1964). There may be a case for establishing a number of types or subspecies of *M. hominis*, but the intraspecies differences which are demonstrable at present would not characterize them adequately. There are differences in the membrane antigens, but the possibility of extracting type-specific antigens, such as are used to characterize strains of *Streptococcus pyogenes*, seems remote. *M. hominis* membrane antigens appear to be proteins, and so far it has not been possible to prepare extracts which give a single precipitating component (Hollingdale & Lemcke, unpublished). Characterization of subspecies must await the definition of new properties by which strains of *M. hominis* can be distinguished.

This work was supported by a Medical Research Council grant which provided the remuneration of MRH.

We are indebted to Dr D. W. Alling (National Institutes of Health, Bethesda, Md., U.S.A.) for giving us details of his method of statistical analysis.

We also thank Dr D. Taylor-Robinson (Clinical Research Centre, Harvard Hospital, Salisbury, Wilts.) and Dr D. G. ff. Edward (Public Health Laboratories, County Hall, London, S.E.1) for supplying strains of *Mycoplasma hominis*.

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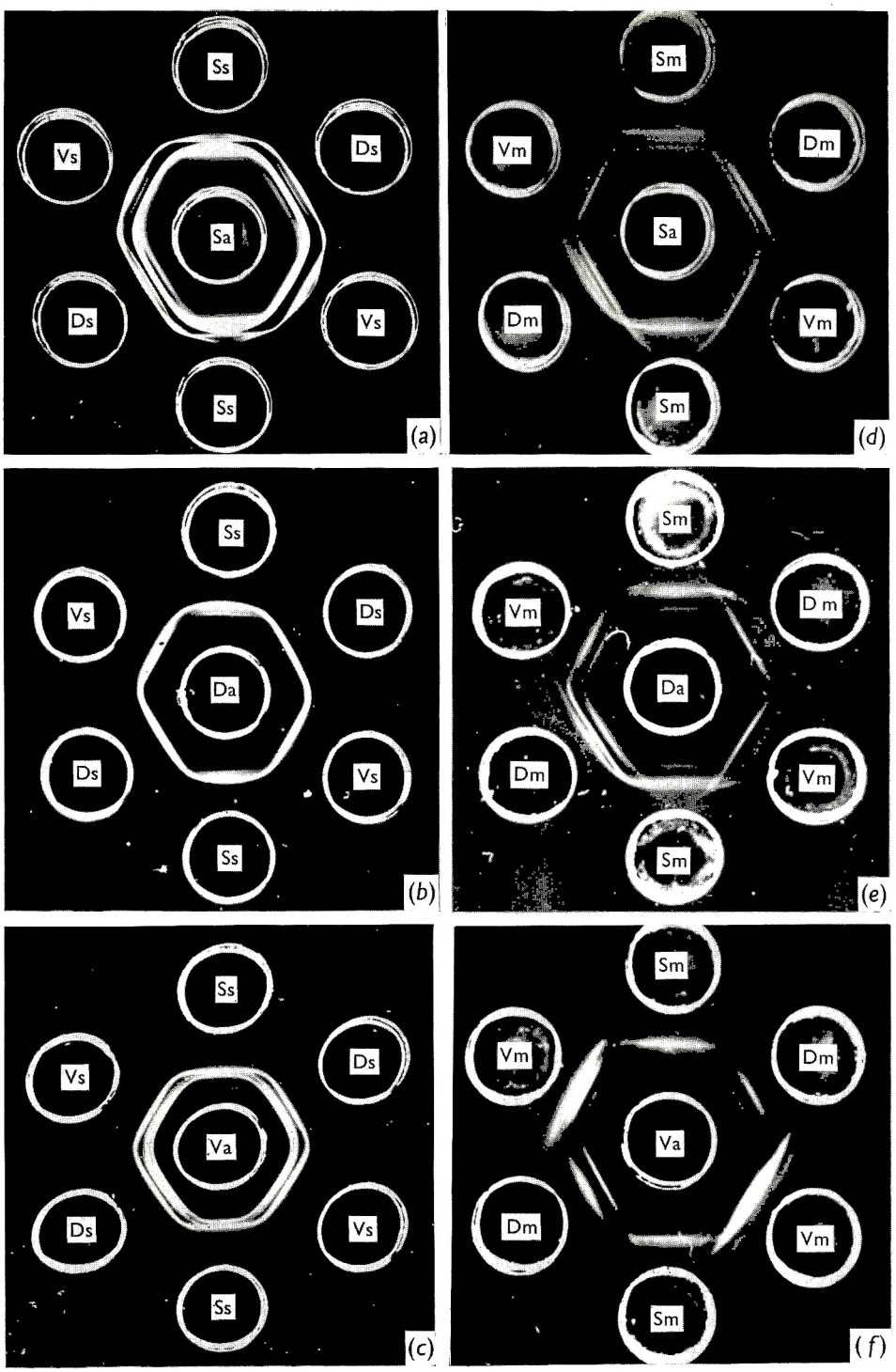
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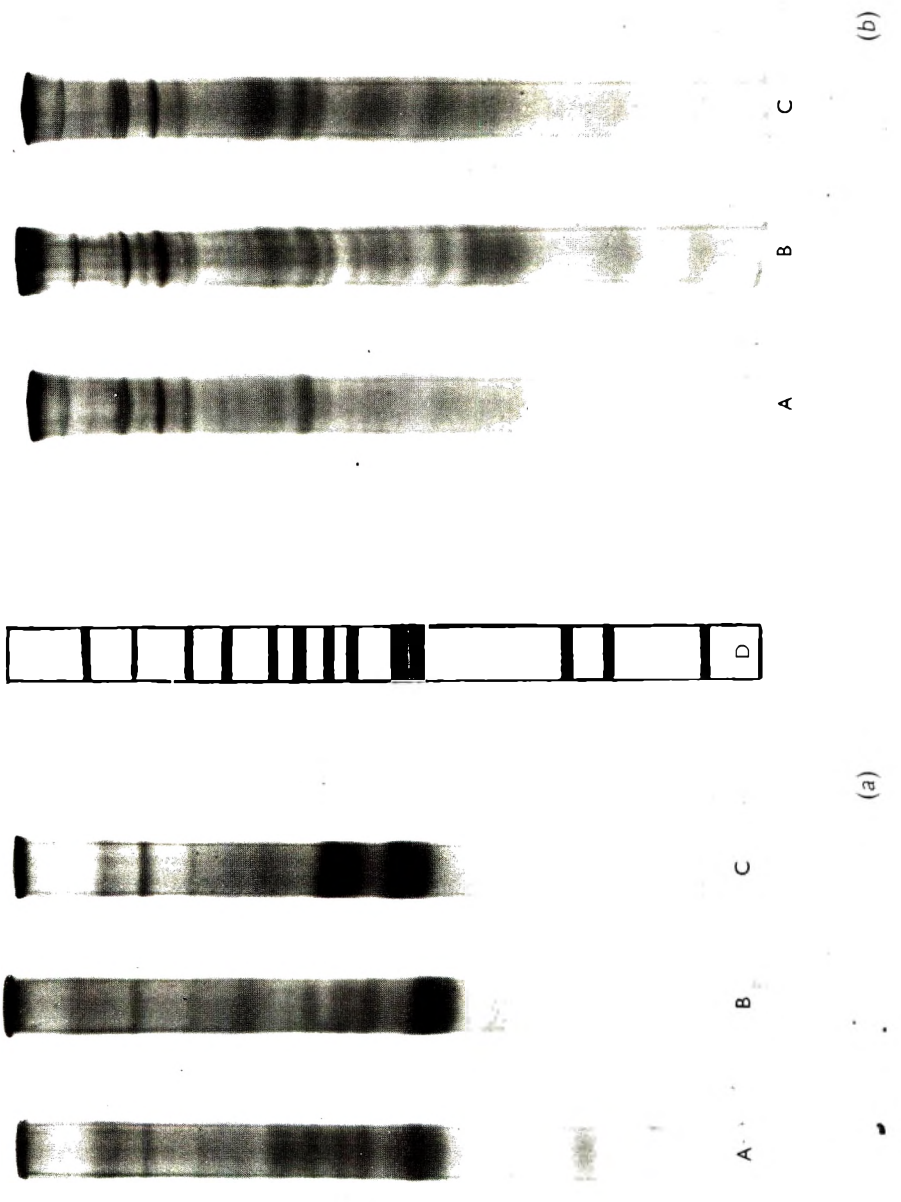
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EXPLANATION OF PLATES

PLATE 1

Gel-diffusion reactions of fractions of three strains of *Mycoplasma hominis* with antisera against whole cells of the same strains. Antisera (undiluted) in centre wells. Antigens at final concentration of 2 mg. protein per ml.

(*a–c*) Soluble fractions of SC4 (Ss), DC63 (Ds) and V2785 (Vs) against antisera to SC4 (Sa), DC63 (Da) and V2785 (Va).

(*d–f*) Membranes of SC4 (Sm), DC63 (Dm) and V2785 (Vm) lysed with Triton X-100 (5 mg. per mg. membrane protein) against same antisera.

PLATE 2

Polyacrylamide gel electrophoresis of fractions of three strains of *Mycoplasma hominis*. *a*, Soluble fractions; *b*, membrane fractions. A, SC4; B, V2785; C, DC63; D, diagram of electrophoretic pattern of soluble fractions.

J. Hyg. Camb. (1970), **68**, 479
With 2 plates
Printed in Great Britain

Serological studies with human papova (wart) virus

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(*Received* 6 *April* 1970)

SUMMARY

Methods for demonstrating antibody to wart virus by complement fixation and passive haemagglutination tests are described and compared with the precipitin test of Almeida & Goffe (1965). The results reveal the much greater sensitivity of the passive haemagglutination method, particularly in the detection of the immunoglobulin M class of antibody. Both complement-fixing and precipitating antibody were detected in sera from patients whose warts had undergone a spontaneous resolution.

The presence of antibody to wart virus was demonstrated in sera from persons who had had warts up to 10 years previously, and in a few cases from those who thought they had never had warts.

The antigenic identity of virus from hand warts and plantar warts of the simple and mosaic types was revealed, and some evidence was obtained for similar identity of the virus from genital warts.

INTRODUCTION

One human tumour that is without doubt due to a virus is the simple wart (*verruca*) or papilloma. This human wart virus is classified in the PAPOVA group (Melnick, 1962) along with other small DNA-containing oncogenic viruses. Much useful knowledge of the role of viruses in neoplastic processes has come from study of two animal viruses, SV 40 and polyoma, also members of the papova group. Research concerned with the activity of the human papova (wart) virus has, however, been severely limited because of the continuing lack of any system for propagating the virus; yet it has much potential value as a model for viral oncogenicity in man.

Electron microscopic studies have revealed the presence of typical papova viruses in various forms of wart—the types commonly seen on the hands and feet (Almeida, Howatson & Williams, 1962), papillomata of the oral mucosa (Frithiof & Wersall, 1967), and condylomata acuminata or genital warts (Dunn & Ogilvie, 1968). Other reports of similar particles, found in human tissues significantly associated with malignant changes, include that of Ruiter & van Mullem, (1966) regarding the lesions of epidermodysplasia verruciformis, and a recent description of the particles in certain cells cultured from a nephroblastoma (Smith, Pinkel & Dabrowski, 1969). There is clearly a need for methods of identifying these viruses more accurately and for studying the host response to them.

Almeida & Goffe (1965) described techniques whereby antibody to wart virus

could be detected in sera, using either (i) direct agglutination, observed in the electron microscope, or (ii) the formation of precipitin lines in agar gel diffusion tests. Sera from 45 % of their 42 patients with warts were found to contain antibody detectable by these methods. In addition, in 13 out of 18 the antibody was entirely of the immunoglobulin M class that does not confer immunity (Goffe, Almeida & Brown, 1966).

The aim of this project was to examine the nature and development of the antibody response to wart virus more fully, and to study its relationship to regression of warts. It was hoped that the antigenic properties of virus from the different types of wart could be examined, and more sensitive methods for demonstrating antibody to the virus found.

MATERIALS AND METHODS

Virus antigens

Wart tissues were collected in saline containing antibiotics (penicillin 200 units/ml. and streptomycin 200 μg./ml.). Material from warts of any one type was pooled, minced finely with scissors and ground in a mortar. The extract was clarified by differential centrifugation, the pellet obtained after ultracentrifugation, (at 100,000g for 90 min.) being resuspended in a small volume of distilled water and used as the antigen.

Electron microscopy

Negative staining was done with 2 % sodium phosphotungstate, pH 7·2. Some antigen preparations were seen to contain large numbers of wart virus particles, and these were counted with reference to a latex suspension of known concentration, using the loop drop method of Watson, Russell & Wildy (1963). Agar blocks cut out from gel diffusion tests were dissected out in a drop of distilled water to which a coated specimen grid was applied and the adherent drop then negatively stained.

Rabbit sera

Two rabbits (Nos. 1, 2) were inoculated with the antigen from simple plantar warts. Rabbit 1 received one subcutaneous dose of antigen emulsified in an equal volume of oil–Arlacel adjuvant, according to the method of Herbert (1967*b*), followed by one intravenous dose, without adjuvant, 10 weeks later: rabbit 2 had three intravenous doses at weekly intervals and a fourth 8 weeks after the third.

A third rabbit was given a cellular antigen prepared as an approximately 10 % suspension from genital wart tissue by passing it through a fine-mesh sieve (Evans, Gorman, Ito & Weiser, 1962). The first inoculation was of 2 ml. intradermally, the second 0·7 ml. subcutaneously, and a final 1 ml. intravenously, with some months between each. Sera were obtained from all the rabbits before and after the inoculations.

Human sera

Sera were obtained from adult patients attending Wart Clinics at the Royal Infirmary, Edinburgh. When possible serial samples were taken. Some sera were also obtained from girls at a local residential college where a survey into wart infection was carried out. The girls were aged 17–23.

All sera were stored at −30° C. and inactivated by heat at 56° C. for 30 min. before use in tests except for the precipitin test, in which they were examined without inactivation.

Precipitin tests

Microscope slides (3 × 1 in.) were coated with 2 ml. of 1 % Ionagar No. 2 (Oxoid) in distilled water and dried. A top layer of 2 ml. 0·7 % agar in phosphate-buffered saline pH 7·2 was set on the precoated slides and wells cut with a diameter 2 mm. and separated from a central well by distance of 3 mm. Tests were incubated in a humidified atmosphere at 37° C. and read at 24 and 48 hr. for the formation of precipitin lines. When required, agar was poured in Petri dishes and larger wells cut, (diam. 5 mm. set 7 mm. apart). These tests were read after 3 days at 37° C. and the precipitin lines recorded photographically using dark ground illumination.

Complement fixation tests

The standard method of Bradstreet & Taylor (1962) was used, with overnight fixation at 4° C. and 3 HD50 of complement. Optimal concentration of antigen was determined by 'chessboard' titration against antiserum to wart virus, and the complement was titrated in the presence of antigen at this concentration. Perspex plates were used, and 0·1 ml. unit volumes. An uninfected control antigen preparation was made from parings of simple callouses in the same way as wart antigen, but the clarified extract was used without ultracentrifugation as no pellet was obtained.

Passive haemagglutination tests

The method used was essentially that of Herbert (1967*a*). Fresh sheep red cells were tanned by incubating a 5 % suspension of cells with an equal volume of tannic acid (BDH) solution, containing 1 mg. in 10 ml. phosphate-buffered saline pH 7·2, at 37° C. for 15 min. The tanned cells were coated with virus antigen by incubating 3 vols. of the 5 % tanned cells in PBS pH 6·4 with one volume of virus antigen and a further 2 vols. of PBS pH 6·4. The optimal amount of antigen for sensitization was previously determined from observation of the concentration that gave the highest titres in sera when several different concentrations were used to coat separate samples of cells. The sensitization had to be carried out at pH 6·4 and not the 7·2 used for other steps in the procedure, and an incubation time of 30 min. at 37° C. was satisfactory. Once sensitized, the cells were washed three times in PBS containing 1 % normal rabbit serum as stabilizer (NRS diluent) and were resuspended in it at a final cell concentration of 1 %.

Sera to be tested were first adsorbed with sheep red cells from the same batch

in order to remove any heterophile agglutinins. A 1/10 dilution of serum was made in a 1 % suspension of the cells and left at room temperature for 30 min. The cells were then removed by centrifugation and the serum inactivated at 56° C. for 30 min. Tests were carried out in disposable plastic plates with conical-bottomed wells (Linbro Chemical Co.), using 0·025 ml. unit volumes. Doubling dilutions of sera were made in the NRS diluent with micro-diluters (Cooke Engineering Co.) and the sensitized tanned cells added to these wells. Controls consisted of the lowest serum dilution plus unsensitized tanned cells, and both sensitized and unsensitized tanned cells alone in the diluent. Tests were left at 4° C. overnight before the pattern of the sedimented cells was read. Serum titre was taken as the highest dilution showing any haemagglutination.

Immunoglobulin determinations

(i) Mercaptoethanol treatment was used to destroy the immunoglobulin M. Serum was incubated with a $\frac{1}{10}$ volume of 0·2 M 2-mercaptoethanol (2-ME) at 37° C. for 1 hr. An equal volume of saline was added to an untreated sample of the serum. Such sera were examined directly in the precipitin and passive haemagglutination tests as the presence of the 2-ME did not appear to interfere with either test.

(ii) Some sera were fractionated on sucrose density gradients to allow the distribution of antibody in the fractions to be determined. Gradients prepared from 40 to 10 % sucrose were left to equilibrate overnight. Using a 1/2 dilution, 0·5 ml. of serum was layered on top and centrifuged at 35,000 rev./min. for 16 hr. in the SW 39 rotor of the Spinco model L. The fractions of 0·48 ml. were collected from the top of the tubes. With each run, a serum containing Paul Bunnell heterophile antibody was included, and a check on its distribution made by doing a Paul Bunnell test on the fractions (Cruickshank, 1965). This precaution, introduced at the recommendation of Pinckard (personal communication), gave a standard for the level where immunoglobulin M should be found in the fractions, as the heterophile antibody belongs to this heavy class (Carter, 1966). The wart sera fractions were examined directly in complement fixation and passive haemagglutination tests as sucrose in the amounts present did not interfere apparently with either test.

RESULTS

Precipitin tests

As had been found by Almedia & Goffe (1965), no antibody was detected by precipitin test in sera from many patients with warts. This is a relatively insensitive test, sera have to be examined undiluted and minimum antigen concentrations of the order of 10^{11} particles/ml. are required for the formation of readily visible precipitin lines. In spite of this, it was possible to demonstrate the appearance of antibody during the course of a wart infection in 78 out of 116, i.e. 68 %, patients whose sera were examined around the time of cure of their warts (Table 1). The 14 who had antibody in early sera are included in this number. In addition it can be seen that in the 147 cases whose sera were examined only early, before there

was any evidence of regression, precipitating antibody was found in only 14, i.e. 10 %. Twenty-seven people were shown to have antibody in a second serum when there had been none in their first. In eight others, increased amounts of precipitin were noted in the second serum, and the replacement of the 2-mercaptoethanol sensitive immunoglobulin M by resistant immunoglobulin G could be seen. The longest period over which any one patient was found to have persisting IgM was 18 months, and it was found on several occasions during that time that there were virus particles to be seen in parings from her mosaic plantar wart.

Table 1. *Incidence of precipitating antibody to wart virus in patients with different types of wart*

Precipitating antibody in serum taken when wart was		Type of wart					
Active	Regressing	SP	MP	H	F	G	Total
−	+	17	9	0	0	1*	27
+	+	2	3	0	0	0	5
+ (IgM)	+ (IgG)	6	2	0	0	0	8
(ND)	+	18	3	3	0	0	24
+	(ND)	8	5	1	0	0	14
−	(ND)	71	25	10	9	18	133
−	−	15	13	6	1	3	38
	Total	137	60	20	10	22	249
With precipitin (%)		37	37	20	0	5	31

SP = simple plantar; MP = mosaic plantar; H = hand; F = facial; G = genital. IgM, mercaptoethanol sensitive antibody: IgG, mercaptoethanol resistant. + = Precipitin present; − = precipitin absent. ND, serum not available.

* History of hand warts 4 years before.

Complement fixation tests

Rising titres of complement-fixing antibody to wart virus were demonstrated in paired sera from 20 out of 30 patients, 67 %. The wart virus antigen fixed complement in the presence of antibody, optimal concentrations of antigen being of the order of 10^9 virus particles/ml. Non-specific fixation with the control antigen (uninfected) was not seen. The complement-fixing antibody titres are compared with precipitating antibody in sera from cases with simple plantar warts showing significant changes in the titres of complement-fixing antibody over the period tested. Complement-fixing antibody was found only in people whose warts were regressing; it appeared later than precipitating antibody and disappeared sooner after cure of the lesion, as is illustrated by some of the cases in Table 2, and the rabbit sera in Table 3.

Table 3 shows the response in the rabbits immunized with wart virus antigen. It illustrates the features of the classical immune response, with the early appearance of 2-mercaptoethanol sensitive IgM which later is replaced by IgG. This was found also in the complement-fixing antibody response. In early sera complement-fixing activity appeared in the heavy fractions of the sucrose density gradients, in the position occupied by the Paul Bunnell IgM antibody, and in the later sera

in the fractions above the middle of the tube where IgG is expected. Complement fixation by IgM has been commonly found to be more efficient in the presence of increased concentrations of antigen (Pike, 1967). This could not be shown with wart virus as the higher concentrations were anti-complementary.

Table 2. *The development of complement-fixing and precipitating antibodies to wart virus in patients with warts*

	Complement-fixing and precipitating antibody in serum			
	Taken when the wart was			
Patient	Active	Regressing	Cured	Taken after stated period after cure
A. D.	< 8 (−)*	128	32 (+)	.
J. P.	< 8 (−)	.	64 (+)	.
C. W.	8 (−)	128 (+)	.	.
E. R.	< 8 (+)	32 (+)	16 (+)	.
V. L.	< 8 (−)	.	64 (+)	< 8 (+) 3 months
M. B.	< 8 (−)	16 (+)	.	< 8 (+) 1 year
A. M.	.	.	64 (+)	< 8 (+) 10 months
H. M.	.	.	32 (+)	< 8 (+) 14 months
A. D.	< 8 (−)	16 (+)	.	< 8 (+) 18 months
H. P.	.	32 (+)	.	< 8 (+) 2 years
S. M.	.	16 (+)	.	< 8 (+) 1 year

* CF antibody given as reciprocal of titre; precipitin result in parentheses.

Table 3. *Antibody response in rabbits after immunization with wart virus*

			Precipitating antibody in serum			
Rabbit no.	Time (weeks)	Inoculation*	Untreated	Treated with 2-ME	CF titre	Class of CF antibody
1	Preinoc.	.	−	−	< 8	.
	0	s.c.	.	.	.	.
	1	.	+	−	< 8	.
	2	.	+ +	+	128	IgM
	3	.	+ +	+ +	512	.
	10	i.v.	.	.	.	.
	11	.	+ +	+ +	512	IgM + IgG
	20	.	+	+	16	.
	24	.	+	+	32	IgG
2	Pre-inoc.	.	−	−	< 8	.
	0	i.v.	.	.	.	.
	1	i.v.	+ +	+	16	.
	2	i.v.	+ +	+ +	64	IgM
	3	.	+ +	+ +	512	.
	4	.	+ +	+ +	128	IgM + IgG
	10	i.v.	.	.	.	.
	11	.	+ +	+ +	512	.
	24	.	+	+	64	IgG

* 10^{11} Particles at each inoculation. s.c. = Subcutaneously with adjuvant; i.v. = intravenously. 2-ME = 2-mercaptoethanol.

This classical pattern of antibody development occurs in patients receiving treatment and in those who do not but whose warts undergo spontaneous regression. Few of the latter come to clinics, but sera were obtained from some who did and precipitating and complement-fixing antibodies were found in sera from four out of six, two with simple plantar warts and two with hand warts.

Passive haemagglutination tests

In view of the limitations imposed by the insensitivity of the precipitin test and the late development of complement-fixing antibody to wart virus, a more sensitive test was sought. An attempt to coat tanned red cells with the virus and to demonstrate agglutination in the presence of specific antibody was successful. This method proves to be very sensitive detector of antibody, particularly of IgM which is a much more efficient haemagglutinin than IgG (Osler, Mulligan & Rodriguez, 1966). This means that early high titres of this antibody were seen in some people whose sera were negative in other tests, and this was confirmed by the significant reduction in titre following treatment with 2-mercaptoethanol. Table 4 shows the results with sera tested by the passive haemagglutination and CF and precipitin tests and showing a significant titre of antibody by at least one of the methods, with examples from persons with each type of wart.

Table 4. *Comparison of antibodies detected by the passive haemagglutination test with precipitins and complement-fixing antibodies*

		Antibodies in serum taken when the wart was							
		Active				Regressing			
Patient	Type of wart	*PHA (U)	PHA (2-ME)	PT	CFT	PHA (U)	PHA (2-ME)	PT	CFT
R. L.	MP	320	10	−	< 8	640	320	+	8
L. B.	MP	160	40	−	< 8	640	320	+	< 8
C. M.	SP	20	ND	−	< 8	2560	640	+	32
M. G.	SP	10240	1280	−	< 8	2560	1280	+	ND
M. P.	SP	20	ND	−	< 8	320	320	+	< 8
B. B.	SP	640	< 10	−	< 8	160	20	−	32
S. S.	H	ND	ND	ND	ND	10240	ND	+	8
J. M.	H	ND	ND	ND	ND	160	ND	−	< 8
B. J.	H	2560	ND	+	< 8	ND	ND	ND	ND
W. C.	H	ND	ND	ND	ND	2560	ND	+	8
M. S.	F	ND	ND	ND	ND	320	ND	−	< 8
V 2	G	640	ND	−	< 8	320	ND	−	< 8
V 7	G	160	ND	−	< 8	ND	ND	ND	ND
V 14	G	1280	ND	−	< 8	ND	ND	ND	ND

* PHA = passive haemagglutination titre in untreated serum (U) and after treatment with 2-mercaptoethanol (2-ME).

PT = precipitin result; CFT = reciprocal of complement fixation titre.

ND = not tested. For type of wart see Table 1.

Antigenic relationships

In all the serological tests, antigen from simple plantar warts was used as it was most abundantly available. When antigen from hand warts or mosaic plantar warts was substituted in the tests no variation in the results was found. The reaction of identity of these three antigens can be seen in Pl. 1 fig. 1. There were insufficient amounts of virus in the facial or genital wart material for use as antigen. However, the rabbit inoculated with genital wart material did develop antibody to wart virus, producing a precipitin that gave a reaction of identity with precipitins in sera from a patient with a simple plantar wart and a rabbit immunized with the virus (Pl. 1, fig. 2).

Specificity of the tests

The precipitin lines formed were in most cases single, curving towards the antigen well. Removal of the precipitate and examination of it by electron microscopy confirmed the belief that it indicated union of virus with antibody. Aggregates of viral particles were seen surrounded by a haze of antibody, or in some areas individual particles with an attached loop of antibody could be seen (Pl. 2, figs. 1, 2), features of antigen–antibody complexes already described by Almeida, Cinader & Howatson (1963). In some tests with human sera two lines could be seen. When these were extracted and examined separately similar aggregates were seen in both; there was no separation of full and empty particles as occurs with enteroviruses in precipitin tests (Conant & Barron, 1967). No particles were seen in agar from areas on either side of the precipitin line.

Complement fixation was also associated with the virus particles, the activity sedimenting with them on ultracentrifugation. No soluble complement-fixing antigens were detected.

Negative staining of washed, saponin-lysed sensitisized red cells as used in the passive haemagglutination test revealed the presence of virus particles on the cell membrane. Pre-incubation of sera with antigen (wart virus) completely inhibited the passive haemagglutination; other antigens (herpes simplex, adenovirus) did not.

Antibody in people not currently infected with warts

Sera from 34 girls who reported never having had warts were examined by precipitin and passive haemagglutination tests for antibody to wart virus. Of these, 25 had PHA titres of 40 or less (13 had 10 or less), 3 had titres of 80, 5 of 160 and one had 320. This last also had precipitating antibody and was the only one to do so.

Sera from a further 76 girls with a past history of warts were also examined. Six had precipitating antibody; three of these had had plantar warts within the previous 2 years and one within 5 years; the other two had had hand warts 3 and 7 years earlier. PHA titres of 80 and over were found in 7 out of 26 with a history of hand warts, and in 20 out of 50 with previous plantar warts. The two longest times since last known infection in these cases were 9 and 10 years.

DISCUSSION

The precipitin test does have the advantage of being easy to use, straightforward, and its results are very satisfactory when positive. However, its limited sensitivity is a drawback, especially in the study of an infection such as warts where the viral antigenic stimulus is small and often much of it is removed by the treatments employed, so that many patients can only develop low titres of antibody. Where the antigenic relation of viruses is being examined, precipitin tests are most valuable as any differences will show up in the formation of crossed lines or spurs or other evidence of reactions of non- or partial identity. The only difficulty lies in the accumulation of sufficient virus for such a test, and this may be easier in certain areas than others, depending on the treatment favoured for any type of lesion.

The significance of the occasionally observed second band of precipitation is not yet clear. It has been noted by others including Le Bouvier, Sussman & Crawford (1966), who found that, as was seen here, antigens composed entirely of full or empty particles gave identical lines, not separate ones. They found the second line formed with some old empty preparations. There were no cross-reactions among the papillomaviruses from man, dog, cattle or rabbits, or polyoma virus of mice.

Complement fixation by wart virus particles, and not by any soluble antigen extracted from the tissues, is confirmed by S. D. Elek (personal communication) and by Almeida, Oriel & Stannard (1969). In 1935 Maderna reported on a complement fixation test in which he used saline extracts of genital warts as antigen, finding some antibody in sera from 24 out of 45 people with this type of wart. It seems unlikely from present experience that the extract he used can have contained enough virus to give this reaction; possibly some other antigen may have been involved.

Complement fixation by IgM as well as IgG antibody is seen with wart virus antisera, and does occur in some other systems, as with herpes simplex virus, (I. W. S. Smith and J. F. Peutherer, (personal communication)), but not in all (Pike, 1967). Complement-fixing antibody is late in developing in several viral diseases, for instance rubella, and does tend to disappear sooner than other types so that its presence can be taken as an index of relatively recent infection. (Sever *et al.* 1965; Schmidt & Lennette, 1966). The results in this study with warts show the same pattern.

Passive haemagglutination is not a test commonly employed in virology as alternatives with adequate sensitivity are usually available, such as neutralization or haemagglutination inhibition tests. PHA has been applied to the study of herpes simplex virus (Scott, Felton & Barney, 1957) and to adenoviruses (Lefkowitz, Williams, Howard & Sigel, 1966). In both these investigations it was found necessary to use a slightly acid pH (6·4) for the sensitization of cells with virus, as noted in this study, and not the neutral pH used with some soluble protein antigens. This test has obvious advantages in detecting particularly IgM antibody to wart virus, and the lower levels of IgG not shown by other tests. However, more work requires to be done, for instance using purer antigen preparations, before the significance

of the titres obtained can be established. It is not altogether surprising that sera from nine out of the 34 girls who thought they had never had a wart did react with the virus in the PHA test. More than one person has been found on examination to have warts of which they were unaware.

The specific inhibition of PHA by pre-incubation with the antigen could be used to determine the smallest amount of virus that will do this, and the test used in this way for detecting virus. Reversed passive haemagglutination, where cells are coated with the antibody and antigen is measured, could also be very useful for this purpose. So far attempts to apply this method in the wart virus system have not been very successful, probably because of an insufficiently high titre of antibody in the sera used.

The question of the antigenic identity of viruses from all types of wart is at present confused. The viruses in what are termed skin warts (hand, feet and face warts) are probably of one type; certainly those from hand, simple and mosaic plantar warts appear identical. (Pl. 1, fig. 1). The evidence regarding the virus from genital warts is different. In this study, sera from people with genital warts (and no history of skin warts) reacted with virus from skin warts in the PHA test only. This was not considered surprising as all the individuals had small warts, and the paucity of virus in this type had already been observed (Dunn & Ogilvie, 1968). A recent report, however, describes the results of complement fixation and immune electron microscopy investigations of the relation of these viruses (Almeida *et al.* 1969). They found that although sera from patients with skin warts fixed complement with and agglutinated genital wart virus, sera from those with genital warts did this only with virus from genital warts, not with skin wart virus. A one-way antigenic cross between the two viruses was postulated. Serum from the rabbit inoculated with genital wart material in this project did react with skin wart virus, however (Pl. 1, fig. 2). Greater quantities of genital wart virus must be obtained before this question can be resolved.

The relation in time of the appearance of antiviral antibody to the regression of warts is interesting. It had not previously been shown that people whose warts underwent spontaneous resolution developed antibody. Indeed Epstein (1967) thought it unlikely that they did so. Antiviral antibody probably plays no part in the cure of warts, which must surely depend on cell-mediated immune responses, involving recognition of altered cell-surface antigens induced by the virus infection, as is seen in the regression of other viral tumours and homograft rejection. However, it is perhaps during the destruction of the infected cells that the virion antigens, normally rather inaccessible inside the cell, are exposed to the antigen recognition system and stimulate the appropriate antibody response. This antiviral antibody would then be of importance in conferring immunity to re-infection with this virus. Inadequate numbers of patients have as yet been followed for a sufficiently long time to allow determination of whether this is indeed so. Such a relationship between antibody and immunity, and not between antibody and tumour regression has recently been revealed in a study of bovine papilloma virus infection (Lee & Olson, 1969).

The author would like to acknowledge with thanks all the constructive interest shown in this project by Dr R. H. A. Swain and Dr Valerie Inglis. Mr R. Hopkins helped to carry out some of the work in establishing the passive haemagglutination test (Hopkins, 1969). The work could not have been undertaken without the support of Dr M. Bunney and Dr D. H. H. Robertson of the Royal Infirmary, Edinburgh, whose patients supplied the specimens. The author is also indebted to the Medical Faculty, Edinburgh University, for a Graduate Research Scholarship.

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EXPLANATION OF PLATES

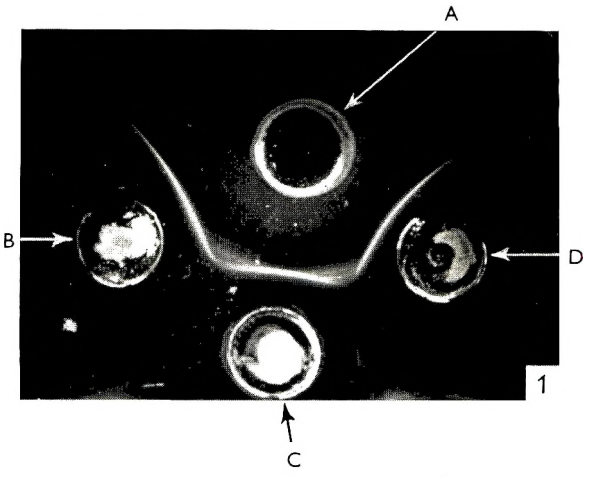
PLATE 1

Fig. 1. Gel diffusion test record. Well A, serum from patient with simple plantar wart; well B, virus antigen from simple plantar warts; well C, virus antigen from mosaic plantar warts; well D, virus antigen from hand warts.

Fig. 2. Gel diffusion test record. Well A, serum from patient with simple plantar wart; well B, serum from rabbit inoculated with genital wart material; well C, serum from rabbit inoculated with virus antigen from simple plantar warts; well D, virus antigen from simple plantar warts.

PLATE 2

Figs. 1, 2. Negatively stained precipitate from gel-diffusion test, showing wart virus particles complexed with antibody. PTA stain. Pl. 2, fig. 1. × 120,000. Pl. 2, fig. 2. × 80,000.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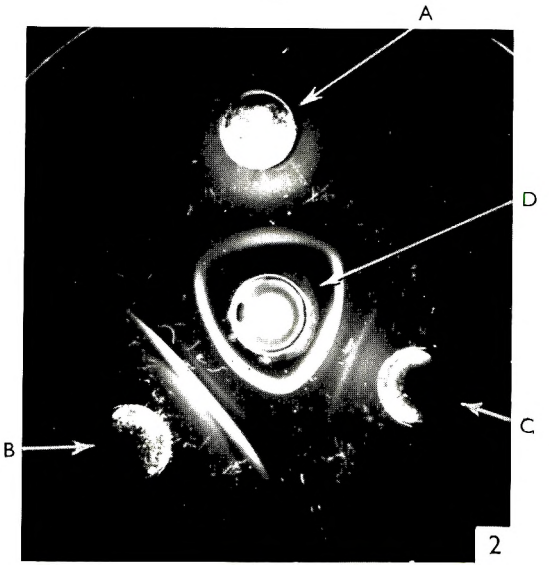
1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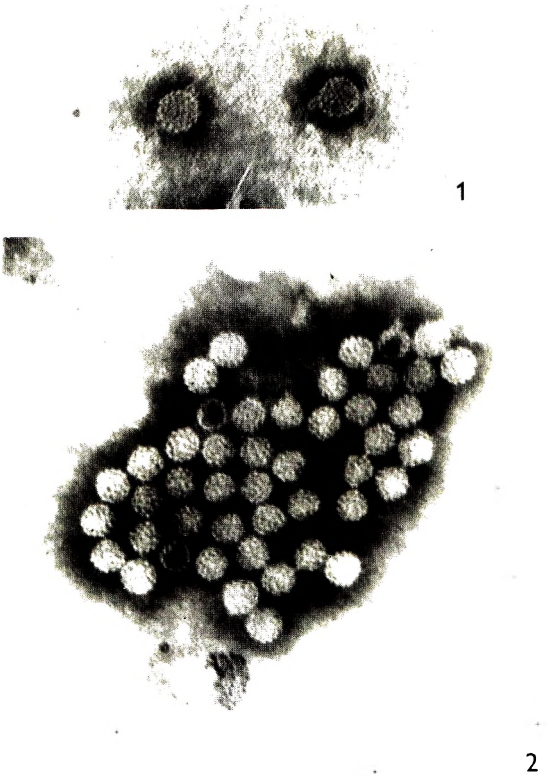

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J. Hyg., Camb. (1970), **68**, 491
Printed in Great Britain

Studies on arboviruses in Egypt

II. Contribution of arboviruses to the aetiology of undiagnosed fever among children

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SUMMARY

Acute blood samples from 120 children, attending the fever hospital in Alexandria and complaining of fever, were collected and examined for haemagglutination-inhibiting (HAI) and complement-fixing (CF) antibodies against the following arbovirus antigens; Sindbis, West Nile (WN), yellow fever, dengue 1, sandfly fever, Quaranfil, Chenuda and Nyamanini. Positive reactions in the acute sera were only detected against Sindbis (4·3%) and WN (4·3%) antigens. The convalescent sera obtained from 48 of these children showed a pronounced HAI titre against WN antigen in 14·6% of them. The same sera showed a lower titre against yellow fever antigen (Asibi strain) which is due to cross-reaction between the two viruses. None of the acute or the convalescent sera showed CF antibodies against Quaranfil, Chenuda or Nyamanini antigens. The convalescent sera were not tested against dengue type 1 antigen. It is suggested that of the known arboviruses in Egypt, WN is the most important from the public health point of view.

INTRODUCTION

In continuation of our study on arboviruses in Egypt (Mayer *et al.* 1967; Mohammed, Sekeyová, Grešiková & El-Dawala, 1968), it was found necessary to evaluate the contribution of these viruses to the aetiology of undiagnosed fever among children. Usually, a large number of children complaining of fever, daily attend the fever hospital in Alexandria. The results of previous serological examination of the human adult population of Alexandria showed the presence of haemagglutination-inhibiting antibodies against sandfly (45%) and West Nile (16%) antigens (Mohammed *et al.* 1968).

In the present investigation, acute and convalescent blood samples were tested with the following arboviruses: Sindbis, West Nile, yellow fever, dengue type 1, Sicilian sandfly, Quaranfil, Chenuda and Nyamanini antigens.

MATERIALS AND METHODS

Human blood sera

Acute-stage blood samples were collected during the months of June to October 1968, from 120 children (60 males, 60 females; 3–13 years of age) attending the fever hospital in Alexandria. Convalescent sera from some of the same children (24 males, 24 females) were collected one and a half months later. Acute sera were stored frozen at $-20°$ C. until the collection of the convalescent sera; both were then tested immediately.

Serological tests

Human sera were tested for the presence of haemagglutination-inhibiting and complement-fixing antibodies.

Complement fixation (CF) test

The standard method using plastic plates was followed. The plates with the sera, antigens and two units of complement were allowed to stand overnight at 4° C. before the addition of the haemolytic system. All sera were absorbed by kaolin before the CF test, since they were found to be anticomplementary.

Haemagglutination inhibition (HAI) test

Antigens for haemagglutination (HA) and haemagglutination inhibition (HAI) tests were prepared by the sucrose acetone extraction method from the brains of infected unweaned mice according to the techniques described by Clarke & Casals (1958). Sera were extracted by acetone and absorbed with goose erythrocytes prior to testing. The HA and HAI tests were carried out by the method of Clarke & Casals (1958) as modified for use in plates.

Virus strains used

Sindbis virus. This is the passage number 2 in unweaned mice at the Rockefeller Foundation Virus Laboratory (RFVL). It has undergone six passages in unweaned mice at the Regional Reference Laboratory (RRL) in Bratislava.

West Nile virus. Egypt 101. Passage number 7 in unweaned mice at the RFVL. It has undergone 12 passages in baby mice at the RRL in Bratislava.

Dengue 1 virus. Originally obtained from the RFVL, passage number 11 in newborn mice, it has undergone a further five passages at the RRL in Bratislava.

Yellow fever virus. Asibi strain. It has undergone four passages in baby mice at the RRL in Bratislava.

Sandfly fever virus. Sicilian phlebotomus fever, Sabin strain, 37 passages at the RFVL, further two passages at the Instituto Superiore di Sanita, Rome, and three passages at the RRL in Bratislava.

Nyamanini virus. (Eg Ar 1304, P-25). It has undergone three passages in baby mice at the RRL in Bratislava.

Quaranfil virus. (Eg Ar 1113, P-6). It has undergone three passages in baby mice at the RRL in Bratislava.

Chenuda virus. (Eg. Ar 1152, P-18). It has undergone two passages in baby mice at the RRL in Bratislava.

The last three viruses were kindly sent by Dr Karabatsos, Yale Arbovirus Research Unit.

RESULTS

In the acute sera from children, haemagglutination-inhibiting antibodies were detected against Sindbis antigen in 4 % and against West Nile antigen in 4 %. No antibodies were found against yellow fever, dengue type 1 or sandfly antigens. Similarly, no complement-fixing antibodies could be observed with Quaranfil, Chenuda or Nyamanini antigens. The results of the HAI and CF tests with acute sera are given in Table 1.

Table 1. *Haemagglutination-inhibiting and complement-fixing antibodies in acute sera of children, Alexandria,* 1968

Antigen	Titre	Number of sera	Positive (%)
Sindbis	< 20	115	—
	20	1	
	40	2	4
	80	2	
West Nile	< 20	115	—
	20	1	
	40	1	4
	80	3	
Yellow fever	< 20	120	0
Sandfly fever	< 20	120	0
Dengue type 1	< 20	120	0
Quaranfil*	< 20	120	0
Chenuda*	< 20	120	0
Nyamanini*	< 20	120	0

* Tested by complement fixation.

Table 2. *Titres of haemagglutination-inhibiting antibodies in acute and convalescent sera which were positive with West Nile antigen, Alexandria,* 1968

Antibody titres in		Age of
Acute serum	Convalescent serum	child years
< 20	640	10
< 20	640	4
< 20	640	5
< 20	640	13
< 20	640	11
< 20	1280	6
< 20	1280	9

In the convalescent sera, on the other hand, seven out of 48 children tested (15 %) showed HAI antibodies against West Nile antigen (Table 2). These same sera showed lower titres against yellow fever antigen, but they and all the other

convalescent sera were negative by the HAI test against Sindbis and sandfly antigens, and by the CF test against Quaranfil, Chenuda and Nyamanini antigens (Table 3).

Table 3. *Haemagglutination-inhibiting and complement-fixing antibodies in convalescent sera of children, Alexandria, 1968*

Antigen	Antibody titre	Number of sera	Positive (%)
West Nile	< 20	41	—
	20	0	—
	40	0	—
	80	0	—
	160	0	—
	320	0	—
	640	5	15
	1280	2	
Yellow fever	< 20	41	—
	20	0	—
	40	4	15
	80	1	
	160	2	
Sindbis	< 20	48	0
Sandfly fever	< 20	48	0
Quaranfil*	< 20	48	0
Chenuda*	< 20	48	0
Nyamanini*	< 20	48	0

* Tested by complement fixation.

DISCUSSION

In the previous serological survey among the urban population of Alexandria, HAI antibodies were found against sandfly fever in 45% and West Nile virus in 16%. The results of the present investigation showed that out of arboviruses known to be present in Egypt, West Nile virus is probably the most important from the public health point of view. The positive convalescent sera showed high titres of HAI antibodies to WN antigen; in five of them a value of 640 and in the other two a value of 1280 (Table 2). It was also noted that although the sera were collected during the period June to October 1968, all positive sera were obtained during August. This is in agreement with the findings of Taylor, Work, Hurlbut & Farag Risk (1956), who described West Nile virus isolations from human blood with the highest percentage during July and August. It should be pointed out that the children with HAI antibodies in their acute sera against WN and Sindbis viruses did not give us convalescent sera.

Cross-reactions with the B group of arboviruses were observed with yellow fever antigen (Asibi strain) in convalescent sera which contained HAI antibodies against West Nile virus. The titre of antibodies against West Nile virus was in all samples higher than with yellow fever antigen. None of the acute or the convalescent sera contained complement-fixing antibodies to Quaranfil, Chenuda or Nyamanini antigens. This finding may indicate that in the sera investigated,

there is a very low contact of humans with these viruses. With the exception of two Quaranfil virus strains (H-2351, H-8762) all of these viruses were isolated from ticks, nestling egrets or pigeon squab (Taylor *et al.* 1966). It is of interest to mention that both Quaranfil and Nyamanini viruses have been also isolated from one and the same egret rookery in South Africa.

The authors are grateful to Mrs V. Bielikova for the excellent technical assistance.

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J. Hyg., Camb. (1970), **68**, 497
Printed in Great Britain

Changes in the antibody status of a population following epidemic infection by influenza virus A2/Hong Kong/1/68

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SUMMARY

The haemagglutinin of influenza virus A2/Hong Kong/1/68 was shown to be markedly different from that of previously isolated A2 virus strains. No haemagglutination-inhibiting (HI) antibody to A2/Hong Kong/1/68 virus was detected in serum specimens collected in 1966 from persons aged 60 years or less. In contrast, HI antibody tests with 270 sera collected in 1968 indicated that 9·6% had demonstrable HI antibody at low titres, and 35·2% of 454 postepidemic (1969) sera had demonstrable HI antibody at relatively high titres. Most sera from persons aged 80 years and more collected in 1968 and 1969 had demonstrable HI antibody to influenza virus A2/Hong Kong/1/68. No HI antibody to the Hong Kong virus was detected in pre-epidemic sera from children aged 6 months to 3 years, whereas 32% of postepidemic sera had HI antibody. The acquisition of HI antibody to A2/Hong Kong/1/68 was not accompanied by an increase in the incidence or titres of HI antibody to heterotypic A2 influenza viruses. For sera from children aged 4–11 years, an increase of HI titre to heterotypic A2 influenza was found.

INTRODUCTION

Although a number of studies have reported the distribution in the population of antibody to influenza viruses, and the relationship of these findings to the sequential appearance of the different families of influenza A virus (Davenport, Hennessy & Francis, 1953; Schild & Stuart-Harris, 1965), little attention has been given to the effects of minor antigenic variations occurring in interpandemic periods. Because of extensive cross-reactions between influenza A viruses of the same family, interpretation of serological data is difficult. Zhdanov (1967) suggested that population studies of serum antibody to interpandemic influenza A viruses may allow prediction of the antigenic nature of future epidemic strains. Thus, a high incidence of antibody would indicate an immune population while the absence of antibody might indicate the likelihood of the virus, or closely related virus, giving rise to an epidemic in the future. Schild & Stuart-Harris (1967) suggested that such studies were more likely to give definitive information if sera from children were used, because as a result of their shorter time of exposure to infection, the pattern of influenza antibody is more restricted in children than in adults.

The arrival of the Hong Kong variant of A2 influenza virus in the United Kingdom during 1968 allowed a serological study of the epidemiology of this pandemic virus in the population. The incidence of haemagglutination-inhibiting antibody to influenza virus A2/Hong Kong/1/68 was estimated in sera collected in 1966, 1968 and 1969 from both children and adults; the largest number were collected shortly before and after the outbreak in 1968 and early 1969. Sera were also tested for HI antibody to four other representative influenza A2 viruses.

METHODS AND MATERIALS

Viruses

Influenza viruses A2/Singapore/1/57 and A2/England/12/64 were the same as used in a previous study (Schild & Stuart-Harris, 1967). Influenza viruses A2/England/10/67; A2/Tokyo/1/67; A2/Hong Kong/1/68 and a recombinant strain containing Fowl Plague haemagglutinin and A2/Hong Kong/1/68 neuraminidase (FPV/A2HK) were obtained from Dr G. C. Schild, World Influenza Centre, Mill Hill, London. Virus pools were prepared in 10-day embryonated eggs by allantoic inoculation of 10^{-3} dilution of seed virus. After 48 hr. incubation at 35° C., allantoic fluids were harvested and stored at − 80° C.

Serum specimens

Sera were obtained in the periods of May–June 1966, September–October 1968 and May–July 1969, from blood donors, women attending antenatal clinics, and from specimens submitted for Wasserman tests. Seventy-nine sera were obtained in August–November 1968, and 75 in May–June 1969 from children admitted to Sheffield Children's Hospital for treatment of accidents and burns, and from specimens submitted for antistreptolysin 'O' testing. All human sera were from persons living in the Sheffield Hospital region. They were stored at − 20° C.

Antisera were obtained by inoculating adult ferrets intranasally with 0·5 ml. of live, egg-grown influenza virus. Ferrets were bled 3–4 weeks after virus infection.

Haemagglutination inhibition (HI) tests

HI tests were carried out in Perspex plates (W.H.O., 1953). Before test, sera were incubated for 18 hr. at 37° C. with five volumes of cholera filtrate (N. V. Philips Duphar, Amsterdam) and subsequently heated at 56° C. for 1 hr. Twofold dilutions of serum in 0·2 ml. volumes were mixed with an equal volume of virus containing eight haemagglutinating units (50 % end-point). After 10 min. incubation at room temperature, 0·2 ml. of fowl erythrocytes (0·5 % suspension in phosphate-buffered saline, pH 7·4) were added, and the HI titre read from the pattern of haemagglutination produced after the cells had settled at room temperature. Fowl erythrocytes were obtained from a single fowl bled at weekly intervals.

RESULTS

Comparison of A2 influenza viruses by HI tests

Table 1 shows the HI antibody titres of five ferret antisera tested against homologous and heterologous A2 influenza viruses. The results indicate that influenza

virus A 2/Hong Kong/1/68 represents a large antigenic deviation from previously isolated A 2 virus strains. Ferret antisera prepared against heterologous A 2 influenza viruses showed only low titres of haemagglutination-inhibiting antibody when tested against A 2/Hong Kong/1/68 virus. Similarly, the A 2/Hong Kong/1/68 ferret antiserum had only low titres of HI antibody against the four heterologous A 2 influenza viruses. Influenza viruses A 2/England/12/64 and A 2/England/10/67 appeared to be closely related antigenically, while the virus strain A 2/Tokyo/1/67 was recognizably distinct from the former two virus strains. All four influenza viruses isolated from 1964 to 1968 were distinct from the prototype influenza virus A 2/Singapore/1/57.

Table 1. *Haemagglutination inhibition titres of ferret antiserum against homologous and heterologous A 2 influenza viruses*

	Ferret antisera against influenza virus				
Influenza viruses	A 2/Sing/ 1/57	A 2/Eng/ 12/64	A 2/Eng/ 10/67	A 2/Tokyo/ 1/67	A 2/HK/ 1/68
A 2/Sing/1/57	**1280***	320	320	< 10	10
A 2/Eng/12/64	40	**1960**	960	480	40
A 2/Eng/10/67	80	1960	**2560**	320	80
A 2/Tokyo/1/67	15	160	320	**1960**	40
A 2/HK/1/68	30	20	60	15	**640**

* Reciprocal HI titre.

Table 2. *Incidence of haemagglutination inhibition antibody to influenza virus A 2/Hong Kong/1/68 in human sera collections*

Date of serum	No. tested	No. positive	Positive (%)	Mean titre
June–Aug. 1966	97	0	0	—
Sept.–Oct. 1968	270	26	9·6	1/26
May–July 1969	454	160	35·2	1/175

Incidence in human sera of HI antibody to influenza virus A 2/Hong Kong /1/68

Sera collected during 1966, the immediate pre-Hong Kong influenza virus period of 1968, and also after the Hong Kong influenza in 1969, were each titrated for HI antibody to influenza virus A 2/Hong Kong/1/68. The results are shown in Table 2. No HI antibody at a titre of 1/6 was found in 97 serum specimens collected in 1966. These sera were all obtained from persons under 60 years of age at the time of collection, 68 samples being from adults aged 20 years or more. Among 270 sera from 1968, 26 (9·6 %) contained HI antibody to influenza virus A 2/Hong Kong/1/68 at a titre of 1/6 or greater. Actual antibody titres were relatively low, the arithmetic mean titre was 1/26, and only one specimen contained antibody at a titre of greater than 1/96. The distribution by age of HI antibody positive sera is shown in Fig. 1. In contrast, of 454 postepidemic serum specimens collected in 1969, 160 (35·2 %) contained HI antibody to influenza virus A 2/Hong Kong/1/68 at a titre of 1/6 or greater, and the arithmetic mean titre of the HI antibody positive

sera was 1/175. Of the serum specimens with HI antibody, 70 (44 %) showed titres of 1/96 or greater.

Fig. 1. shows the incidence of HI antibody to influenza virus A2/Hong Kong/1/68 at 1/6 or greater in pre- and postepidemic serum specimens, grouped by age. No HI antibody to influenza virus A2/Hong Kong/1/68 was detected in sera collected in 1968 from persons aged 9 years or less. Variable percentages of sera with HI antibody to Hong Kong influenza virus (3–19 %) were found in the age groups 10–79 years. In the postepidemic sera collected in 1969, the incidence of HI antibody increased with age from 30 % in children of 9 or less to a maximum of 40 % for persons aged 20–29 years. It declined in each older decade to 21 % for persons aged 60–69 years and 24 % in those of 70–79 years.

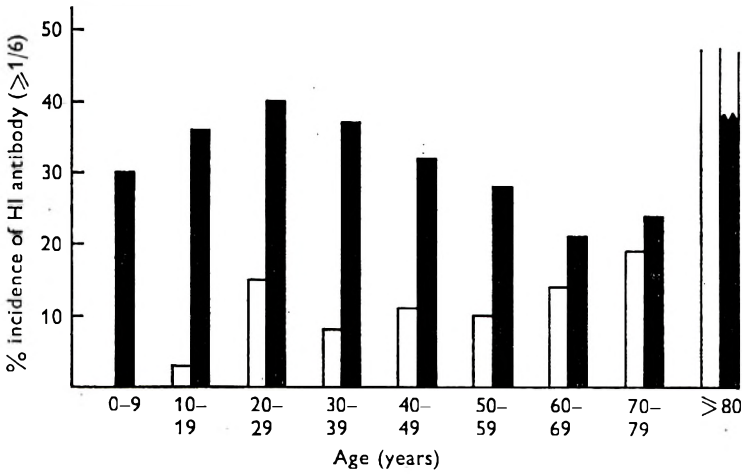

Fig. 1. Percentage incidence of haemagglutination-inhibition (HI) antibody to influenza virus A2/Hong Kong/1/68 in human sera. □, Pre-epidemic (1968) sera; ■, postepidemic (1969) sera.

Only three pre-epidemic (1968) sera were available from persons aged 80 years or more; these contained HI antibody at titres of 1/18, 1/24 and 1/36. In view of the small number of samples, ten further sera collected in 1964 from persons aged more than 80 years were tested; eight contained HI antibody. Fourteen postepidemic sera (1969) were available from persons aged 80 years or more, and 12 contained detectable HI antibody to influenza virus A2/Hong Kong/1/68. The arithmetic mean titre of these HI positive sera was 1/28; this finding was in striking contrast to the results obtained for younger persons (Table 2).

Relationship of influenza virus A2/Hong Kong/1/68 HI antibody to HI antibody against other A2 influenza viruses

A possible explanation for the small number of serum specimens collected in 1968 with HI antibody to influenza virus A2/Hong Kong/1/68, as well as the relatively low titre of this antibody, was that inhibition might be due to cross-reacting antibody to heterologous A2 influenza viruses. To investigate this possibility, sera with and without HI antibody to A2/Hong Kong/1/68 influenza virus

were tested for HI antibody to other A2 influenza viruses. The results are shown in Table 3. Among sera with HI antibody to A2/Hong Kong/1/68, the arithmetic mean titre of HI antibody to A2/Singapore/1/57; A2/England/12/64; A2/England/10/67 and A2/Tokyo/1/67 was 1/71; 1/24; 1/22 and 1/23, respectively. The corresponding titres against these same viruses in sera without detectable HI antibody to A2/Hong Kong/1/68 were 1/19; 1/16; 1/10 and 1/22, respectively. Thus, sera containing HI antibody to A2/Hong Kong/1/68 had higher mean titres of HI antibody to three of the four A2 influenza viruses tested than sera without demonstrable HI antibody to A2/Hong Kong/1/68.

Table 3. *Haemagglutination-inhibition (HI) antibody to A2 influenza viruses in sera with and without HI antibody to influenza virus A2/Hong Kong/1/68*

	HI antibody to A2 influenza virus strain			
HI antibody to A2/Hong Kong/1/68	Virus strain	No. tested	No. positive sera (titres ⩾ 1/6)	Mean HI titre*
Positive (⩾ 1/6)	A2/Sing/1/57	20	20	71
	A2/Eng/12/64	17	13	24
	A2/Eng/10/67	18	16	22
	A2/Tokyo/1/67	13	13	23
Negative (< 1/6)	A2/Sing/1/57	40	38	19
	A2/Eng/12/64	40	31	16
	A2/Eng/10/67	37	20	10
	A2/Tokyo/1/67	40	38	22

* Reciprocal of arithmetic mean titre.

A further explanation for the presence of low titres of HI activity against influenza virus A2/Hong Kong/1/68 in the 1968 sera was non-specific interference in the HI test caused by the presence in the sera of antineuraminidase antibody. A reaction between neuraminidase-inhibiting antibody, acquired during earlier influenza infection by virus with an identical neuraminidase, might cause steric interference of haemagglutination (Webster & Pereira, 1968); this would be interpreted falsely as indicating the presence of specific HI antibody to A2/Hong Kong/1/68 virus. Therefore, sera collected in 1968 which inhibited A2/Hong Kong/1/68 virus were tested for their ability to inhibit haemagglutination by the hybrid influenza virus FPV/A2HK, which has the same neuraminidase as the former virus. No haemagglutination inhibition of this virus was found in the sera at a titre of 1/6 or greater. This result indicated that the presence of inhibitory action against influenza virus A2/Hong Kong/1/68 in pre-epidemic sera was not due to steric inhibition by antineuraminidase antibody.

HI antibody to A2 influenza viruses in sera from children

Haemagglutination-inhibition tests using five A2 influenza viruses were carried out on 79 pre-epidemic sera (1968) and 75 postepidemic sera (1969) from children 6 months to 11 years. Fig. 2 shows that no detectable HI antibody to influenza A2/Hong Kong/1/68 was found in pre-epidemic sera from children aged 6 months

to 3 years whereas among 35 postepidemic sera, 11 (32%) contained HI antibody at titres of 1/12 or greater. The acquisition of HI antibody to influenza virus A2/Hong Kong/1/68 was not accompanied by an increase in the incidence or titres of HI antibody to other A2 influenza viruses (Fig. 2). No detectable HI antibody to influenza virus A2/Hong Kong/1/68 was found in pre-epidemic sera (1968)

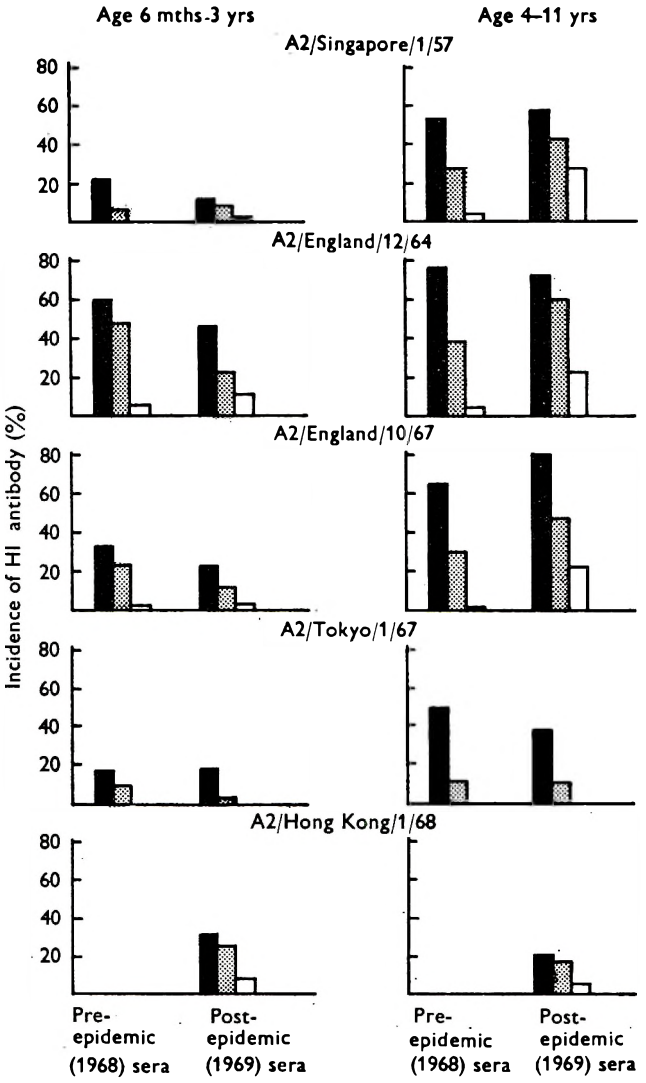

Fig. 2. Percentage incidence of haemagglutination-inhibiting (HI) antibody to A2 influenza viruses in pre- and postepidemic children's sera. HI antibody at titres of: ■, ⩾ 1/12; ▦, ⩾ 1/48; □, ⩾ 1/192.

from children aged 4–11 years, whereas 20% of postepidemic sera (1969) from similarly aged children contained antibody at titres of 1/12 or greater. During the epidemic period there was no significant increase in incidence of HI antibody to other A2 influenza viruses. However, the incidence of HI antibody at titres equal to or greater than 1/48 and 1/192 to influenza viruses A2/Singapore/1/57;

A 2/England/10/64 and A 2/England/12/67, increased during this period. HI antibody titres to influenza virus A 2/Tokyo/1/67 were unaffected by the influenza epidemic of 1968–9.

DISCUSSION

Tests made in our laboratory with five A 2 influenza virus strains and homologous ferret antiserum agreed with the findings of Coleman *et al.* (1968) that the haemagglutinin of influenza virus A 2/Hong Kong/1/68 was widely divergent from that of previously isolated A 2 influenza viruses. Only low levels of cross-reactivity with the other A 2 influenza viruses were found.

The results of haemagglutination tests with pre-epidemic human sera collected in 1968 and influenza virus A 2/Hong Kong/1/68 were different for persons aged less than 11 years, 12–79 years and 80 years or more. The low titres of HI antibody found in sera from persons aged 10–79 years could have three explanations. First, since the neuraminidase of influenza virus A 2/Hong Kong/1/68 is identical with that of earlier occurring influenza strains (Schulman & Kilbourne, 1969), steric inhibition of haemagglutination might have been caused by a reaction between the neuraminidase of influenza virus A 2/Hong Kong/1/68 and serum anti-neuraminidase (Webster & Pereira, 1968). No evidence to support this view was found.

Secondly, the HI activity to influenza virus A 2/Hong Kong/1/68 in pre-epidemic sera from persons aged 10–79 years represented cross-reacting HI antibody induced by previous infecting A 2 influenza viruses. Sera with demonstrable HI antibody to influenza virus A 2/Hong Kong/1/68 had higher mean HI titres to three or four A 2 influenza viruses tested than did specimens with no HI antibody to A 2/Hong Kong/1/68 virus. The largest difference was found for HI antibody to influenza virus A 2/Singapore/1/57, though ferret antiserum against influenza virus A 2/Singapore/1/57 showed little cross-reactivity with A 2/Hong Kong/1/57 virus. If the HI antibody to influenza virus A 2/Hong Kong/1/68 found in sera collected in 1968 was cross-reacting HI antibody, it was acquired since 1966, as sera collected in that year contained no detectable HI antibody against influenza virus A 2/Hong Kong/1/68.

A third explanation is that the introduction of influenza virus A 2/Hong Kong/1/68 into the population predated the serum collection. This explanation cannot be excluded. However, the absence of detectable HI antibody in children less than 11 years of age and the low mean titre of HI antibody in seropositive specimens did not support this view.

Most of the small number of specimens from individuals aged 80 years or more contained HI antibody to influenza virus A 2/Hong Kong 1/68, and this result is in agreement with previous findings (Masurel, 1969; Marine & Workman, 1969; Zakstelskaja, 1969). The presence of pre-epidemic HI antibody to A 2/Hong Kong/1/68 in this age group has been interpreted as evidence of influenza infection by a virus antigenically similar to or identical with this influenza virus in the years 1890–5 (Davenport, Minuse, Hennessy & Francis, 1969). The alternative view that it merely represents heterotypic antibody built up by successive A 2 virus infec-

tions has to be considered. However, the sharp change from a relatively low incidence of HI antibody in those aged less than 80 years to a high incidence for older persons, argues against this view. In addition, Masurel (1969) detected a high incidence of HI antibody to A2/Hong Kong/1/68 virus in sera from persons aged 70 years and more which had been collected before the first A 2 virus isolation in 1957.

No indication of the epidemic caused by influenza virus A 2/Hong Kong/1/68 in 1968–9 could have been obtained from observations of children's sera collected before the epidemic, and using A 2 viruses available at that time. Haemagglutination tests with postepidemic human sera collected in the summer of 1969 showed that only one-third of the population had experienced clinical or subclinical infection by Hong Kong influenza virus. The occurrence of an influenza epidemic in Sheffield in the following winter (1969–70) showed that this figure was indeed too low to give population immunity. Further surveillance of the immune status of the population is required to determine the level of immunity necessary to protect the community against successive influenza epidemics by a particular virus strain.

We wish to thank The Wellcome Trust for support for one of the authors (S.J.M.), and Professor Sir Charles Stuart-Harris and Professor M. G. McEntegart for their advice and criticism, and Mrs. K. Spinks for excellent technical assistance.

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J. Hyg., Camb. (1970), **68**, 505
Printed in Great Britain

Trials with a live attenuated rubella virus vaccine, Cendehill strain

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(*Received* 6 *May* 1970)

SUMMARY

This report summarizes closed, family, and open studies conducted sequentially over a 10 month period with the Cendehill rubella virus vaccine in more than 16,000 children and adolescents. This strain of rubella was attenuated by serial propagation on primary rabbit kidney cell cultures. Inoculation of the Cendehill vaccine produced seroconversion in 97% of the 3589 susceptible (seronegative) vaccinated persons. There was no spread of the virus to susceptible controls living in close contact with those vaccinated. The vaccine was well tolerated. No arthritis or arthralgia occurred in 860 female subjects 13–18 years of age who were included in the study. The Cendehill vaccine would appear to meet the requirements of an acceptable vaccine.

INTRODUCTION

The Cendehill strain of rubella virus was isolated in May 1963, at the University of Louvain, Belgium, from a patient with clinical signs of rubella. It was attenuated by serial propagation in primary rabbit kidney cell cultures derived from a select colony of animals bred and reared in isolation under pathogen-free conditions. Details on the Cendehill strain and its biological characteristics, the resultant vaccine, and safety test methods have been dealt with by others at two international symposia on rubella vaccines (Huygelen *et al.* 1969; Prinzie, Huygelen, Gold & Farquhar, 1969).

The studies in Jamaica began in March 1968. Before this, vaccine trials had been conducted in Switzerland (Martin duPan, Peetermans, Huygelen & Prinzie, 1967, 1968; Majer, 1967). Our initial studies were conducted in a closed setting in order to ascertain that the virus did not spread to susceptible contacts in the local environment. Upon fulfilling this prerequisite and confirming the findings of the Swiss studies that the virus was not being disseminated, the investigation was extended first to a family setting, and then to large scale studies in children in the community. This paper reports pertinent findings from these studies.

METHOD

Closed study

This study was conducted with 54 rubella susceptible (seronegative) children residing in two institutions, the Maxfield Home and the Alpha School. The children were from 3 to 17 years old. They ate, played, attended classes, and slept together in the respective dormitories for boys or girls.

Twenty-eight were vaccinated with the Cendehill rubella vaccine, receiving a subcutaneous dose of 0·5 ml. (5000 TCID 50); the rest served as unvaccinated controls. At the Maxfield Home quarters were very crowded and the beds were very close to each other, a condition which should have allowed for efficient transmission of virus, if transmission were to occur. Specimens were taken with swabs from the nose and throat of the vaccinated children daily from days 9 to 15, and from the controls on days 2, 20, 29 and 32, for virus isolation studies; the method of Plotkin *et al.* (1968) was used. Blood specimens were taken before vaccination and, subsequently, on day 45 from the vaccinated children and on day 65 from the controls, to determine rubella antibody titres. A modification of the haemagglutination inhibition (HI) test, described by Stewart *et al.* (1967), was used. All of the children, vaccinated and controls, were interviewed and examined daily throughout the study for clinical symptoms and signs by a physician and nurse.

Family study

We selected only families which had at least two seronegative children, and had no rubella seronegative women of child-bearing age in the immediate families or in other families with whom they had frequent contact. We screened 103 families and found 67 which qualified under these restrictions. These families had 115 seronegative children, and we vaccinated 52 of them, and reserved the other 63 as controls. Virus isolation tests were carried out on all those vaccinated, from throat and nose specimens collected 11–12 days after the vaccination day. Physicians and nurses from our research team visited the homes frequently during the study, and examined the vaccinated children, controls, and other members of the family. Repeat blood samples for the determination of HI titres were taken from all of the vaccinated and control children 2 months after vaccination.

Open study

In this study, we drew blood samples for determination of HI titres and then vaccinated the subjects on the same day. Our medical teams visited a total of 19 primary and secondary schools in and around Kingston and vaccinated 14,610 children with the Cendehill vaccine. One thousand five hundred and sixty-one children constituted a control group and received an injection of sterile saline solution to provide a baseline for the monitoring of lymphadenopathy, fever, or other reactions which may have been provoked by agents other than rubella virus.

Registered nurses visited all the schools twice a week for 3 weeks to check on absenteeism due to illness. These cases were more closely followed up at home and school. All seronegative vaccinated children or controls who were absent because

of illness were visited in their homes by one of our physicians. Test results subsequently showed that 5207 of those vaccinated were seronegative; 3847 (almost 74 %) were available for retesting and follow-up 2 months after vaccination. Serological data by age groups for more than 11,000 of these children are shown in Table 1.

RESULTS

Serological tests

Seroconversion occurred in 100 % (closed), 98 % (family) and 97 % (open) of the seronegative vaccinated children; there was no spread of the virus to the seronegative controls. Details of the postvaccination serological results are shown in Tables 2–4.

Table 1. *Distribution of rubella antibodies in* 11,609 *Jamaican children and adults*

Age years	Seropositive		Seronegative	
	Number	%	Number	%
< 5	3	60	2	40
5–9	3414	62	2101	38
10–14	4263	72	1694	28
15–20	96	73	36	27
Totals	7776		3833	

Table 2. *Serological results of the closed study using the Cendehill vaccine. Seronegative persons only*

Group	Seroconversion	GMT*	Haemagglutination inhibition after vaccination	
			No. of children	Titres
28 Vaccinated	100 %	1/77	2	16
			7	32
			7	64
			8	128
			4	256
26 Controls	0	0	26	< 8

* Geometric mean titre.

Virus isolation

As Table 5 shows, no rubella virus was isolated at any time from the specimens taken from the nose and throat of the 26 seronegative controls in the closed study. Rubella virus was isolated once from five of the seronegative vaccinated children in the closed study, 11–13 days after vaccination, and from one in the family study, 12 days after vaccination.

Clinical symptoms

Vaccine-related side effects and other clinical symptoms have not been problems in our studies with the Cendehill vaccine. In the closed study, an almost equal number of vaccinated persons and controls had lymphadenopathy before and during the study. Lymphadenopathy, however, is common in Jamaican children and, therefore, was not considered to be related to vaccination. During this study, sporadic outbreaks of measles, mumps and chicken pox occurred in

Table 3. *Serological results of the family study using the Cendehill vaccine. Seronegative persons only*

Group	Seroconversion	GMT	Haemagglutination inhibition after vaccination	
			No. of children	Titres
52 Vaccinated	98 %	1/56	1	< 8
			1	8
			6	16
			14	32
			19	64
			9	128
			1	256
			1	1024
63 Controls	0		63	<8

Table 4. *Serological results of the open study using the Cendehill vaccine*

Group	No. of subjects	No. of sero-negatives	Number tested after vaccination	Sero-conversion		GMT
				No.	%	
Vaccine	14,610	4711	3509	3410	97·1	1/51
Placebo	1,561	496	338	0	—	—

Table 5. *Results of virus isolation tests*

	Specimens examined	Positive specimens*	
		Vaccinees	Controls
Closed study	274	5	0
Family study	76	1	Not taken

* Taken 11–13 days after vaccination.

both vaccinated children and controls, but did not interfere with the stimulation of rubella antibodies in those vaccinated. Nor did the intercurrent infections aggravate the benign clinical response to the vaccine. In the family study, one vaccinated child developed a rubella-like reaction characterized by rash and temperature elevation.

Dengue fever was endemic during the open study, and approximately 175 subjects reported symptoms such as: rash, which occurred primarily on the face and trunk, but in a few subjects, on the limbs; temperature elevations, which ranged from 101·8–103·2° F. in eight of the subjects; and regional lymphadenopathy, which was postcervical in only one of the subjects. The incidence of these effects was *ca.* 1% in the seronegative and seropositive vaccinated children, and was *ca.* 0·4% in the seronegative and seropositive placebo controls. This difference is not statistically significant. In the entire group of almost 5000 seronegative persons vaccinated in this study, only one was considered to have had a rubella-like reaction; namely, a rash on the head and trunk which developed on the 17th post-vaccination day, persisted for 6 days, and was accompanied by a temperature elevation. There was no evidence of arthralgia or arthritis in the 860 vaccinated females, aged 13–18 years (275 seronegatives, 585 seropositives) who were included in these studies.

DISCUSSION

Our findings in large scale clinical trials bear out the earlier reported preliminary findings that the Cendehill rubella virus vaccine evokes a good immunogenic response and does not spread virus to susceptible persons in close contact with those vaccinated. Preliminary studies carried out 24 months after vaccination suggest that the Cendehill vaccine provides long-lasting immunity (Prinzie *et al.* 1969). Recently, we retested 14 of the vaccinated children from our first, closed study and found that relatively high antibody titres had persisted for 12 months. With two possible exceptions, there were no side effects or reactions attributable to the vaccine. Rash, lymphadenopathy, and fever have occurred with about equal frequencies in rubella susceptible and rubella immune vaccinated children and in children who were not vaccinated. Although our series disclosed no problems from arthralgia or arthritis, joint pains have been reported by others in adult women receiving rubella vaccines, but these manifestations have occurred less frequently and have been milder with the Cendehill than with the HPV-77 strain (Dudgeon, Marshall, Peckham & Hawkins, 1969; Cooper *et al.* 1969; Horstmann *et al.* 1969). The degree of attenuation of the Cendehill vaccine may account for its causing fewer joint manifestations (Gold, Prinzie & McKee, 1969; Farquhar & Corretjer, 1969).

On the basis of our experience, along with that of others, the Cendehill rubella virus vaccine appears to be a highly effective and safe vaccine. Based on our findings there is no contraindication to administering the vaccine during times when mumps, chicken pox, and measles are prevalent.

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THE QUARTERLY JOURNAL OF MEDICINE

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NEW SERIES · VOL. XXXIX · No. 154 · APRIL 1970
(Vol. LXIII of the continuous series)

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OXFORD UNIVERSITY PRESS

Corrigendum

E. C. Anderson, P. B. Capstick, G. N. Mowatt and F. B. Leech.
J. Hyg., Camb. (1970) **68**, 159–72.

On page 170, first line of footnote,

For $P = (N - n^m/N)$

Read $P = \{(N-n)/N\}^m$

The Journal of Hygiene Volume 68, No. 3 September 1970

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Printed in Great Britain at the University Printing House, Cambridge