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The preservation of bacteriophage H 1 of *Corynebacterium ulcerans* U 103 by freeze-drying

By J. D. DAVIES and M. J. KELLY
Department of Pathology, University of Cambridge, Cambridge

(*Received* 25 *March* 1969)

Corynebacteriophage H 1 was found to be extremely labile when stored in suspension at 4° C. and also when stored at low temperatures after conventional slow rates of cooling. This seemed a suitable organism to use for a detailed investigation of its sensitivity to freezing and drying under different conditions with the intention of applying the results, in the light of recent research work on the factors influencing survival during and after freeze-drying, to maintain maximum survival in the freeze-dried product. Bacteriophages, though comparatively simple in structure, represent well-defined organized biological entities, and it is hoped that the result of such studies will eventually prove to be useful in enabling the damage which occurs during freezing, thawing and drying to be localized at a structural or biochemical level.

MATERIALS AND METHODS

Preparation and maintenance of phage

The initial sample of bacteriophage H 1 of *Corynebacterium ulcerans* U 103 was obtained in the form of a broth lysate which had been frozen rapidly and stored at −70° C. After thawing rapidly at 37° C., 0·5 ml. of this lysate was used to inoculate a 10 ml. volume of a susceptible culture of *C. ovis* E 1144 B in digest broth, which had been incubated on an inclined revolving stage for 2 hr. at 37° C. (to prevent clumping), and lysis was allowed to proceed for 5 hr. at 37° C.

This lysate was then used to inoculate 20 ml. of a 2 hr. culture of *C. ovis* prepared as before. A further 5 hr. was allowed for complete lysis to occur. Bacterial debris was removed by centrifugation, and 2 ml. volumes of the supernatant were dispensed into neutral glass tubes, cooled rapidly by direct immersion in liquid nitrogen and stored at −196° C.

Preparation of experimental samples

When required, a tube containing 2 ml. frozen lysate was thawed rapidly by agitation in a water bath at 37° C., and diluted 1/10 with digest broth. Equal volumes of this suspension and of each double-strength additive were thoroughly mixed, and 0·1 ml. samples of each mixture dispensed into 0·5 ml. freeze-drying tubes (Johnson and Jorgensen, London). Double-strength solutions (%, w/v) of additives were prepared in distilled water and sterilized by autoclaving, except solutions containing peptone, which were Seitz-filtered.

Assay

The bacteriophage was assayed by a top agar technique similar to that described by Adams (1959). The indicator bacteria (*C. ovis* E 1144 B) were taken from an overnight culture and subcultured for 2–3 hr. on digest agar slopes at 37° C. The culture was then transferred to 5 ml. of digest broth and placed in a Mickle shaker for 5 min. at the minimum speed. Any remaining clumps of bacteria were removed by light centrifugation.

Experimental samples of 0·1 ml. and 0·15 ml. of the indicator suspension were added to 2·5 ml. of 0·7 % digest agar which was melted and maintained at 46° C., care being taken during the mixing to prevent any bubble formation. The mixture was then poured as a thin layer on top of nutrient agar in a Petri dish. The number of plaques produced were counted after 24 hr. incubation at 28° C. Triplicate samples were used for each experimental condition and triplicate counts were made on each sample so that each result given represents the mean of nine counts.

Rate of cooling

Rates of cooling were used varying from 1 to 900° C./min. and different ranges of rate required different methods.

A cooling rate of 1° C./min. was obtained by cooling the sample in 0·5 ml. freeze-drying tubes in an aluminium block placed on the thermo-electrical refrigerated stage of the freeze-drying apparatus (Greaves & Davies, 1965).

For rates between 1 and 5° C./min. the method described by Nagington & Greaves (1962) was convenient. The apparatus consists of a hollow cylinder of polystyrene with a polystyrene base, fitting into the neck of a Linde LNR-25 B liquid nitrogen refrigerator. Sample tubes are placed in the cylinder, and the rate of cooling is determined by varying the depth of insertion of the cylinder in the refrigerator neck.

Rates of 5–40° C./min. were obtained by a modification of the previous method, the sample tubes being placed in a large Pyrex tube (3 cm. × 15 cm.) insulated externally with cotton wool. This tube was placed in a Linde storage cylinder with perforated base, which was lowered to about 1 in. above the surface of the liquid nitrogen in a Linde LNR-25 B refrigerator. The rate of cooling was adjusted by altering the thickness of the cotton-wool insulation. For rates of 40–70° C./min. the insulated tube was dispensed with, and sample tubes were placed directly in the Linde storage cylinder which was held at varying distances above the liquid nitrogen surface in the Linde LNR-25 B refrigerator.

In the higher ranges, 100–350° C./min. was obtained by immersing the lower halves of the sample tubes in ethanol which had been cooled to temperatures between −60 and −100° C. by pouring liquid nitrogen on the surface of the ethanol contained in a Dewar bowl and stirring until the nitrogen had boiled off. For a rate of 450° C./min. the lower halves of the sample tubes were immersed directly in liquid nitrogen, and for the highest rate, 900° C./min., the sample tubes were insulated externally with a coating of kieselguhr in a glycerol–methanol mixture as adhesive before immersing the lower halves of the tubes in liquid nitrogen. The

kieselguhr coating acted by preventing the formation of an insulating layer of gaseous nitrogen, and so enabled a rapid rate of freezing to be achieved.

Temperature measurement was by means of a 'Virtis' thermistor probe (10–700–7 p) attached to a Virtis (Gardiner, New York) sample temperature recorder (10–702–1). The probe was placed in a control sample situated between the experimental samples. Using the freezing techniques described, the rate of cooling was not constant over the whole range of temperatures and for this reason the rate of cooling was measured over the linear range $-10°$ to $-25°$ C.

In all cases samples were thawed rapidly by immersing and shaking in a water-bath maintained at 37° C. unless otherwise stated.

Freeze-drying

The apparatus used was that described by Greaves & Davies (1965). This consisted of a two-stage thermoelectric refrigerator mounted on a thick brass base plate which was water-cooled on the opposite side. A recessed 'O' ring in this plate gave a vacuum seal for the 'bell jar' top of the desiccator. The desiccator chamber was connected to a 'Megavac' pump, via a vacuum valve and a phosphorus pentoxide trap. The tubes containing samples to be dried were placed in aluminium blocks on the second stage and a fixed temperature was maintained throughout the drying period. Samples were dried under a pressure reaching 0·002 mm. Hg after 48 hr. (72 hr. for those dried at $-35°$ C.) After primary drying the cooling stage was switched off and the sample allowed to warm slowly to room temperature. Air was then admitted slowly and the samples were either immediately rehydrated with 1 ml. digest broth or were submitted to a secondary drying overnight on a vacuum manifold at room temperature. After secondary drying all samples were sealed *in vacuo* (0·01 mm. Hg) and kept in the dark at room temperature until required.

RESULTS

Preliminary tests showed that the survival of corynebacteriophage H 1 suspended in digest broth dropped by four log units every 5 days when stored at 4° C. Rapid cooling (450° C./min.) by immersion in liquid nitrogen followed by a rapid thaw at 37° C. gave 66% survival, whilst cooling slowly (1° C./min.) to $-40°$ C. followed by a rapid thaw gave 26% survival. The higher survival obtained after rapid freezing was in agreement with that obtained in a study on the freeze-thaw sensitivity of the coliphage T 4 (Steele, Davies & Greaves, 1969). However, with the T 4 bacteriophage it has been found that osmotic effects mask damage at slower cooling rates (Leibo & Mazur, 1967). It has also been suggested that osmotic damage was one of the main factors in preventing the successful freeze-drying of the coliphage T 4 (Davies, 1967). It was therefore necessary to investigate the sensitivity of the corynebacteriophage to osmotic damage before attempting to interpret any freeze-thaw or freeze-drying results.

Osmotic sensitivity

Samples of 0·1 ml. of bacteriophage suspension in broth were added to 10 ml. volumes of saline solutions of increasing strength, mixed and allowed to stand at

room temperature for 15 min. Samples were then taken for assay, after suitable dilution.

The results (Table 1) show that the corynebacteriophage H 1 is resistant to osmotic shock. As osmotic shock is considered to be involved in rehydration

Table 1. *Survival of the corynebacteriophage after exposure to varying concentrations of saline*

Concentration of NaCl	Percentage survival
0 (distilled water)	71
0·175 M	93
0·25 M	102
0·5 M	101
1 M	92
2 M	99
3 M	99
Saturated	98

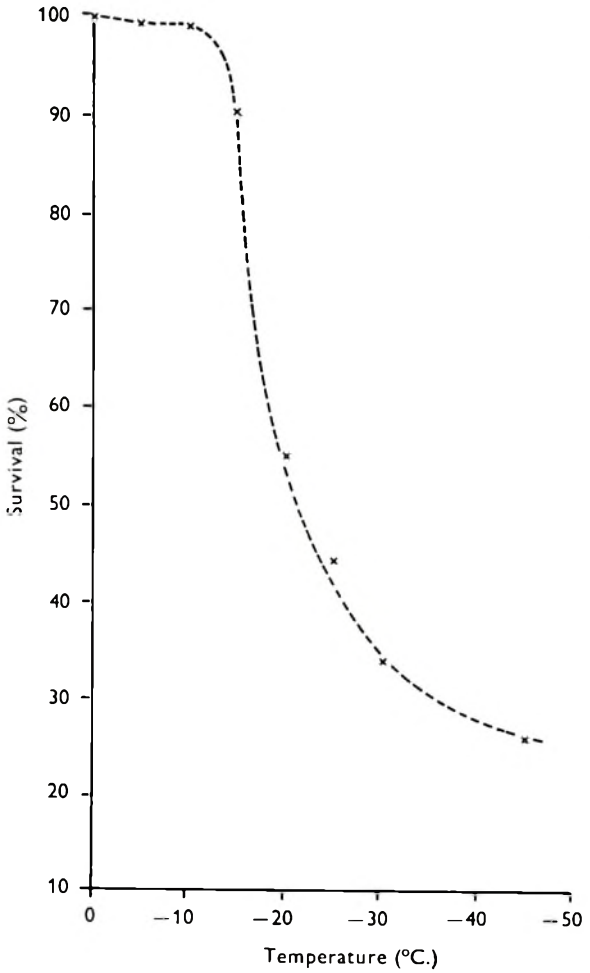

Fig. 1. The effects of cooling broth suspensions of corynebacteriophage at 1° C./min. to different subzero temperatures.

damage it seemed reasonable that if freezing damage could be eliminated it might be possible to freeze-dry the corynebacteriophage and still obtain a high survival.

Freezing

A preliminary experiment had shown that a slow rate of cooling was more damaging to the corynebacteriophage than rapid cooling. It was therefore of interest to determine whether there was in fact a critical temperature at which

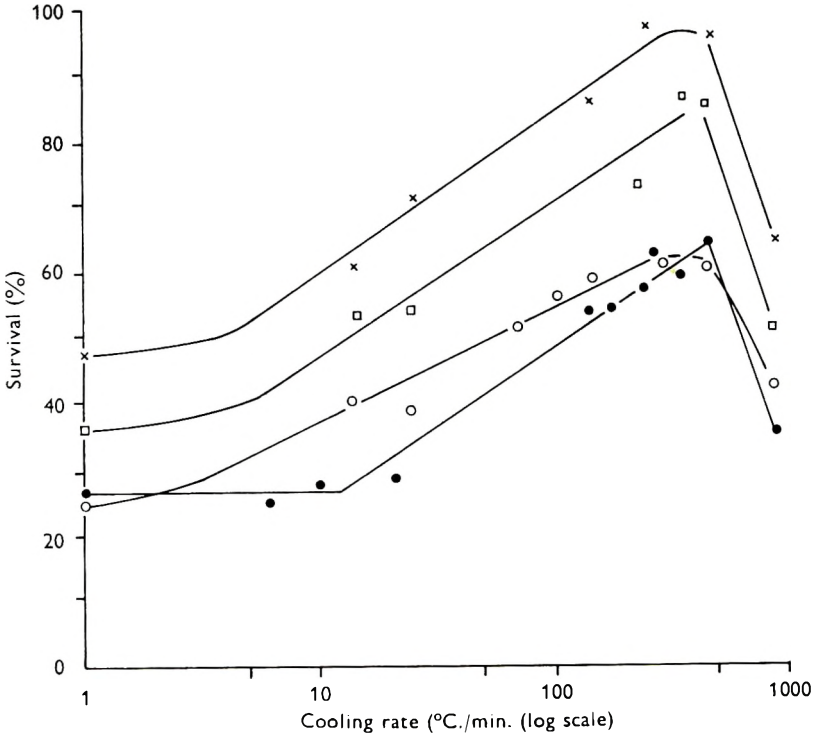

Fig. 2. The effects of different cooling rates on suspensions of corynebacteriophage in various additives. Suspending media: ×, 20 % peptone; □, 10 % peptone; ○, 10 % sucrose; ●, broth lysate.

damage started. Samples were cooled at 1° C./min. and ice formation induced at −5° C. The temperature was held at −5° C. for 10 min. to allow equilibration before continuing to cool at 1° C./min. Samples were removed at temperatures between −10° and −45° C., thawed rapidly, and viability assessed. The results (Fig. 1) showed that the damage began to occur at −14° C. and this increased with further lowering of the temperature. It is probable that such damage is caused by an increasing concentration of salt in the suspending medium, the effects being somewhat greater during cooling (Davies, 1969) and it is therefore likely that the physical or chemical consequences of such an increase in ion concentration at each temperature are time-dependent and should therefore be reduced by increasing the rate of cooling (Fig. 2). An increase in survival was found to occur with increasing rates of cooling; the maximum survival being obtained at

450° C./min. At 900° C./min., however, there was a drop in the survival, and it is thought that this can be explained by the formation and presence of ice crystals within the organism or by the removal of structural water from the protein or nucleic acid components at the high rate of cooling used. Adding various protective agents to the suspension increased the survival and this was particularly noticeable when the concentration of peptone was increased until at 20 % it was possible to achieve 95 % survival when cooling at 450° C./min. It was subsequently found that the addition of 10 % sucrose and 2 % sodium glutamate to 20 % peptone did not affect the survival of corynebacteriophage obtained with peptone alone when cooled at 450° C./min. and further studies were made with mixtures of these three additives.

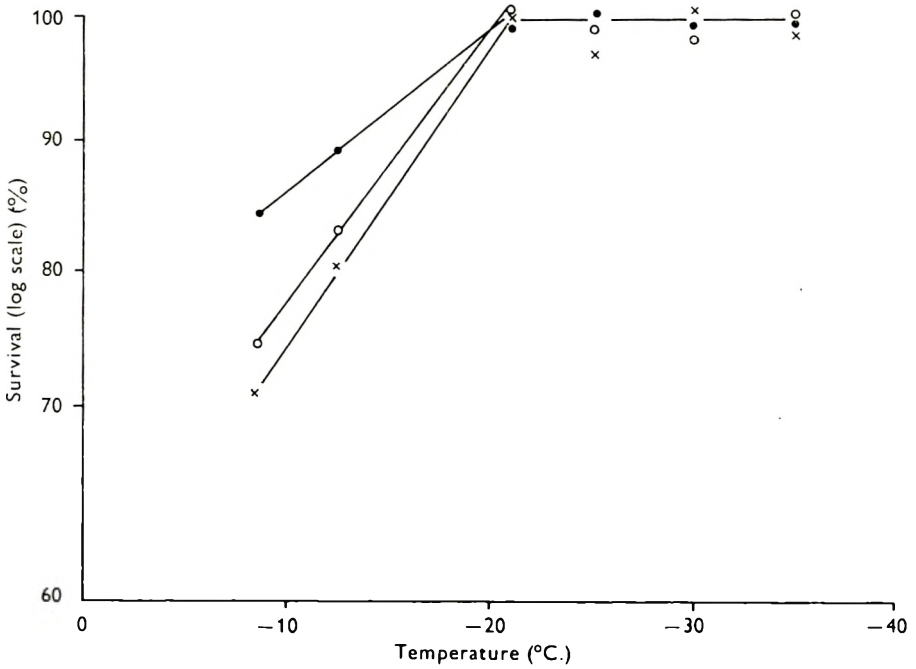

Fig. 3. The effects of cooling at 450° C./min. and subsequent equilibration at different subzero temperatures over a period of 15 min. followed by a rapid thaw. Suspending media: ×, 20 % peptone; ○, 20 % peptone + 10 % sucrose; ●, 20 % peptone + 10 % sucrose + 2 % sodium glutamate.

Freeze-drying

After cooling at 450° C./min. to −196° C. samples suspended in 20 % peptone, 20 % peptone + 10 % sucrose, and 20 % peptone + 10 % sucrose + 2 % sodium glutamate, were transferred to the drying block which was maintained at a fixed temperature. The samples were allowed to equilibrate for 15 min. On studying the effects of different temperatures it was found that provided the temperature was below −21° C. there was no damage during the warming to a suitable drying temperature (Fig. 3). The damage which occurred at temperatures above −21° C. may have been caused by the growth of ice crystals or alternatively by a high concentration of a solute at a low temperature.

After drying at the various temperatures it was found that in the suspending medium 20 % peptone + 10 % sucrose + 2 % glutamate survival was relatively constant (Fig. 4). On the other hand, if the glutamate was absent the survival in 10 % peptone or 10 % peptone + 10 % sucrose increased logarithmically with decreasing temperatures, both media giving over 97 % survival after drying at − 35° C.

Storage

The survival immediately after drying is not in itself a sufficient guide to the survival likely to be achieved after long-term storage. The results of storing samples, which had been freeze-dried at − 25° C., in the dark at room temperature are shown in Table 2. Survival after 3 months storage did not alter significantly in

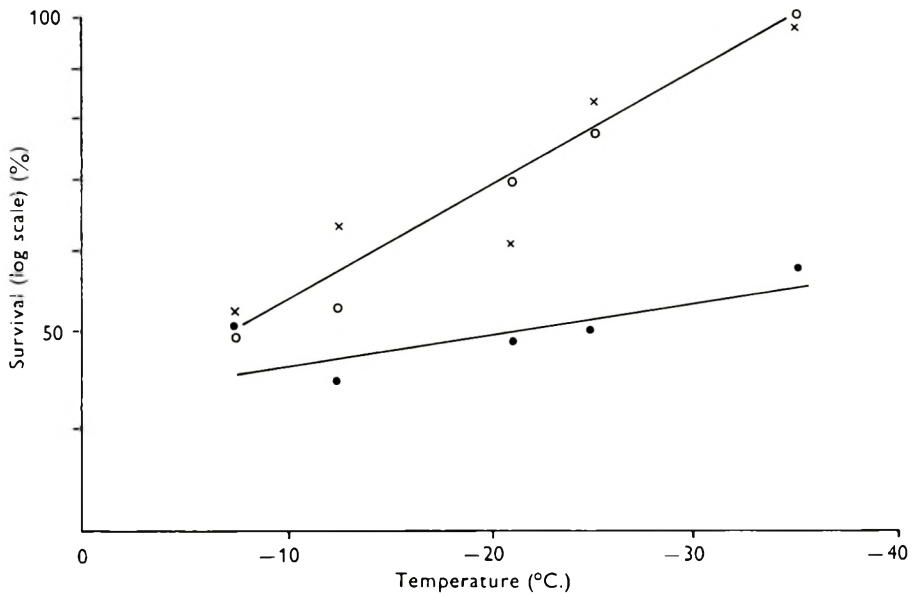

Fig. 4. The survival of corynebacteriophage after cooling at 450° C./min., equilibration at a particular temperature for 15 min. followed by drying from this temperature for 48 hr. (72 hr. for the samples dried at − 35° C.) with a final pressure of 0·002 mm. Hg. Samples were allowed to warm to room temperature and were rapidly rehydrated with digest broth. Suspending medium: ×, 20 % peptone; ○, 20 % peptone + 10 % sucrose; ●, 20 % peptone + 10 % sucrose + 2 % sodium glutamate.

Table 2. *Percentage survival of the corynebacteriophage after freeze-drying at − 25° C. and after storage of the dried product at room temperature in the dark*

	Suspending fluid used for freeze-drying		
Time of storage (days)	20 % peptone	20 % peptone, 10 % sucrose	20 % peptone, 10 % sucrose, 2 % sodium glutamate
0	84	80	53
40	82	85	55
92	75	82	54

those samples dried in 20% peptone + 10% sucrose or 20% peptone + 10% sucrose + 2% sodium glutamate. There was, however, some evidence of a drop in survival in samples dried in 20% peptone alone.

In order to investigate the significance of this drop in survival, samples which had been dried at −35° C. and sealed *in vacuo* were subjected to heat-stability tests. Samples were tied in weighted muslin bags and immersed in water maintained at different temperatures for periods of 1 hr. (Fig. 5). In 20% peptone

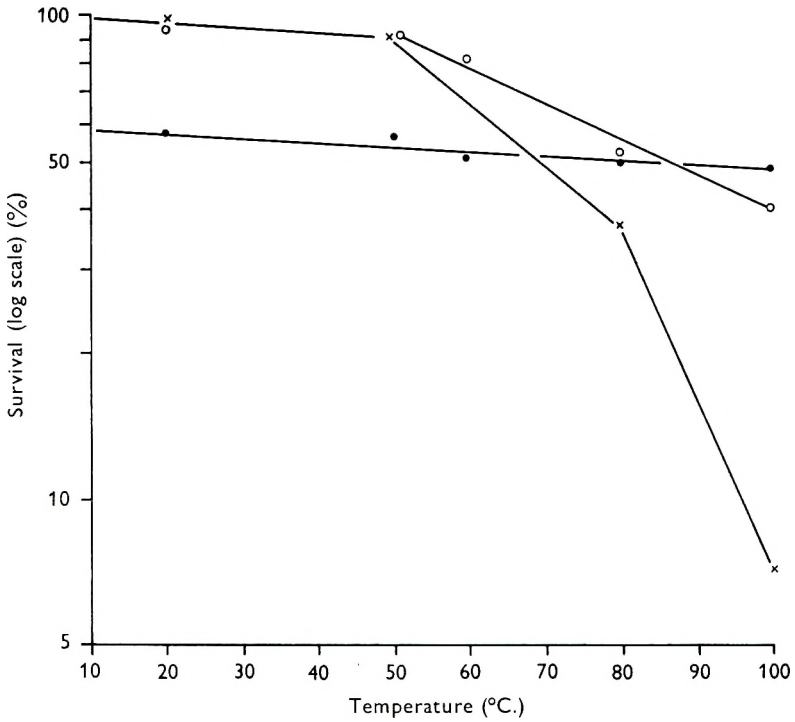

Fig. 5. The effects of heating for 1 hr. at different temperatures on the survival of suspensions of corynebacteriophage freeze-dried at −35° C. and sealed *in vacuo* (0·01 mm. Hg). Suspending media: ×, 20% peptone; ○, 20% peptone + 10% sucrose; ●, 20% peptone + 10% sucrose + 2% sodium glutamate.

survival remained constant at temperatures up to 60° C. Above this temperature survival began to drop, the rate of decline being even more evident at temperatures above 80° C. The curve appears to consist of two components, suggesting that two types of mechanism are involved in the drop in survival at high temperatures. With the addition of 10% sucrose there appears to be only one cause of damage, which again occurs at temperatures above 50° C. The addition of 2% glutamate to the mixture, however, whilst decreasing the immediate survival after drying, seems to stabilize the dried organisms against further damage brought about by increasing temperatures.

Maintaining the samples at 100° C. for different periods of time gave similar results to those described above (Fig. 6). Suspensions in 20% peptone showed a

steep drop in survival over the first 30 min., with a more gradual logarithmic decline during the following 4–5 hr. The initial drop was reduced by the addition of 10 % sucrose but the slope of the second part of the curve remained unaltered. The addition of 2 % sodium glutamate to the mixture stabilized survival throughout the heat treatment and seemed in particular to reduce the second type of damage.

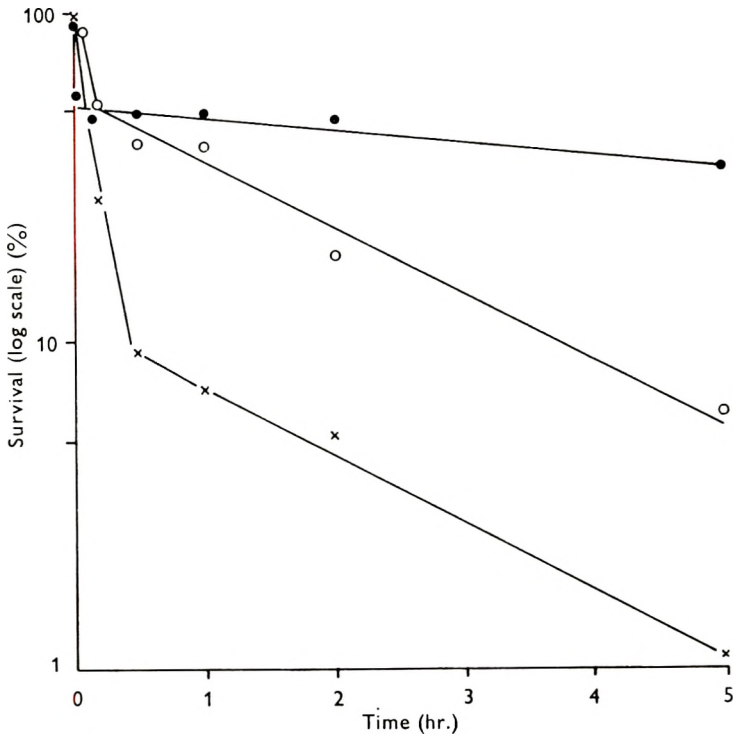

Fig. 6. The effects of storage at 100° C. for different periods of time on the survival of corynebacteriophage suspension freeze-dried at −35° C. and sealed *in vacuo*. Suspending media: ×, 20 % peptone; ○, 20 % peptone + 10 % sucrose; ●, 20 % peptone + 10 % sucrose + 2 % sodium glutamate.

DISCUSSION

It has proved possible to eliminate freezing damage in the corynebacteriophage H 1 by selecting a suitable concentration of protective agent and adjusting the cooling rate so as to prevent damage from increasing salt concentration whilst also preventing damage caused by the formation of minute crystals at the high rate of cooling. The concentration of peptone required to give maximum survival suggests that the protective action was the result of the formation of glass-like structure (Davies, 1966) though recent work has shown that the peptides present in the solution may also exert a specific effect on the protein membrane (Davies, 1969).

The addition of sucrose to the freeze-drying medium followed the suggestion by Fry & Greaves (1951) that sugars help to bind water and prevent over-drying.

This suggestion has received further support in the results of the heat-stability testing where peptone alone gives a rapid drop in survival. Sucrose also has the advantage that it does not interfere in the protection afforded by sodium glutamate (Muggleton, 1958). It is not yet known why the addition of sodium glutamate should cause a drop in the survival immediately after drying, though the fact that it stabilizes the organisms against the secondary damage during heat treatment suggests that this damage is caused by carbonyl-amino group interaction.

Although it is impossible to predict long-term storage without detailed accelerated storage tests, it is reasonable to assume from our heat-stability tests that, for storage at room temperature (20° C.) for reasonable lengths of time, 20 % peptone + 10 % sucrose would give satisfactory results. If, however, higher temperatures were likely to be encountered 20 % peptone + 10 % sucrose + 2 % glutamate mixture would give the optimal survival over long-term storage though it must be emphasized that this would be at the expense of an immediate high survival. The final choice of media thus depends upon the conditions to be encountered in the dried state.

The high survival obtained after drying at low temperatures has confirmed that drying in the absence of glutamate should take place from as low a temperature as practical (Greaves & Davies, 1965). Research is now in progress to determine whether drying at lower temperatures determines the removal of water from ice crystals before the removal of water bound by the suspending medium. This in turn may influence the stability of the structural water within the macromolecules of the organism.

The successful preservation of the corynebacteriophage H 1 by freeze-drying has served to emphasize that by adjusting such factors as suspending medium, cooling rate and drying temperature to suit different species of organisms it is now possible to achieve maximum survival over prolonged periods in the dried state.

SUMMARY

This paper describes an investigation into the successful preservation at room temperature of the bacteriophage H 1 of *Corynebacterium ulcerans* U 103 which was extremely labile when in suspension at 4° C.

Cooling at a rate of 1° C./min. showed that the survival decreased logarithmically at temperatures between − 14 and − 45° C. Survival of broth suspensions of the corynebacteriophage were found to increase proportionally with an increase in the rate of cooling though there was a marked drop in survival at rates of approximately 900° C./min. The addition of peptone solutions was found to increase the survival over the range studied, whereas the addition of sucrose solutions had only a slight effect.

By avoiding freezing damage by cooling at rates of 450° C./min. in (*a*) 20 % peptone solution, (*b*) 20% peptone and 10% sucrose, and (*c*) 20% peptone, 10 % sucrose and 2 % sodium glutamate, a study was made of the drying stage of the freeze-drying process. On drying at controlled temperatures it was found that there was no damage on rewarming to temperatures below − 21° C.

after cooling to $-196°$ C., but that the survival immediately after drying in the absence of glutamate, showed a logarithmic relationship with the temperature of drying, lower temperatures giving better survival.

On storage for a period of 3 months at room temperature *in vacuo* and darkness, there was no appreciable loss in survival in the mixtures though suspensions in peptone alone showed a slight decrease. At higher temperatures this decrease in survival could be differentiated into two types of damage, each of which could be influenced by the presence of sucrose or glutamate.

We are grateful to Professor H. R. Carne for providing the initial sample of bacteriophage H 1 of *Corynebacterium ulcerans* U 103, and to Professor R. I. N. Greaves for his interest and encouragement during the course of this investigation.

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The antigens of *Mycoplasma hominis*

By M. R. HOLLINGDALE and RUTH M. LEMCKE
*Department of Microbiology, Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W.*1

(*Received* 3 *April* 1969)

Mycoplasma hominis is the only species of human origin, other than *M. pneumoniae*, to be implicated in human disease. Despite this, little is known of its antigenic structure. Indeed, only a few species of *Mycoplasma* have been examined in this respect, although it is already clear that the chemical structure of their antigens is far from uniform. A polysaccharide hapten is present in *M. mycoides* var. *mycoides* (Plackett & Buttery, 1958; Buttery & Plackett, 1960; Plackett, Buttery & Cottew, 1963), whereas a hapten of *M. pneumoniae* contains several active glycolipids (Plackett, Marmion, Shaw & Lemcke, 1969) and *M. laidlawii*, type B contains a glucolipid hapten (Plackett & Shaw, 1967). *M. pulmonis*, on the other hand, apparently has no lipid hapten but contains protein and polysaccharide antigens (Deeb & Kenny, 1967).

M. hominis is a potential pathogen causing inflammatory pelvic disease in women (Marmion, 1967) and generalized blood-stream infection following childbirth (Stokes, 1955; Tully & Smith, 1968), abortion (Tully *et al.* 1965; Harwick, Iuppa, Purcell & Fekety, 1967) or hysterectomy (Stokes, 1959). We have fractionated the antigens of *M. hominis* and determined their behaviour in a variety of serological tests.

MATERIALS AND METHODS

Strains

Strain SC4 was isolated from the urethra of a male patient. It was isolated on rabbit serum agar, so that antisera could be prepared after only a few subcultures. The strain was cloned twice from single colonies. Stocks of the fourth subculture were frozen at $-30°$ C., and lyophilized ampoules prepared from the sixth, so that cultures which had undergone fewer than ten subcultures could be used for fractionation. SC4 was identified as *M. hominis* by growth-inhibition tests on agar (Huijsmans-Evers & Ruys, 1956; Clyde, 1964) with antisera against authentic strains of *M. hominis*, *M. pneumoniae*, *M. orale*, type I, *M. fermentans* and *M. salivarium*.

Media and cultural conditions

Liquid medium consisted of Difco PPLO broth (without crystal violet) supplemented with Oxoid yeast extract (0·5 %, w/v), sodium deoxyribonucleate (0·002 %, w/v), penicillin (50 units/ml.) and human plasma (20 %, v/v). The plasma was citrated plasma from out-dated transfusion blood, treated by a modification of the method of Maizels (1944) to remove fibrin. Plasma was mixed with sterile kaolin

(160 g./l.), shaken thoroughly, allowed to stand for 24 hr. at 4° C. and shaken again. The kaolin was allowed to settle out at 4° C. for 5–6 days, and the plasma siphoned off, clarified and sterilized by Seitz filtration, and stored at 4° C. It was sometimes necessary to refilter the treated plasma before use owing to the precipitation of residual fibrin, but this did not affect the growth of the mycoplasma. Unlike media containing horse serum, this medium contained very little extraneous material that sedimented on centrifugation of mycoplasma cultures; in fact not more than 5 % of the dry weight of the cells.

For fractionation, *M. hominis* SC4 was grown for 18 hr. at 37° C. without shaking. For seeding, 1 vol. of a similar 18 hr. culture was added to each 1000 vol. of medium. Determinations of the optical density at 580 mμ and of the colony-forming units per ml. from counts on agar, showed that the logarithmic phase of growth ended at 16–18 hr. Cells were harvested at 15,000 *g* for 30 min. in an MSE 1800 centrifuge (or, in the case of a batch prepared at Porton, in a Sharples centrifuge) and washed in an appropriate buffer, first in 20 % of the original volume of culture and then in 10 %. At first, a 0·02 M phosphate buffer containing 0·01 M Mg^{2+} ion at pH 7·1 (Marmion, Plackett & Lemcke, 1967) was used to prevent loss of cell contents by spontaneous lysis. Later, however, it was found that the release of protein from the cells by alternate freezing and thawing was increased when no Mg^{2+} ion was present. Thereafter, for all disruption procedures, cells were washed in 0·15 M sodium chloride, or in sodium chloride/tris buffer (0·15 M sodium chloride, 0·05 M tris) at pH 7·4. Centrifuged deposits not used immediately were stored at −30° C.

When material for the inoculation of rabbits was required, unheated rabbit serum (10–15 %, v/v) was substituted for human plasma in the medium. For growth-inhibition tests, Difco PPLO agar replaced the broth, and unheated horse serum (Burroughs Wellcome no. 3) the human serum or plasma, because with this medium the zones of inhibition were more distinct.

Disruption and fractionation of Mycoplasma hominis *by physical methods*

Osmotic lysis

The method of Razin (1963) was used, except that the suspensions were incubated at 37° C. for 30 min. not at room temperature.

Alternate freezing and thawing

Washed cells suspended in deionized water to approximately one-tenth of the original culture volume were frozen by alcohol and dry ice at −25° C. or below and thawed at 37° C. After 10 freeze–thaw cycles, unbroken cells were removed by centrifugation at 8000 *g* for 30 min. Only a little more protein was released after ten more cycles.

Gas cavitation under nitrogen

Washed cells from 2·5 l. of culture were suspended in 30 ml. dilute sodium chloride/tris buffer (0·0075 M sodium chloride, 0·0025 M tris), equilibrated for

20 min. with stirring, under nitrogen (1600 lb./in.^{2}) in a modified bomb calorimeter, which was held in ice and water, and the pressure then released (Wallach & Ullrey, 1962). Suspensions were treated six times and centrifuged at 8000 *g* for 30 min. The resulting pellet was resuspended in the same volume of buffer and treated six times more before centrifugation at 8000 *g*. The 8000 *g* supernatants were pooled for further fractionation and the deposits of unbroken cells discarded.

Ultrasonic treatment

Washed cells from 2·5 l. of culture were suspended in 10 ml. dilute sodium chloride/tris buffer, and treated for 2 min. with a Branson S 75 Sonifier (Branson Instruments Inc., Stamford, Connecticut, U.S.A.) at 20 kc./sec. The suspension was held in ice and water throughout. After centrifugation at 8000 *g* for 30 min., the resuspended pellet was treated again, and the supernatants pooled.

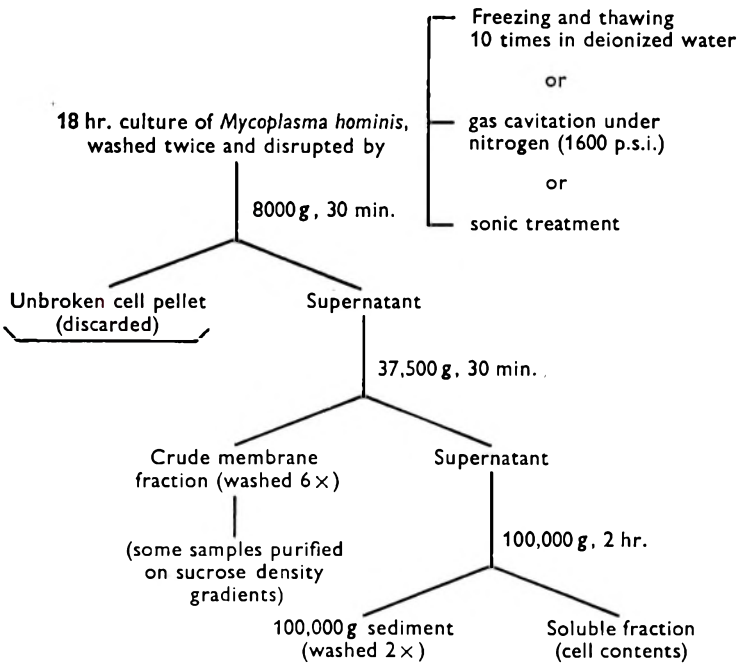

Fig. 1. Scheme for the disruption and fractionation of *M. hominis*.

Fractionation of disrupted cells

The 8000 *g* supernatants were centrifuged according to the scheme in Fig. 1. The crude membrane fraction gave a yellow-brown transparent pellet, which was washed six times in dilute sodium chloride/tris buffer. The soluble fraction (cell contents) was concentrated by pressure dialysis at 4° C. The material sedimenting at 100,000 *g* usually formed a small transparent pellet which was washed twice in buffer.

Purification of membrane fraction

Washed crude membrane fraction (0·4 ml. volumes containing 1–2 mg. of membrane protein) in dilute sodium chloride/tris buffer containing sucrose (10 %, w/w) was layered on top of a sucrose gradient prepared from buffered sucrose solutions containing 55, 50, 36 and 23 % (w/w) sucrose. Tubes were centrifuged at 20,000 rev./min. for 20 min. at 1° C. in a Spinco model L centrifuge on an SW 39 L rotor. When layered on top of a similar gradient, whole cell suspensions at the equivalent protein concentration passed to the bottom of the tube. Membrane preparations gave a discrete band approximately half-way up the tube and sometimes a small amount of sediment, presumably of unbroken cells, at the bottom. Fractions were collected by upward displacement on an ISCO Model 180 Density Gradient Fractionator (Instrumentation Specialities Co. Inc., Lincoln, Nebraska, U.S.A.) with a buffered sucrose solution (60 %, w/w) containing potassium hydrogen phthalate (0·15 %, w/v). The optical density at 245 mμ of the displaced material was plotted continuously on an ISCO Model UA 2 Analyser. The fractions containing the purified membrane were pooled, dialysed against buffer overnight at 4° C., and then centrifuged at 37,500 *g* for 40 min.

Lysis of membranes by detergents

Successive 0·1 ml. volumes of sodium lauryl sulphate, sodium deoxycholate, or Triton X-100 were added to 3 ml. amounts of a suspension of membranes at 0·5 mg. protein per ml. in dilute sodium chloride/tris buffer. The final concentration was 9 mg. detergent per mg. membrane protein. After each addition of detergent, the mixtures were incubated at 37° C. for 15 min. and the optical densities at 500 mμ determined in a Unicam SP 500 spectrophotometer. The readings were corrected for dilution by the detergents.

Protein estimations

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

Since SC4 was isolated on rabbit serum agar, it was possible to raise antisera against whole cells from cultures which had been subcultured less than seven times and had never been in contact with the serum of other species. For subcutaneous (s.c.) inoculation, washed cells from 200 ml. of 2- to 3-day cultures were suspended in 2 ml. saline (0·15 M) and emulsified in 3 ml. of Drakeol–Arlacel adjuvant (Lemcke, 1965). Rabbits received two such doses at an interval of 13 days. Thirteen days after the second s.c. inoculation, 13 ml. of a washed saline suspension from 400 ml. of culture was given in six intravenous (i.v.) doses, (1, 1, 2, 2, 3 and 4 ml.), on alternate days. Rabbits were bled 5–7 days after the last inoculation. Sera were stored at −30° C. without preservative.

For antiserum to purified membranes, a suspension containing 3·5 mg. protein

was inoculated i.v. in six doses on alternate days. At first, an attempt was made to prepare antiserum to soluble fraction produced by gas cavitation, but the resulting antiserum reacted strongly in gel-diffusion with detergent-lysed membranes. Accordingly, freeze-thaw soluble fraction was used. The response to i.v. inoculation of this fraction was negligible even after the administration of 10 mg. protein, but good antiserum resulted when 5 mg. protein in Drakeol–Arlacel adjuvant was given in two s.c. inoculations separated by an interval of 13 days.

Serological tests

Complement fixation

The quantitative method of Fulton & Dumbell (1949) was adapted to the Microtiter System (Cooke Engineering Co., Arlington, Va.). In titrations with antiserum to whole cells, the amount of complement fixed by the fractions or extracts at a concentration of 0·5 mg. protein per ml. was compared with that fixed by whole cells at the same concentration. With lipid extracts and the aqueous phase of the phenol extract, comparisons were made with the antigens at 1·0 mg. dry weight per ml. Pre-immunization serum was included in all titrations. The logarithms of the units of complement fixed by each antigen were plotted against dilutions of antiserum, and the areas under the curve calculated. The 'area' of fixation by whole cells at 0·5 mg. protein per ml. or 1·0 mg. dry weight per ml. was recorded as 1·0 and the amount of complement fixed by fractions or extracts then expressed in terms of this unit fixation.

For comparing the complement-fixing activity of antisera against the purified membrane and soluble fractions, graded dilutions of a whole-cell antigen were titrated against graded dilutions of each antiserum. Since there was an antigen concentration at which all the antisera had a maximum serum titre, the titres recorded were those obtained at this optimal antigen concentration.

All sera were absorbed with packed sheep red blood cells to remove Forssman type antibody and, when anticomplementary, held with 1/10 guinea-pig serum overnight at 4° C. before inactivation at 56° C. for 30 min. Complement was obtained as guinea-pig serum, separated, pooled and stored in small quantities at −30° C.

Growth inhibition on agar

Tests were made by the method of Clyde (1964), using preimmunization sera as controls.

Metabolic inhibition

The test designed by Purcell, Taylor-Robinson, Wong & Chanock (1966) for the inhibition of arginine metabolism by antibody was used, except that the medium contained human instead of horse serum. Preimmunization sera were included in all tests. For absorption of metabolism-inhibiting (MI) antibody, 0·1 ml. volumes of whole-cell antiserum were absorbed for 48 hr. at 4° C. by whole-cell suspensions, fractions or extracts containing 1 mg. protein, and precipitates were removed by centrifugation. With the chloroform–methanol extract, whole-

cell antiserum (0·1 ml.) was absorbed by a dispersion containing 1 mg. dry weight of lipid and compared with antiserum similarly absorbed with 1 mg. dry weight of whole cells. Control antiserum was held in the same way with buffer or suspending fluid.

Indirect haemagglutination

A suspension of washed sheep erythrocytes (4 %, v/v) was treated with an equal volume of tannic acid (Mallinckrodt, 1 in 20,000, w/v) in phosphate-buffered saline, pH 7·2 (PBS), for 30 min. at room temperature. After three washes, the tanned cell suspensions (2 %, v/v) were treated for 45 min. at room temperature with an equal volume of a sonicated suspension of SC4 in buffered saline. The sensitized erythrocytes were washed once in PBS and twice in PBS containing 1 % (v/v) normal rabbit serum (NRS diluent). For test, 0·1 ml. amounts of erythrocytes (1 %, v/v) in NRS diluent were mixed with an equal volume of antiserum in the same diluent. Preimmunization sera were also included in the test. The results were read after 3 and 18 hr. at room temperature. Since maximum titres were obtained when erythrocytes were sensitized with sonicated suspensions of SC4 at 0·125–0·063 mg. protein per ml., fractions were compared at the same concentrations. Membrane fractions were sonicated briefly to obtain a fine dispersion. All sera, including the normal rabbit serum used in the NRS diluent, were first absorbed with packed sheep erythrocytes. Absorption of indirect haemagglutinating (IHA) antibody by whole cells and fractions was carried out as described for the absorption of MI antibody.

Gel diffusion

The method described by Lemcke (1964) was used. Whole-cell lysates and fractions were used at 2 mg. protein per ml.

Immunoelectrophoresis

Slide cover glasses (8·2 cm × 8·2 cm) were covered with 7 ml. of Difco Noble agar (1 %, w/v) in barbitone buffer, pH 8·6 (Oxoid), at an ionic strength of 0·025 and containing sodium azide (0·1 %, w/v). Antigen wells were 2 mm. in diameter and electrophoresis was at 4° C. for 90 min. at 150 mV. in barbitone buffer, ionic strength 0·05. Antiserum was in troughs 1 mm. in width and precipitin arcs were allowed to develop at 4° C.

Chemical extractions

Chloroform–methanol

Washed whole cells were extracted as described for *M. pneumoniae* by Marmion *et al.* (1967). After evaporation of the solution to dryness at 35° C. *in vacuo*, the lipid was taken up in a little chloroform and stored at −10° C. For serological tests, the chloroform was evaporated by a stream of nitrogen and the lipid dispersed in veronal-buffered saline, pH 7·2 (CFT diluent, Oxoid), by shaking with glass beads or by sonic treatment for 2 min. at 4° C. The dry weight of the lipid in the dispersion was determined and serological activity was recorded in terms of dry weight.

Aqueous acetone

Washed whole cells were extracted with aqueous acetone (80 %, v/v) on a magnetic stirrer for 10 min. at room temperature. The insoluble residues, deposited at 8000 *g*, were re-extracted in the same way. The supernatants were pooled and filtered through glass wool. Acetone was removed by evaporation *in vacuo* and finally with a stream of nitrogen. The lipid was dispersed in the remaining water by sonic treatment for 1 min. at 4° C. and the dry weight determined. The residue remaining after lipid extraction was washed, resuspended in CFT diluent and the complement-fixing activity determined at 1 mg. dry weight per ml.

Phenol

Two methods were used: (i) Cells previously extracted with chloroform–methanol were washed, suspended in water and extracted with warm aqueous phenol as described by Marmion *et al.* (1967). After removal of the phenol by dialysis, the aqueous phase was lyophilized, and dissolved in CFT diluent for serological tests. (ii) Cells suspended in water were shaken vigorously with an equal volume of aqueous phenol (90 %, w/w) for 1 hr. at 4° C. (Gierer & Schramm, 1956; Westphal, Luderitz & Bister, 1952). After separation, the aqueous and phenolic layers were dialysed against water to remove the phenol. The clear aqueous phase was lyophilized. The insoluble material which separated from the phenolic phase as the phenol was removed was also lyophilized; it was partly soluble in sodium lauryl sulphate, sodium deoxycholate and dilute sodium hydroxide, and all three solutions gave identical precipitin lines in gel-diffusion. Accordingly, the rest of the material was extracted at 37° C. for 30 min. with 0·06 N sodium hydroxide, clarified by centrifugation for 10 min. at 3000 *g* and dialysed against 0·001 M tris-HCl buffer, pH 7·4 at 4° C. for 24 hr.

Potassium hydroxide

The method of Warnaar *et al.* (1965) for extracting an antigen from vaccinia virus was used on a suspension of *M. hominis* containing 2 mg. protein per ml.

RESULTS

Disruption of cells

The cell materials absorbing at 260 mμ, released by osmotic lysis, increased as the salt concentration decreased from 0·256 M to 0·022 or 0·014 M and thereafter decreased as the concentration decreased to 0·006 M (Fig. 2). In this respect, *M. hominis* behaved differently from several species examined by Razin (1963), who found that maximum lysis of *M. laidlawii* and *M. bovigenitalium* occurred at the lowest salt concentration tested (0·006 M) and of *M. mycoides* var. *capri*, at the highest concentration (0·256 M). These differences may reflect some fundamental species differences in the composition or structure of the membranes. Nevertheless, even at the tonicities which allowed the maximum lysis of *M. hominis*, the amount of materials absorbing at 260 mμ was only twice that released in the 0·256 M suspensions. Similarly, the optical density at 500 mμ was reduced by less

than one-third. Osmotic lysis was, therefore, unlikely to give satisfactory yields of fractions. Of the three other methods, sonic treatment released most protein (67–88 %) from the cells, compared with 45–65 % by gas cavitation and 31–52 % by freezing and thawing.

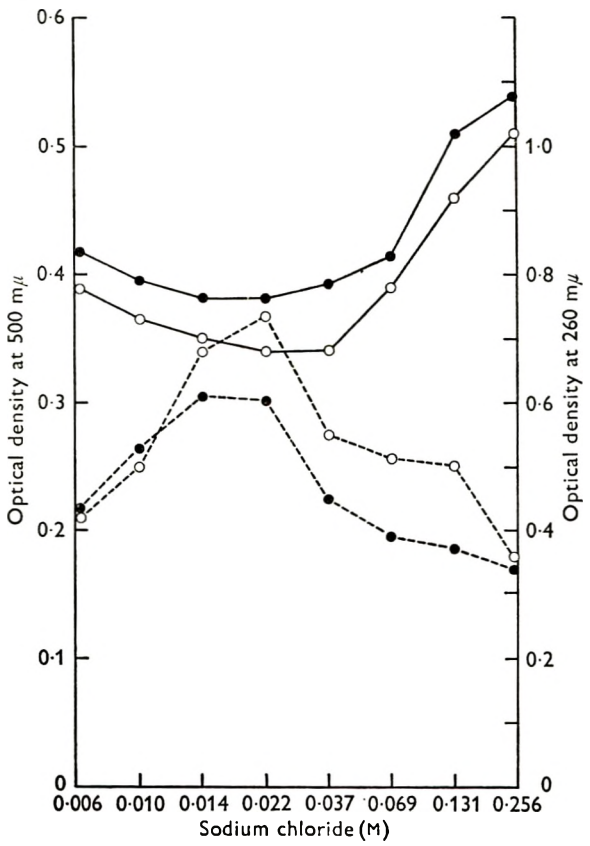

Fig. 2. Lysis of an 18 hr. culture of *M. hominis* SC4 in salt solutions of decreasing tonicity. —, Optical density at 500 mμ; ---, optical density at 260 mμ. Expt. 1. (●); Expt. 2. (○). The concentrations of sodium chloride include the amount of salt added with the suspension.

Fractions obtained by physical disruption

Serology

The complement-fixing activities of fractions from typical experiments with SC4 disrupted by freezing and thawing, gas cavitation and sonic treatment are shown in Table 1. All the fractions fixed complement with antiserum to whole cells, and the corresponding fractions produced by the three methods differed little.

The results of metabolic inhibition, using antisera absorbed with whole cells and fractions from five different experiments, are summarized in Table 2. Crude and purified membrane fractions absorbed MI antibody even more strongly than whole cells at the same protein concentration. Soluble fractions absorbed at least 32 times less effectively than membranes, but the 100,000 *g* sediments were nearly as active as the membranes.

Both crude and purified membranes were highly active in sensitizing erythrocytes to agglutination by antisera against whole cells or purified membranes (Table 3). The membranes were nearly as active as sonicated suspensions of whole cells at the same protein concentration, whereas the soluble fractions were much less so.

Table 1. *Complement fixation, with antiserum to whole cells, of fractions of* Mycoplasma hominis *SC*4 *containing* 0·5 *mg. protein per ml., expressed as fractions of the amount of complement fixed by whole cells*

Method of cell disruption	Whole cells	Crude membrane	Soluble fraction	100,000*g* sediment
Freezing and thawing	1·00	1·05	0·92	NT
Gas cavitation	1·00	0·86	1·00	NT
Sonic treatment	1·00	0·85	1·00	0·78

Table 2. *Absorption of metabolism-inhibiting (MI) antibody from antiserum to whole cells (titre* 1280–2560) *by fractions of* Mycoplasma hominis *SC*4

	Factor by which titre decreased after absorption by				
		Membrane			
Method of cell disruption	Whole cells	Crude	Purified	Soluble fraction	100,000*g* sediment
Gas cavitation (Expt. 9)	128	256	NT	4	32
Gas cavitation (Expt. 11)	128	> 256	NT	4	NT*
Sonic treatment (Expt. 1)	128	> 256	NT	8	128
Sonic treatment (Expt. 2)	128	> 128	NT	2	> 256
Sonic treatment (Expt. 12)	128	128	128	2	NT

Titre = highest dilution of the serum inhibiting a colour change of 0·5 pH units.
NT = not tested.
* Insufficient to test.

Table 3. *Agglutination, by antisera to whole cells and to purified membrane, of tanned erythrocytes sensitized with sonicated cells or fractions of* Mycoplasma hominis *SC*4 *at* 0·125 *mg. protein per ml.*

	Titres	
Erythrocytes sensitized with	Antiserum to whole cells	Antiserum to purified membrane
Sonicated cells	5120	2560–5120
Crude membrane	5120	2560
Purified membrane	1280–2560	640–1280
Soluble fraction (gas cavitation)	20–40	10–20
Soluble fraction (freeze–thaw)	160	NT

NT = not tested.

Both crude and purified membranes strongly absorbed IHA antibody from antiserum to whole cells (Table 4). A soluble fraction produced by freezing and thawing absorbed very little, but one produced by gas cavitation decreased the titre 128-fold, probably because of the presence of membrane components, since a similar fraction produced by gas cavitation stimulated the formation of antibody to membrane antigens (see above).

Table 4. *Absorption of indirect haemagglutinating antibody by whole cells or fractions of* Mycoplasma hominis *SC*4

(Sera titrated against tanned erythrocytes sensitized with a sonicated suspension of SC4 at 0·125 mg. protein per ml.)

Antiserum to whole cells absorbed with	Titre of haemagglutinating antibody
Nil (control)	2560
Whole cells	< 10
Crude membrane	10
Purified membrane	20
Soluble fraction (freeze–thaw)	1280
Soluble fraction (gas cavitation)	10–20

With antiserum to whole cells, soluble fractions, whether produced by freezing and thawing, gas cavitation or sonic treatment, gave a characteristic pattern in gel-diffusion or immunoelectrophoresis (Pl. 1, fig. 1*a*, *b* and Pl. 2, fig. 1*a*, *b*). In the gel-diffusion results, the precipitin lines were numbered 1–6, starting at the antiserum well (Pl. 1, fig. 1*b*). The lines 4 and 5 were heaviest and sometimes appeared as a single broad band; line 6 was heavy, and sometimes double. In contrast, membrane fractions were inactive (Pl. 2, fig. 1*a*) unless first lysed with detergents such as sodium lauryl sulphate, sodium deoxycholate, Triton X-100 or Brij 58, or with dilute sodium hydroxide (0·1–0·05 M). Fractions treated with the first three detergents gave the most precipitin lines. According to electron micrographs, Triton X-100 produced the greatest degree of membrane breakdown, although the reduction in optical density at 500 mμ was greatest with sodium deoxycholate and sodium lauryl sulphate (Fig. 3). Moreover, some batches of sodium lauryl sulphate and sodium deoxycholate gave diffuse, non-specific zones of precipitation which were not entirely absent with serum absorbed three times with detergent. Thus, Triton X-100 seems the detergent of choice for lysing membranes for gel-diffusion tests. The intensity and resolution of the precipitin lines was best when 5 mg. per mg. of membrane protein was used.

The gel-diffusion pattern of detergent-lysed membranes with whole cell antisera was quite distinct from that of soluble fractions (Pl. 1, fig. 2). The gel-diffusion pattern of the soluble fraction was not affected by the addition of sodium lauryl sulphate or Triton X-100 at the concentration used to lyse membranes and whole cells. Whole cells lysed with detergents gave lines characteristic of both fractions (Pl. 1, fig. 2). The lines given by detergent-lysed membranes developed close together and were more difficult to resolve than those given by soluble fractions,

but where the resolution was good, at least three lines were detectable (Pl. 1, figs. 2, 3). Immunoelectrophoresis of membrane lysed with the anionic detergent, sodium lauryl sulphate, gave a strong arc, which was probably complex, toward the anode (Pl. 1, fig. 5*b*). With the non-ionic detergent Triton X-100, crude membranes gave a strong arc and purified membranes a double arc near the origin (Pl. 1, fig. 5*a*). In addition, both crude and purified membrane preparations gave an arc at the cathode end (Pl. 1, fig. 5*a*). When membranes are lysed with sodium lauryl sulphate the active components migrating toward the anode are probably complexes of detergent and membrane protein which migrate according to the charge on the detergent ion. Membranes purified on sucrose density gradients gave the same gel-diffusion pattern as crude membrane fractions (Pl. 1, fig. 3). However, in immunoelectrophoresis crude, but not purified membranes, gave a faint, diffuse arc in the position of the main arc of the soluble fraction (Pl. 1, fig. 5*a*).

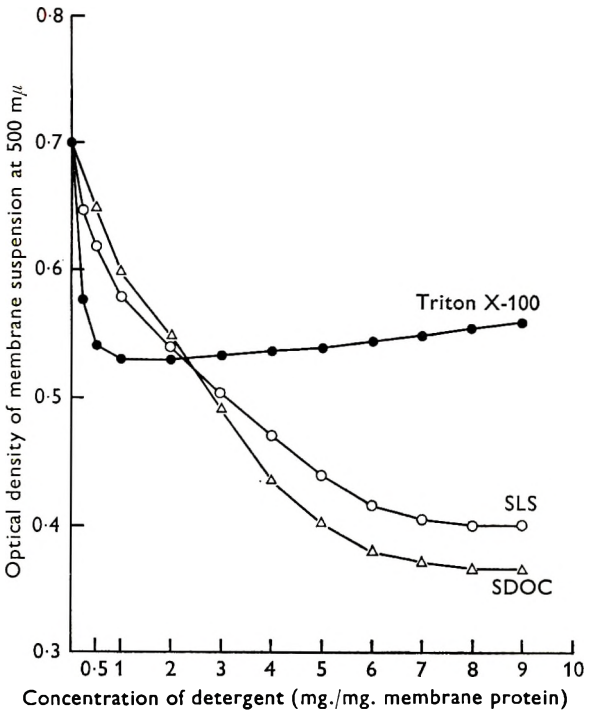

Fig. 3. Decrease in optical density at 500 mμ of *M. hominis* SC4 membrane suspensions with increasing concentrations of sodium lauryl sulphate, sodium deoxycholate and Triton X-100.

Soluble fractions from some batches of sonically treated cells proved to be contaminated by membrane components. Besides the pattern characteristic of soluble fractions, there were one or two additional bands near the antigen well, which linked up with some given by detergent-lysed membrane (Pl. 1, fig. 4). Sonic treatment evidently rendered some membrane components unsedimentable at 100,000*g*, and is therefore not suitable for preparing soluble fraction free of membrane components.

Some of the resuspended 100,000 *g* sediments gave lines characteristic of the soluble fraction, in particular, line 6. However, when lysed with detergents, the deposits invariably gave strong lines characteristic of the membrane fraction. Repeated washing of one preparation removed the line 6 component, leaving only the membrane components to be revealed by detergent lysis. The material sedimenting at 100,000 *g* therefore consists largely of membranes, and this undoubtedly accounts for its activity in absorbing MI antibody (Table 2). Moreover, since 100,000 *g* sediments contain no reactive components different from those in the membrane and soluble fractions, the 37,500 *g* centrifugation stage of the fractionation process can be omitted, sedimenting the membranes at 100,000 *g* and increasing the number of washes.

Table 5. *Serological reactivity of antisera produced by inoculation of membranes (3·5 mg. protein) or soluble fraction (5 mg. protein) of* Mycoplasma hominis *SC4 into rabbits*

(The activity of a typical antiserum to whole cells is included for comparison.)

Antiserum to	Zone of growth-inhibition (mm.)	Titre			No. of precipitin lines with	
		Metabolic inhibition	Indirect haemag-glutination	Complement-fixation*	Detergent-lysed membrane	Soluble fraction
Purified membrane	3–5	160	2560–5120	160	3	0
Soluble fraction (freeze–thaw)	0	< 10	160	1280–2560	2†	5
Whole cells	4–6	2560–5120	5120	2560	3	6

* Antigen = whole cell suspension of SC4. † Very faint.

Immunogenicity

Of the antisera against purified membranes and the soluble freeze–thaw fraction, only that against the membranes had growth- and metabolism-inhibiting activities (Table 5). In the indirect haemagglutination test, the membrane antiserum had a titre of 2560–5120, whereas that of the antiserum to the soluble fraction was only 160 (Table 5). In gel-diffusion tests, the membrane antiserum reacted only with detergent-lysed membrane and not with soluble fractions, but antiserum to the soluble fraction gave two faint lines with both crude and purified membranes (Table 5). It is highly probable, therefore, that the IHA activity of this serum was due to small amounts of antibody to membrane antigens. The absence of MI antibody and the small amounts of IHA antibody in the antiserum to the soluble fraction was not due to a general failure of the immune response in the rabbit; the complement-fixing titre of this antiserum was higher than that of the membrane antiserum (Table 5). Preimmunization sera gave no reaction in growth-inhibition and gel-diffusion, and had titres of < 10 in the other tests.

Extracts of whole cells by chemical methods

On the average, 22·2 % of the dry weight of the original cells was recovered in chloroform–methanol extracts, which contained 0·9 % protein. In the aqueous acetone extract, 16·5 % of the dry weight of the cells was recovered, and 3·2 % of this was protein. Neither extract had much complement-fixing activity compared with whole cells (Table 6), confirming the results of Kenny (1967) with chloroform–methanol extracts of *M. hominis*. Chloroform–methanol extracts, dispersed with glass beads or by sonic treatment, were equally active. The low reactivity of the chloroform–methanol extracts with homologous serum is apparently specific, in that they did not react with antisera to *Mycoplasma orale* type 1, *M. salivarium* and *M. arthritidis*. This is contrary to Kenny's observation that a chloroform–methanol extract of *M. hominis* cross-reacted with antisera to other mycoplasma species. Kenny attributed the cross-reactions to lipids adsorbed by the mycoplasmas from the calf or horse serum in the medium. The reaction of the lipid extracts with homologous antiserum is unlikely to be due to an antigen of the Forssman type (Provost, Perreau & Queval, 1964), since all antisera were absorbed by sheep erythrocytes before titration.

Table 6. *Serological activity of extracts of* Mycoplasma hominis *SC* 4 *with antiserum to whole cells of SC* 4

Suspension or extract under test	Complement-fixing activity	No. of precipitin lines (gel-diffusion)	Titre in metabolic inhibition test after absorption by suspension or extract*
Whole-cell suspension	1·00	NT	20
Chloroform–methanol	0·15	1	1280–2560
Aqueous acetone	0·12	1†	NT
Residue after aqueous acetone extraction	0·57	NT	NT
Aqueous phenol (at 68° C.)–aqueous phase	0·00	0	NT
Aqueous phenol (at 4° C.)–aqueous phase	0·00	0	NT
Aqueous phenol (at 4° C.)–alkaline extract of phenolic phase	0·68	4	160
Potassium hydroxide	0·81	4	80

* Titre of unabsorbed serum in metabolic inhibition test = 2560.
† Very faint. NT = not tested.

The chloroform–methanol extract did not absorb MI antibody (Table 6). In gel-diffusion tests with whole cell antisera, it gave a single, rather diffuse line, but this did not link up with any of the lines given by the soluble fraction or detergent-lysed membranes. The aqueous acetone extracts gave only a faint reaction in gel-diffusion.

The aqueous phases after warm or cold aqueous phenol extraction contained, respectively, 4·1 and 15·8 % of the dry weight of the cells extracted and gave a

positive Molisch reaction for carbohydrate. However, neither of the extracts was active in complement-fixation or gel-diffusion tests (Table 6).

The solid from the phenolic phase after cold aqueous phenol extraction, represented 31 % of the dry weight of the cells extracted and contained 81 % protein. An extract of the solid with dilute sodium hydroxide contained 27 % of this protein and was appreciably active in complement fixation and in absorbing MI antibody (Table 6). In gel-diffusion, the extract contained at least three components in common with detergent-lysed membranes, and one component corresponding to line 1 of the soluble fraction (Pl. 2, fig. 2*a*, *b*).

The soluble material extracted from whole cells with dilute potassium hydroxide contained, on the average, 11·6 % of the original cell protein. Like the phenolic extract, it was active in complement fixation, gel-diffusion and the absorption of MI antibody (Table 6). It contained two gel-diffusion components (Pl. 2, fig. 3*a*, *b*) in common with detergent-lysed membranes, one of which was also shared with the phenolic extract (Pl. 2, fig. 2). Components corresponding to lines 1 and 3 of the soluble fraction were also present, but it is clear that the serologically reactive components in the phenolic and potassium hydroxide extracts were derived mainly from the membrane. It is noteworthy that none of the major precipitating components of the soluble fraction, i.e. those which are associated with lines 4, 5 and 6, was present in any of the extracts.

DISCUSSION

The diversity of antigenic structure within the genus *Mycoplasma* is emphasized by our results with *M. hominis*. Whereas a major lipid hapten can be extracted from *M. pneumoniae* with chloroform–methanol or acetone (Kenny & Grayston, 1965; Marmion *et al.* 1967; Soběslavský, Prescott, James & Chanock, 1966, 1967), comparable extracts of *M. hominis* have little serological activity and clearly do not contain any major hapten or antigen. Nor is there any serologically reactive polysaccharide comparable to that in *M. mycoides* var. *mycoides*; the aqueous phases from phenol extractions of *M. hominis* were consistently inactive, whereas similar extracts of *M. mycoides* contain the galactofuranose polysaccharide hapten (Plackett *et al.* 1963). In contrast, components of *M. hominis* active in complement-fixation, metabolic inhibition and gel-diffusion tests behave as proteins in that they pass into the phenolic phase during aqueous phenol extraction and can be extracted from the cells with dilute alkali. Thus, *M. hominis*, the only species that does not ferment carbohydrates and whose antigens have been studied, has antigenic determinants which are different from those of the carbohydrate-fermenting species. What is known of *Mycoplasma* species antigens suggests that the mycoplasmas comprise a heterogeneous group, a supposition compatible with the pronounced differences in the DNA base ratios within this genus (Neimark, 1967; McGee, Rogul & Wittler, 1967).

It was possible by gel-diffusion and immunoelectrophoresis to distinguish the antigens characteristic of the membrane of *M. hominis* from those of the soluble cell contents, and to identify them in any fraction or extract. Moreover, the anti-

gens which stimulate the production of growth-inhibiting and MI antibody are in the membrane rather than in the soluble fraction, judging from the ability of membrane fractions to stimulate MI antibody formation and to absorb MI antibody from antiserum to whole cells. The association of MI antibody with membrane components is also suggested by the ability of the phenolic and potassium hydroxide extracts to absorb MI antibody, since both contained mainly membrane components. It seems that IHA activity also is associated with the membrane. Since all three methods used for disruption resulted in some contamination of the soluble fractions with membrane, the low level of activity of soluble fractions in inducing or absorbing IHA antibody and in sensitizing erythrocytes for agglutination, is probably only due to membrane fragments.

The antigens associated with metabolic inhibition and indirect haemagglutination are apparently in the membrane of other mycoplasmas. Thus, Williams & Taylor-Robinson (1967) found that MI antibody was induced by membrane fractions, but not by the cell contents, of *M. gallisepticum* and *M. pneumoniae*. Moreover, in *M. pneumoniae* the lipid hapten, which is undoubtedly derived from the membrane, is active in blocking growth-inhibiting, MI and IHA antibody (Soběslavský *et al.* 1966, 1967; Lemcke, Plackett, Shaw & Marmion, 1968).

It is apparent from our fractionation studies that the cells of *M. hominis* are more difficult to disrupt than those of *M. laidlawii*, which lyse in solutions of low tonicity and from which membranes can be prepared by osmotic shock. Kim, Clyde & Denny (1966), using the criterion of cell viability, also found that a strain of *M. hominis* was relatively resistant to osmotic lysis, sonic treatment and alternate cycles of freezing and thawing. Of the three methods used to disrupt cells, sonic treatment released the most protein from the cells and gave the highest yield of membrane fraction, but gel-diffusion tests showed that the soluble fractions produced by this method were frequently contaminated with membrane components that did not sediment at 100,000 *g*. It is of interest in this connexion that Pollack, Razin, Pollack & Cleverdon (1965) found membrane-associated enzymes in the 'soluble' fraction of sonically treated cells of *M. laidlawii*. Nevertheless, although sonic disruption is unsuitable for preparing soluble fractions, it seems to be the only practical method for preparing membranes in sufficient quantities for antigenic analysis. Soluble fractions produced by gas cavitation under nitrogen were also contaminated with membranes, although in amounts too small to be demonstrable in gel-diffusion tests or by immunoelectrophoresis. Freezing and thawing proved to be the best method for producing soluble fractions, although even they were not entirely free from membrane antigens.

It is, however, towards the isolation and characterization of the membrane antigens that current work is being directed, since they rather than soluble fractions are associated with metabolic inhibition and indirect haemagglutination. The corresponding antibodies may be of greater significance than complement-fixing antibodies in the diagnosis of naturally occurring human infections with *M. hominis*. Thus, Taylor-Robinson *et al.* (1965) found that indirect haemagglutination was more sensitive than complement fixation for detecting antibody to an oral strain of *M. hominis* in the serum of patients of all ages. Jones & Sequeira

(1966), who measured the complement-fixing and MI antibodies to *M. hominis* in about 3000 sera, concluded that MI antibody, although occurring less frequently than complement-fixing antibody, is probably produced only in response to severe infection. The characterization of the antigens associated with metabolic inhibition and indirect haemagglutination should help to elucidate the significance of these antibodies in human infection with *M. hominis*.

SUMMARY

A genital strain of *Mycoplasma hominis* was fractionated by differential centrifugation after disruption of the cells by alternate cycles of freezing and thawing, by gas cavitation under nitrogen, or by ultrasonic treatment.

Antigens of the cell membrane were distinct from those in the soluble cell contents, judging from metabolic inhibition (MI), indirect haemagglutination (IHA), gel-diffusion and immunoelectrophoresis tests, and by the antibody response in rabbits inoculated with these fractions. The antigens which gave rise to MI and IHA antibody were located in the cell membrane.

Extraction of whole cells of *M. hominis* by various chemical methods suggested that the active components were protein in nature and that there was no lipid hapten, as in *M. pneumoniae*, or polysaccharide, as in *M. mycoides* var. *mycoides*.

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EXPLANATION OF PLATES

PLATES 1–2

Gel-diffusion and immunoelectrophoresis reactions of fractions and extracts of *M. hominis* SC4 with antiserum to whole cells of SC4. Antiserum in centre wells in gel-diffusion tests.

PLATE 1

Fig. 1 (*a*). Precipitin patterns given by soluble fractions produced by freezing and thawing (Sf), by gas cavitation (Sg) and by sonic treatment (Ss). (*b*) System of numbering precipitin lines given by soluble fractions with antiserum to whole cells.

Fig. 2. Precipitin patterns of soluble fraction (Sg), detergent-lysed membrane (Mt) and detergent-lysed cells (Ct). Detergent was Triton X-100, 5 mg. per mg. of membrane or cell protein.

Fig. 3. Precipitin patterns of crude membrane fraction (Mt), membrane purified on a sucrose density gradient (Mp) and cells (Ct) after treatment with Triton X-100 (5 mg. per mg. of membrane or cell protein).

Fig. 4. Gel-diffusion reactions showing presence of membrane components in soluble fractions produced by sonic treatment of *M. hominis* SC4. Soluble fractions (Ss), crude membranes lysed with sodium lauryl sulphate, 5 mg. per mg. membrane protein (Ms). Arrow indicates common component.

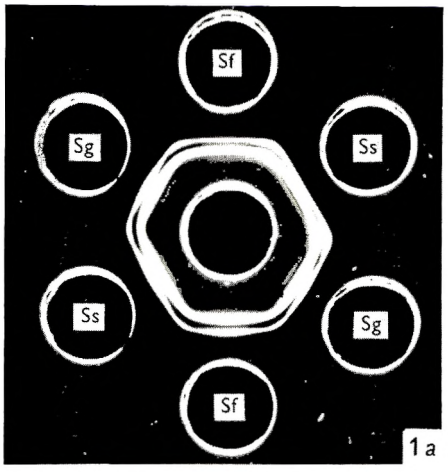
Fig. 5 (*a*) and (*b*). Immunoelectrophoresis of membranes lysed with detergents. (*a*) Purified membranes (Mp) and crude membrane (Mt) lysed with Triton X-100. (*b*) Crude membranes lysed with sodium lauryl sulphate (Ms), soluble fraction included as a control (Sg). Both detergents at 5 mg. per mg. membrane protein. Endosmosis (drift toward cathode) of components in soluble fraction stronger than in Pl. 2, fig. 1.

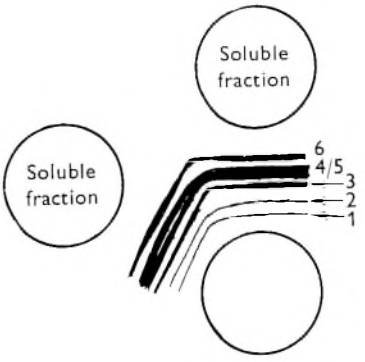
PLATE 2

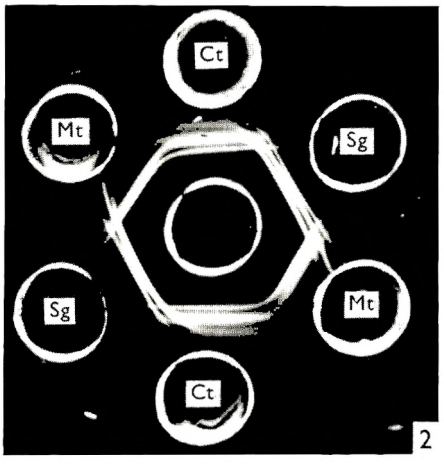
Fig. 1 (*a*) and (*b*). Immunoelectrophoresis of soluble fractions produced by gas cavitation (Sg) and by sonic treatment (Ss), and of membrane not treated with detergent (M).

Fig. 2 (*a*) and (*b*). Comparison of precipitin lines given by phenolic extract (P, derived from phenolic phase after cold, aqueous phenol treatment) with those given by soluble fraction (Sg) and membrane lysed with 5 mg. sodium lauryl sulphate per mg. membrane protein (Ms). Potassium hydroxide extract (K) included for comparison.

Fig. 3 (*a*) and (*b*). Comparison of precipitin lines given by potassium hydroxide extract (K) with those given by soluble fraction (Sg) and membrane lysed with 5 mg. sodium lauryl sulphate per mg. membrane protein (Ms).


Sf
Sg
Ss
Ss
Sg
Sf
1a


Soluble fraction
Soluble fraction
6
4/5
3
2
1
1b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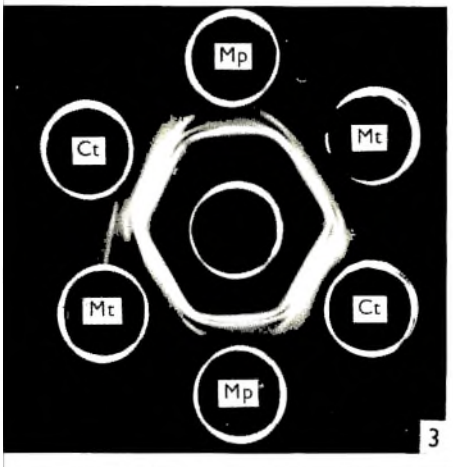
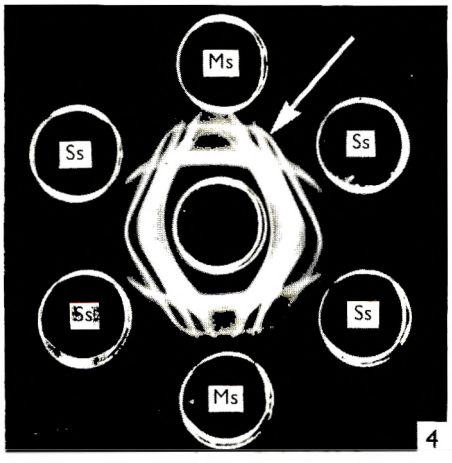
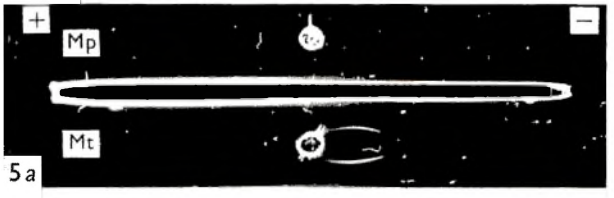
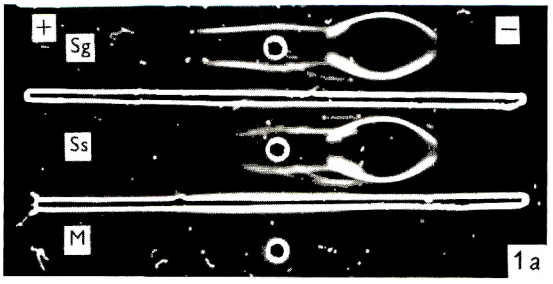
Mt
Sg
Sg
Mt
Ct
2


Mp
Ct
Mt
Mt
Ct
Mp
3

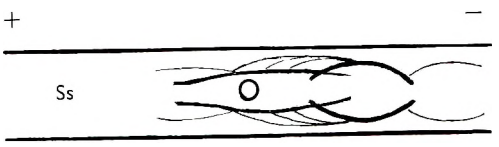

Ms
Ss
Ss
Ss
Ss
Ms
4


+
−
Mp
Mt
5a

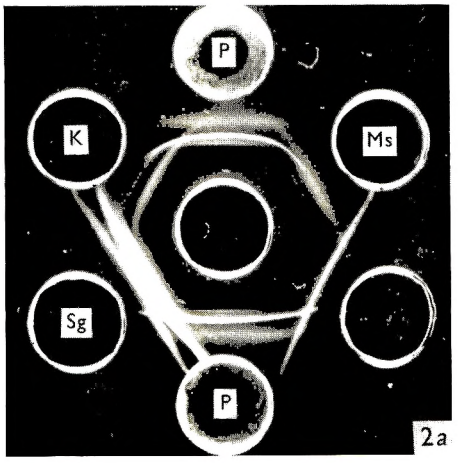

+
−
Ms
Sg
5b



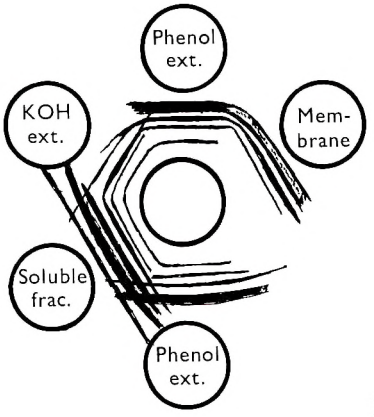
1a



1b



2a



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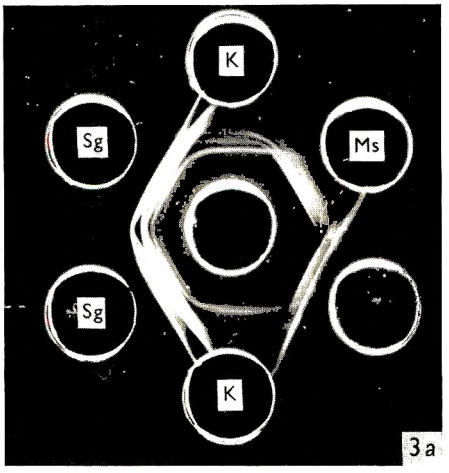
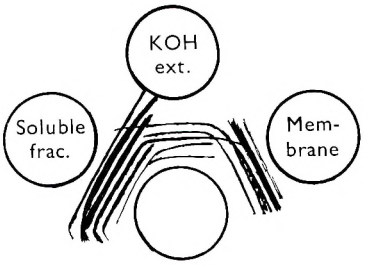


3a



3b

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Antibody response following smallpox vaccination and revaccination

By A. W. DOWNIE, L. St VINCENT, A. R. RAO and C. H. KEMPE
Department of Pediatrics, University of Colorado Medical Center and the Infectious Disease Hospital, Tondiarpet, Madras

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The measurement of antibody following vaccination has been recorded in many publications. The inhibition of vaccinia haemagglutinin, because of its technical simplicity, is a method which has been commonly employed in recent years, although the complement-fixation and neutralization techniques have also been used. Some kind of neutralization test is believed to give a better measure of protective antibody than the other tests (Elisberg, McCown & Smadel, 1956; McCarthy, Downie & Bradley, 1958; Espmark & Rabo, 1965). The more recently introduced precipitation test in agar gel, useful as a diagnostic test in smallpox, is usually negative for antibody in postvaccination sera. All four techniques for measuring antibody were used in our studies.

The present study was made as a background to the work on smallpox reported in the following papers (Downie *et al.* 1969*a*, *b*; Kempe *et al.* 1969). Because of differences in technical methods the titres of antibodies in postvaccination sera reported from different laboratories or even from the same laboratory at different times may not be directly comparable. This difficulty may be partly overcome by the use of an international reference serum of known antibody content. However, in the tests reported below on postvaccination sera the same techniques and reagents were used as in the studies on smallpox sera and the results have been used to assess the significance of our antibody measurements in smallpox. Three different groups of vaccinated individuals were studied: U.S. Army recruits bled 2–4 weeks after vaccination or revaccination, blood donors in Madras with histories of repeated vaccinations and young adults admitted as chickenpox patients to the Infectious Disease Hospital in Madras.

MATERIALS AND METHODS

Sera

The 210 sera from U.S. Army recruits had been collected for the preparation of vaccinia immune gamma globulin in 1962. The small samples used in the present investigations had been set aside at the time of collection and had been stored frozen at $-20°$C. The Madras sera, 45 from blood donors and 42 from chickenpox patients, had been collected in 1960 and stored frozen since that time. All sera were inactivated by heating at 58°C. for 20 min. before use in the tests described below.

Requests for reprints should be addressed to Dr C. H. Kempe, 4200 East Ninth Avenue, Denver, Colorado 80220.

Tests for precipitins

All sera were tested undiluted against vaccinia soluble antigen prepared from vaccinia-infected rabbit skin as described by Downie & Kempe (1969). The antigen was used in the optimal dilution of 1/4. Agar in a concentration of 1 % was used to prepare a layer 1 mm. thick on microscope slides. Using a plastic template, cups 4 mm. in diameter were cut in three rows so that the distance between neighbouring cups was 1·5 mm. Antigen was placed in the centre row of cups, and sera in the outside rows. Slides were kept in a moist container at room temperature and the tests read after 4, 24, and 48 h.

Haemagglutinin inhibition

Blood cells from two fowls were used throughout in a concentration of 0·5 % stabilized with 1 % normal rabbit serum. Haemagglutinin was prepared from vaccinia-infected chorioallantoic membranes (Downie & Kempe, 1969). To two-fold serial dilutions of serum from 1/10 upwards equal volumes of haemagglutinin diluted to contain four doses were added; after incubation for 1 hr. at 37°C. one volume of fowl red cells was added and the results read after the tests had stood 1–2 hr. at room temperature. These tests were carried out by the micro method in plastic plates.

Complement fixation

The same vaccinia rabbit soluble antigen was used as in the precipitation-in-agar-gel tests. The optimum concentration, determined by block titration (Downie & Kempe, 1969) was 1/80. The complement used was pooled preserved guinea-pig serum kept at 4°C. (Richardson, 1941). Two and a half doses of complement were used throughout. Sensitized cell suspension was prepared by mixing 3·0 % washed sheep cells with an equal volume of sheep cell haemolysin diluted to contain 4 M.H.D. Serum dilution, complement and antigen each in 0·1 ml. volumes were mixed and kept overnight at 4°C. before adding 0·2 ml. of sensitized cells next day and incubating the mixtures at 37°C. for completion of the tests. A vaccinial immune rabbit serum was titrated with each batch of tests.

Neutralization tests

These were made in monkey kidney monolayers in Leighton tubes. Sera were tested in threefold dilutions from 1/10 upwards. Equal volumes of serum dilutions and vaccinia virus suspensions were mixed and held in a water bath at 37°C. for 2 hr. before adding 0·1 ml. volumes to monolayers in Leighton tubes. The tubes were incubated at 37°C for 40 hr., the medium removed and the cell sheets stained with carbol fuchsin before counting plaques. Three or four tubes were used for each serum-virus mixture. The vaccinia virus suspension, prepared from infected monkey kidney tissue cultures, was diluted before use so that 30 to 70 plaques were produced in control tubes. A serum with known antibody content or the International Standard immune serum (pooled convalescent smallpox serum) was titrated with each batch of tests.

The mean counts for each serum dilution–virus mixture were plotted on graph paper as a percentage of the negative serum–virus control; the titre of the serum was determined by the point where the graph crossed the 50 % line.

The titres recorded for all techniques indicate dilutions of sera before admixture with virus or antigen.

RESULTS

Precipitins

None of the postvaccination sera produced a precipitin line in the tests in agar gel. Among the 45 Madras blood donors there were seven persons who had suffered from smallpox years previously; these sera also were negative for antibody by the precipitation test.

Table 1. *Incidence of haemagglutinin-inhibiting antibodies in three groups of vaccinated or revaccinated individuals*

	Percentage of sera		
		Madras	
Titre	U.S. Army recruits (210)	Chickenpox (42)	Blood donors (45)
< 10	59	55	13
10	34	36	56
20	6	7	22
40	1	2	9

Haemagglutinin-inhibition antibody

The results of these tests for the three groups of sera are shown in Table 1. The numbers of sera in the three groups differed and the results have been expressed as percentages for comparative purposes.

There is no significant difference in the titres shown by the Army recruits and the chickenpox patients in Madras. The titres for the blood donors in Madras are higher than those for the first two groups. The results were not significantly affected by the inclusion of the seven blood donors with a history of smallpox. Five of these had an HI titre of 1/10, one had a titre of 1/20, and one had a titre of 1/40.

Complement-fixing antibody

The results of tests on the sera expressed in percentages are shown in Table 2.

Again there is little difference in the results between the sera of the U.S. Army recruits and the sera from the Madras chickenpox patients. The sera from the blood donors in Madras show rather more positive results but the difference is not significant. Of the seven donors with a history of smallpox, five had a titre of less than 1/5, one had a titre of 1/5, and one had a titre of 1/10.

Neutralization tests

The titres for individual sera are shown in Fig. 1. The results are shown for the second batch of 100 sera from U.S. Army recruits, as there was insufficient serum remaining from the first batch of 110 sera to permit testing at a dilution of 1/10.

Table 2. *Complement-fixing antibody in three groups of vaccinated or revaccinated individuals*

	Percentage of sera		
		Madras	
Titre	U.S. Army recruits (210)	Chickenpox (42)	Blood donors (45)
<5	93	95	89
5	4	0	2
10	3	5	9
20	0	0	0

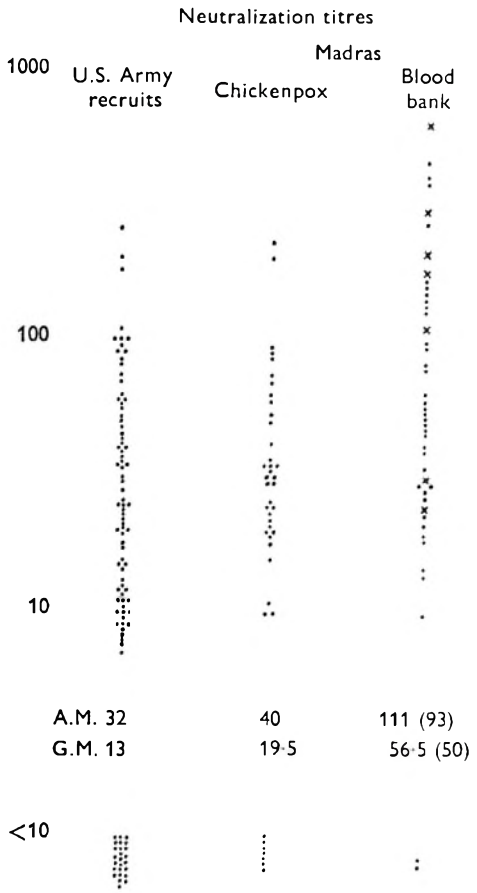

Fig. 1. Neutralizing antibody in three groups of vaccinated or revaccinated individuals. A.M. = arithmetic mean; G.M. = geometric mean. Figures in parentheses give the A.M. and G.M. after exclusion of seven persons with history of smallpox. × , History of smallpox.

It can be seen that the majority of the sera neutralized vaccinia virus in a dilution of 1/10 or higher although many were negative in the H.I. and complement fixation tests—an observation reported by others (Herrlich, Mayr & Munz, 1956; McCarthy *et al.* 1958). The neutralizing titres were slightly higher for the chickenpox sera than for the Army recruits—arithmetic means 40 and 32, geometric means 19·5 and 13 respectively. The sera from Madras blood donors showed significantly higher levels of antibody—arithmetic mean 111, geometric mean 56.5. If the results of the seven sera from patients with a past history of smallpox are omitted from this group, the arithmetic mean for the remaining 38 was 93 and the geometric mean 50, titres significantly higher than those from the first two groups.

DISCUSSION

The results in general suggest that the neutralization test permits the detection of antibody which may not be detected by the HI and CF tests with the techniques used. It has been shown in previous studies that neutralizing antibody may persist for years after vaccination, when HI and CF tests for antibody have become negative, and that after revaccination a marked rise in neutralizing antibody may not be accompanied by a corresponding rise in HI and CF antibody (McCarthy *et al.* 1958). The differences in mean titre of neutralizing antibody between the three groups of sera may be explained in part by the previous history of smallpox vaccination. We have no information about previous vaccination in the Army recruits, but vaccination 2–4 weeks before the collection of blood samples had been successful. Of the chickenpox patients, a history of revaccination was available for only 20 of the 42 individuals in the series, whereas 41 of 45 blood donors had a history of revaccination. Many of the chickenpox patients were boys who had come from outside Madras to work with Madras families, whereas the blood donors were mostly native to Madras where vaccination and revaccination were more commonly carried out.

It seems a little surprising that the titres in the Army recruits were relatively low as these recruits were bled 2–4 weeks after vaccination or revaccination, the time when maximum antibody response might be expected; the time since vaccination or revaccination of the other two groups was variable although usually longer. The HI and CF antibody response following primary vaccination is not usually great and revaccination may fail to induce increase in titre in these antibodies. After successful revaccination the neutralizing antibody titres are generally considerably higher than after primary vaccination, (McCarthy *et al.* 1958), but the titres in the Army recruits are not dissimilar to those previously recorded in revaccinated individuals.

SUMMARY

Three groups of post-vaccination sera were studied for vaccinial antibody by precipitation, haemagglutinin-inhibition, complement-fixation and neutralization tests. All sera were negative by precipitation and many by haemagglutinin-inhibition and complement-fixation tests, but most showed neutralizing activity

at serum dilutions of 1/10 or higher. The differences in antibody titres between the three groups of sera were most probably related to the past history of revaccination.

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Antibody response in non-haemorrhagic smallpox patients

By A. W. DOWNIE, L. St VINCENT, L. GOLDSTEIN, A. R. RAO and C. H. KEMPE

Department of Pediatrics, University of Colorado Medical Center, and the Infectious Disease Hospital, Tondiarpet, Madras

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Various studies have been made on the antibody response in smallpox. In some of these haemagglutinin inhibition tests (HI) have been used for measurement of antibody (Collier & Schönfeld, 1950) in others, complement-fixation tests (CFT), precipitation tests (pt.) and HI tests (Herrlich, Mayr & Mahnel, 1959) or CF, HI and neutralization tests (Downie & McCarthy, 1958). In the present study, all four tests were used and the findings have been used for a comparative study of cases of haemorrhagic smallpox and for assessment of possible subclinical infection in contacts of smallpox patients and the antibody response in minimal infections.

MATERIALS AND METHODS

The sera were collected from patients in the Infectious Diseases Hospital in Madras during the years 1963–6 and were held frozen at $-20°$ C. until examinations were completed. There were 130 paired sera and 33 single specimens from patients taken mostly during the first 14 days of illness. In addition, there were 53 specimens collected during convalescence or after recovery. With five exceptions these 53 samples were collected between 2 and 4 weeks after the onset of illness. In all, 216 sera from 151 individuals were studied. Fourteen of the patients died, 11 of these had not been vaccinated. In all there were 21 patients who had not been vaccinated. This is a much smaller proportion of unvaccinated patients in this series than in the general population of smallpox patients in the hospital during this period (Rao, 1968). Most unvaccinated smallpox cases were in children, whereas blood for antibody studies was collected mostly from adults. Of the 151 patients studied only two were below 17 years of age: these two were unvaccinated males aged 2 and 6 years, respectively, who died of their disease.

The techniques used in carrying out precipitation, HI, CFT and neutralization tests for antibody were described in the previous paper (Downie, St Vincent, Rao & Kempe, 1969). All sera were examined routinely for antigen by precipitation and complement-fixation tests using suitable dilutions of vaccinia immune rabbit serum. Blood specimens were examined for the presence of virus from 19 patients only.

Requests for reprints should be addressed to Dr C. H. Kempe, 4200 East Ninth Avenue, Denver, Colorado 80220.

RESULTS

Examination for virus and virus antigen in blood

Of the 19 patients whose blood was examined for virus, seven died, all between the 10th and 17th day of illness. Blood was examined for virus from one patient on the 2nd day of illness, from another on the 3rd day, from three on the 4th, from two on the 5th, from three on the 6th, from two on the 7th, from two on the 9th, from one on the 11th, from one on the 13th, from one on the 17th day of illness, and in two patients the date of blood culture was not recorded. From only two specimens was virus isolated and in both the amount of virus was small, for virus was recovered only by subinoculation of the egg membranes inoculated with the specimen. One of the positive specimens was collected on the 6th day of illness from a patient with a confluent eruption, the other on the 4th day from an unvaccinated patient showing a discrete eruption. Both these patients recovered.

All serum specimens subsequently examined for antibody, 216 in all, were examined for the presence of antigen by precipitation and CF tests with an anti-vaccinial serum prepared in rabbits. Undiluted sera were tested for antigen by precipitation, and sera in a dilution of 1/10 by CFT. None of the sera showed antigen by these methods.

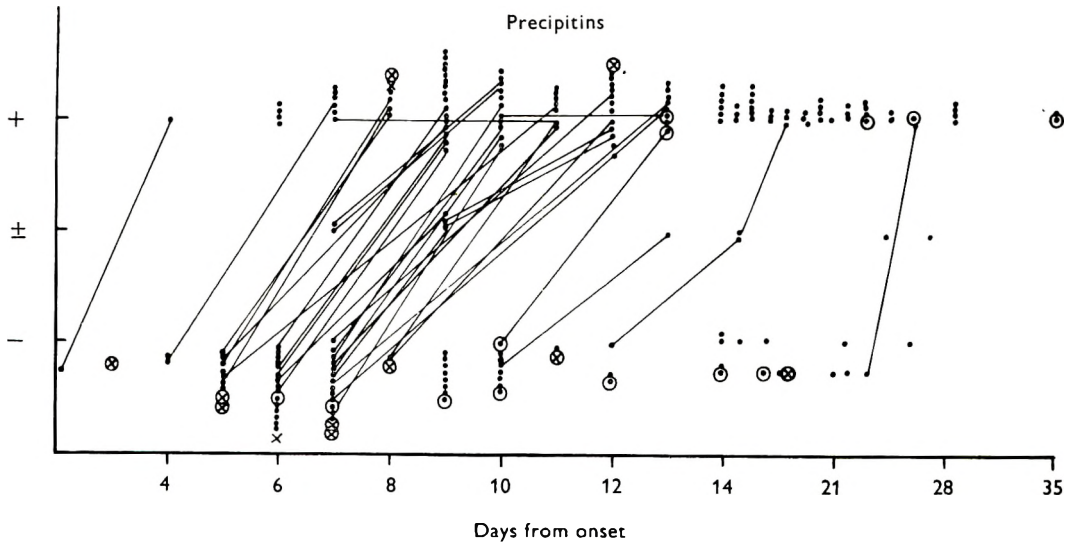

Fig. 1. Precipitating antibody in the sera of smallpox patients. ○, Unvaccinated; ×, fatal cases.

Tests for antibody

Precipitation in agar gel

The results of these tests are shown in Fig. 1. The sera positive for antibody by this test usually showed precipitation lines between the sera and antigen within 5 hr., but a few showed lines only after 20 hr. The sera recorded as giving a ± result showed only a faint line after 48 hr.

It will be seen from the figure that the majority of sera tested before the 8th day of illness were negative, but from the 8th day onwards most of the sera showed

precipitating antibody. Sera from unvaccinated patients were less likely to be positive, there being nine negative results as against seven positives from the 8th day onwards. Three sera from patients who died showed no precipitins as against two which were positive during this period. The lines in the figure connect first and second specimens of serum. Where both specimens gave the same result in the test lines were not drawn between the two specimens; consequently the number of lines in the figure is less than the number of paired specimens examined. This also applies to Figs. 2–4.

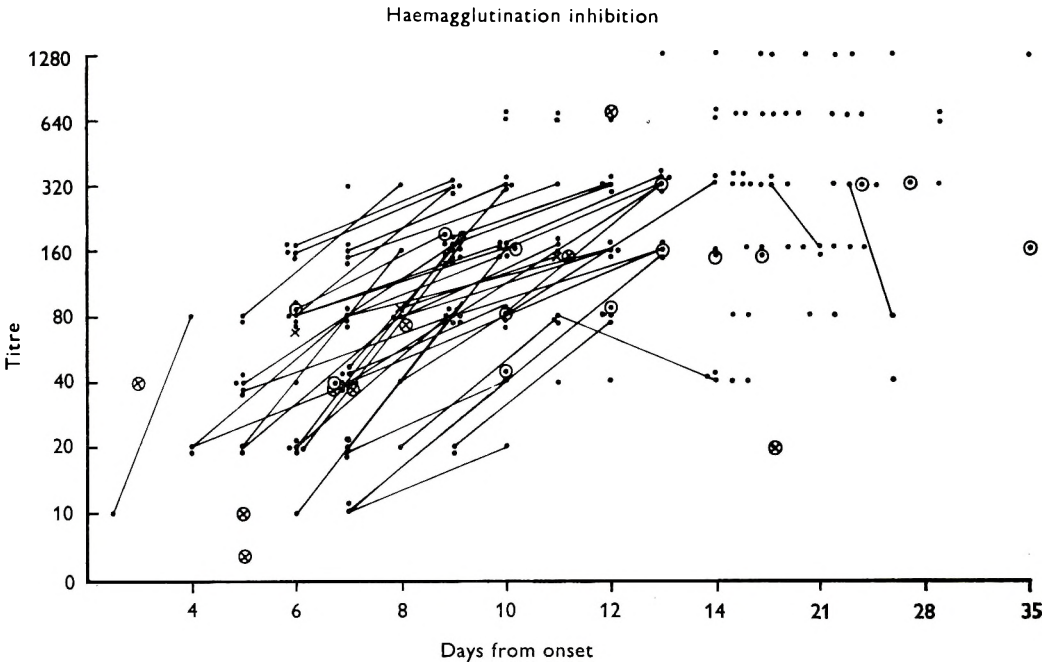

Fig. 2. Haemagglutinin-inhibiting antibody in the sera of smallpox patients.
○, Unvaccinated, ×, fatal cases.

Haemagglutination inhibition tests

It is apparent from the results shown in Fig. 2 that antibody shown by this test appears rather earlier than that revealed by precipitation in agar gel. Maximum titres tend to be reached between the 12th and 21st day from onset by which time all show significant titres (1/20 or more by the technique used). Sera from unvaccinated patients showed HI antibody from the sixth day onwards although the titres tended to be somewhat lower than those attained by the sera of previously vaccinated patients.

Complement-fixation test

With one exception, no sera tested before the 6th day of illness gave a positive test for antibody by this technique, but from the 8th day most were positive (Fig. 3). Maximum titres were attained after the 14th day and the only three sera negative at this time were from unvaccinated patients. As with the precipitation

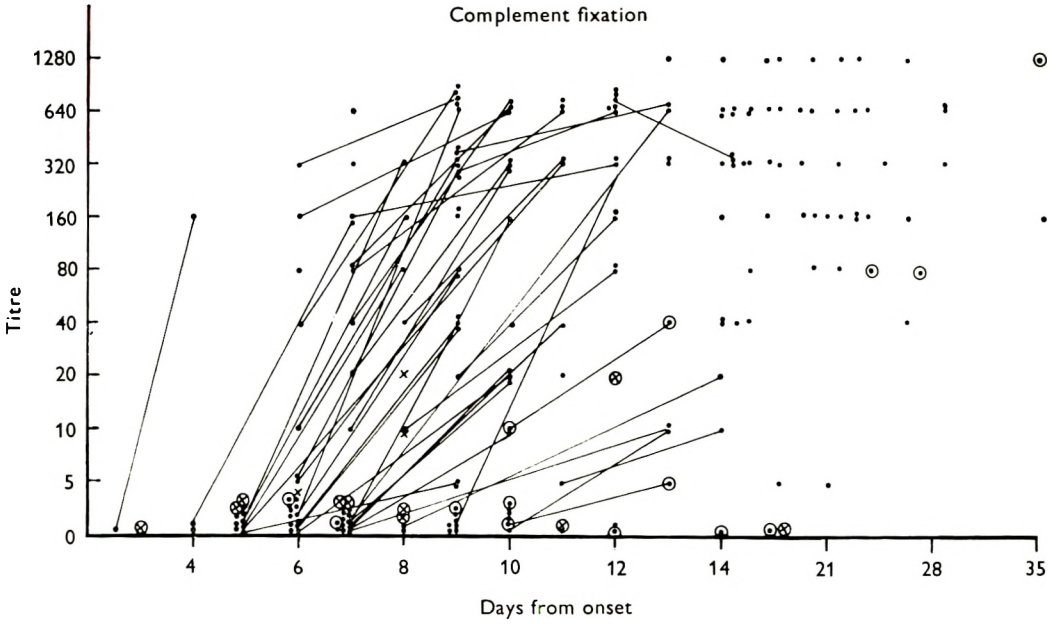

Fig. 3. Complement-fixing antibody in the sera of smallpox patients.
○, Unvaccinated, ×, fatal cases.

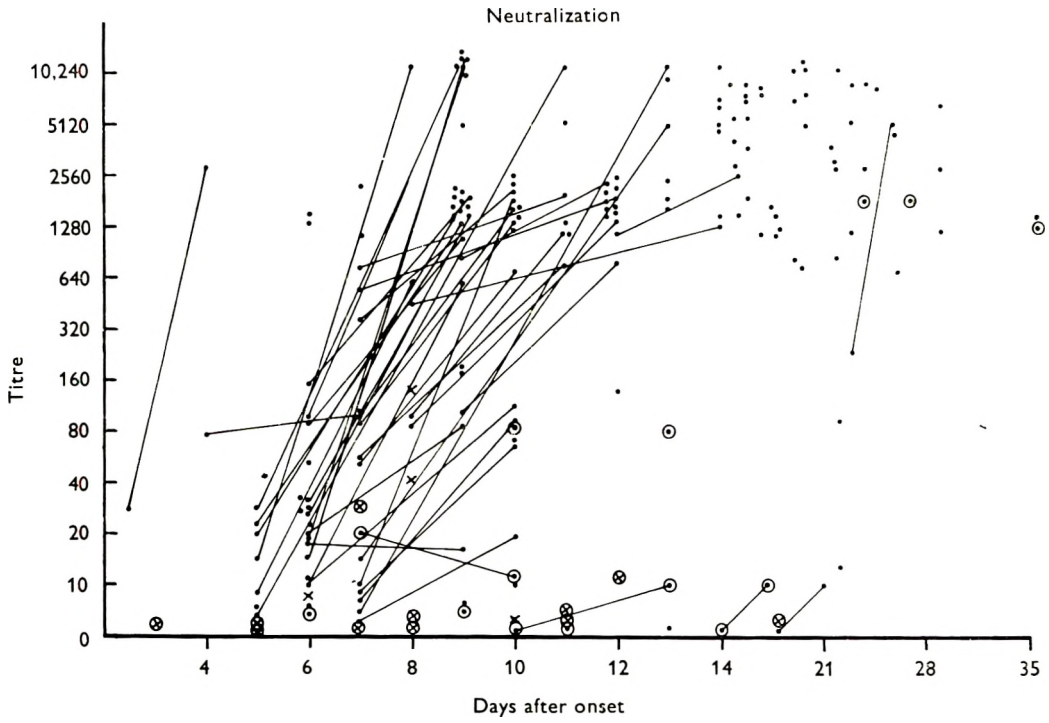

Fig. 4. Neutralizing antibody in the sera of smallpox patients.
○, Unvaccinated; ×, fatal cases.

tests, more than 50% of sera from unvaccinated individuals examined after the 8th day were negative. As will be seen in Fig. 3, only three of 13 patients who died showed CF antibody and these three had titres of 1/10 or 1/20.

Neutralization tests

The results of titrations of neutralizing antibody are shown in Fig. 4. Most of the sera tested showed neutralizing activity from the 5th day of illness and maximum titres were frequently reached by the 9th day. Many of the sera shown grouped at 1280 from the 9th to the 12th day of illness were not tested in higher dilutions. The few sera showing little or no neutralizing activity from the 8th day onwards were obtained from patients who suffered severe and fatal infections, most of them lacking a history of previous vaccination.

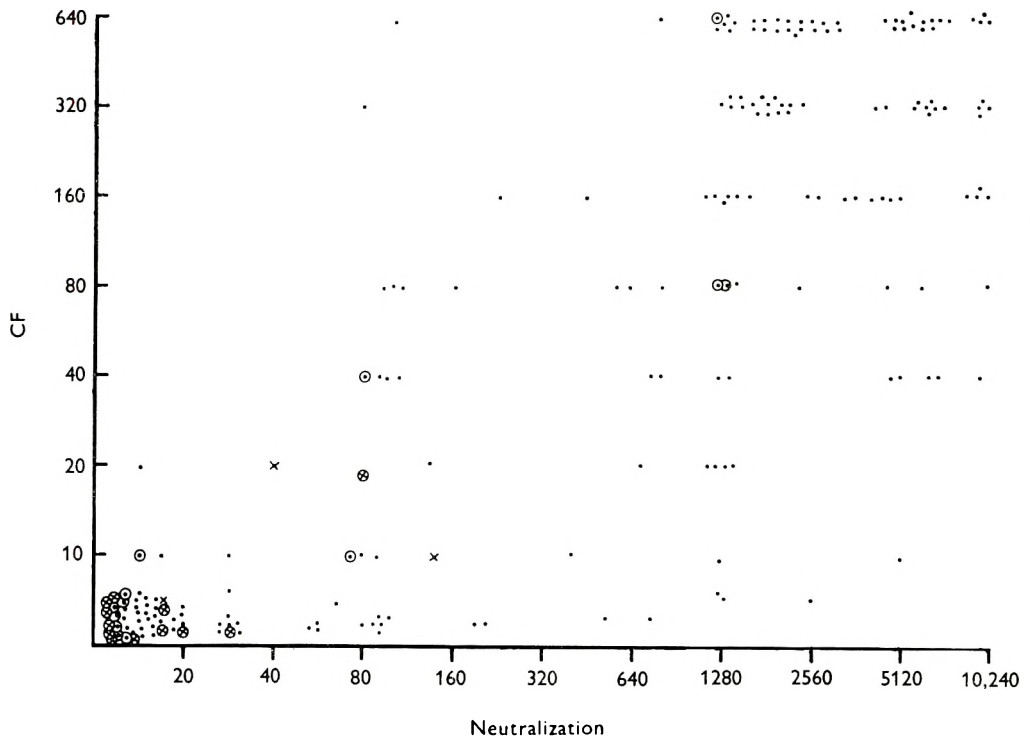

Fig. 5. Comparison of complement-fixing and neutralizing antibody titres in the sera of smallpox patients. ○, Unvaccinated; ×, fatal cases.

Correlation of results of individual tests

The titres for complement fixing and neutralizing antibody on individual sera are shown in Fig. 5. A rough correlation of the results of these two tests is apparent, although some sera showing good neutralization had low CF titres. From this figure too it can be seen that most of the sera from fatal and unvaccinated cases had low antibody titres by both tests.

There is not, however, such good correlation between the HI and the CF or neutralization tests (Figs. 6, 7). While many sera showed high titres by all three

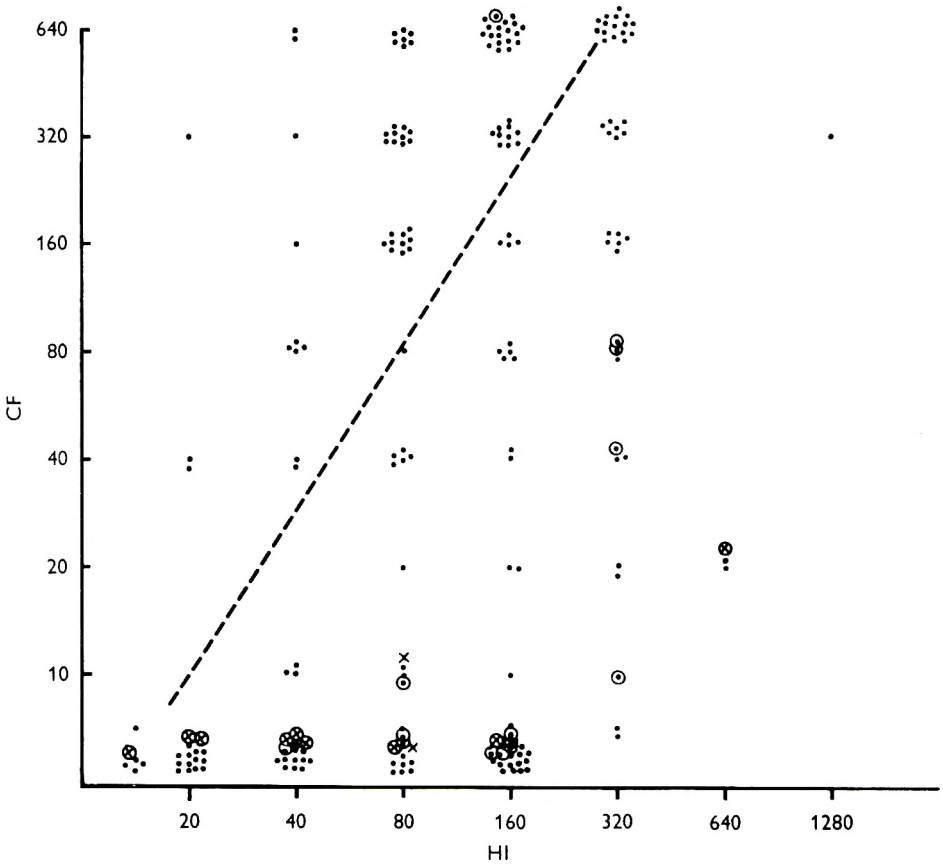

Fig. 6. Comparison of complement-fixing and haemagglutinin-inhibiting antibody titres in the sera of smallpox patients. ○, Unvaccinated; ×, fatal cases.

Table 1. *Comparison of results of precipitation in agar gel and complement-fixation tests for antibody in the sera of smallpox patients*

Complement-fixation titre	Precipitation		
	−	+	++
640	1	3	39
320	1	3	27
160	3	2	18
80	2	2	11
40	2	5	7
20	3	1	4
10	3	1	4
5	6	2	1
0	57	0	3
Totals	78	19	114

tests, other sera, including those from unvaccinated patients, had reasonably good titres by the HI test but little antibody when tested by the CF and neutralization techniques.

The comparison of the results of precipitation-in-agar-gel tests with those of CF

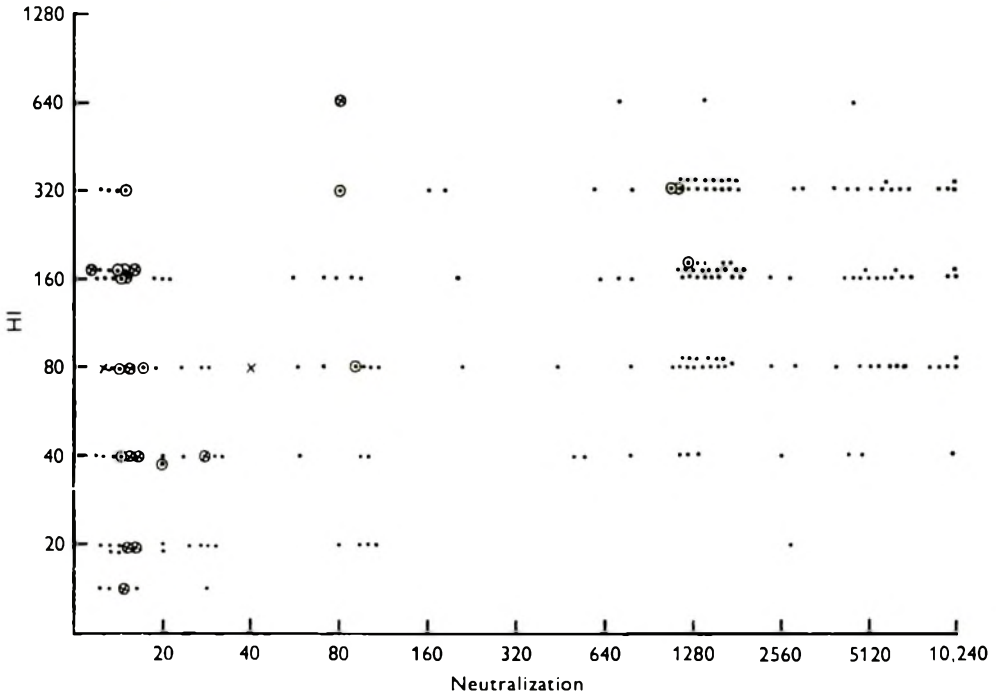

Fig. 7. Comparison of haemagglutinin-inhibiting and neutralizing antibody titres in the sera of smallpox patients. ○, Unvaccinated; ×, fatal cases.

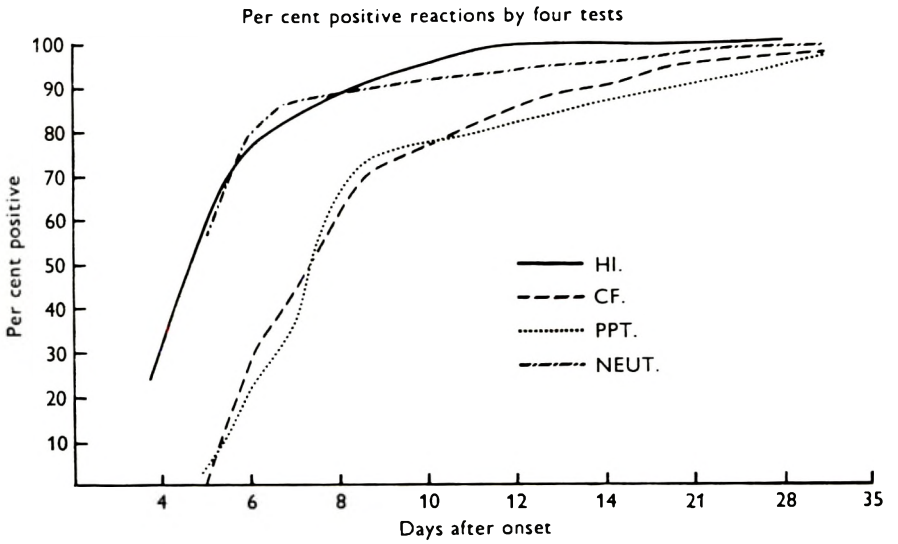

Fig. 8. Time of appearance of antibody as determined by four techniques, in relation to day of disease.

and neutralization tests are shown in Tables 1 and 2. Reasonable correlation is shown by the results of the three tests in that the majority of sera with high CF and neutralization titres gave rapid precipitation with vaccinial antigen in agar gel. Most of the sera showing low or absent titres by CF and neutralization were also negative by precipitation. However, there were a few sera which, although showing little or no antibody by CF and neutralization techniques, gave good precipitation with a vaccinial antigen. (A few sera examined by the precipitation and CF tests were not tested for neutralizing antibody and this accounts for the different totals of sera shown in the two tables.)

Table 2. *Comparison of results of precipitation in agar gel and neutralization tests for antibody in the sera of smallpox patients*

	Precipitation		
Neutralization titre	−	+	+ +
10,000	0	1	12
1000 to < 10,000	0	8	79
100 to < 1000	9	4	13
10 to < 100	37	1	7
< 10	31	0	4
Totals	77	14	115

DISCUSSION

Only a relatively small number of examinations were made for the presence of virus in the blood of these non-haemorrhagic smallpox patients. However, the finding of virus, even in low concentration on the 4th and 6th day of illness in two patients who recovered, is contrary to the experience of Downie *et al.* (1953) who found no virus after the 2nd day in 25 patients who recovered. It appears that the presence of small amounts of virus in blood as late as the sixth day of illness is not incompatible with recovery. On the other hand, earlier findings were confirmed in that virus antigen was not detected in the serum of any of the patients in this series, including those who died.

It is apparent from Figs. 1–4 that antibody response is detected earlier in the disease by HI and neutralization tests than by precipitation and CF techniques. This is illustrated in Fig. 8, in which the percentage of positive results by the four techniques is shown in relation to the day of disease. These findings are in agreement with those of Downie & McCarthy (1958), who tested sera for HI, CF, and neutralizing antibody and with those of Herrlich *et al.* (1959) who used precipitation, HI and CF tests. Although there were fewer unvaccinated patients in the present series, the low antibody titres in these patients by precipitation, CF and neutralization tests are in accord with the findings in the two previous studies quoted. On the other hand, relatively high titres of HI antibodies were frequently observed in unvaccinated and fatal infections in all three series. This and the lack of correlation of HI titres with CF and neutralization titres shown in Figs. 6 and 7 suggest that HI antibodies do not provide a good indication of immune response

and are of low prognostic value—a point which is also illustrated by the study of haemorrhagic cases. The CF antibody titres recorded by Herrlich *et al.* (1959) in various types of smallpox were considerably lower than those recorded by Downie & McCarthy (1958) and in the present series—a result presumably attributable to the less sensitive technique which they employed. This is reflected in their failure to demonstrate CF antibodies in eight cases of alastrim examined after the 12th day of illness, whereas such antibody was found in all cases of alastrim examined after the ninth day by McCarthy & Downie (1953).

The data of Downie & McCarthy (1958) indicate the decline in HI and CF titres with passage of time after smallpox infection. We have no information on the persistence of precipitin except that positive reactions are obtained up to 35 days after onset of illness and are not found a few years after recovery from smallpox. Tests for precipitins were negative in the sera of six cases of generalized vaccinia observed in Denver.

In the retrospective diagnosis of missed cases of smallpox and in minimal or subclinical infections, the above findings, together with those in the previous paper, suggest that with the techniques used, the presence, in a single specimen of serum, of a positive precipitin-in-agar-gel test, a CF titre of 1/20 or greater, an HI titre of 1/80 or higher and a neutralizing titre of 1/500 or greater are indicative of recent smallpox infection. The majority of smallpox sera examined gave a positive precipitation test in agar gel and titres in the other tests greater than those mentioned. Where sera taken early and late in the course of illness are available rises in titre will strengthen the evidence of smallpox infection. But in missed cases such paired samples of sera will rarely be available. In unvaccinated patients the antibody levels mentioned above may not be reached, but in these the disease is likely to be severe and unlikely to be overlooked. Minimal infections generally occur in vaccinated persons in whom antibody titres reach a high level.

SUMMARY

Two hundred and sixteen sera from 151 patients suffering from smallpox (non-haemorrhagic) were examined for antibody by precipitation in agar gel, by haemagglutinin inhibition (HI), complement fixation (CF) and neutralization tests. Most of the patients were adults and the majority had been vaccinated earlier in life. HI and neutralizing antibodies showed rising titres from the 6th day of illness while the majority showed precipitins and CF antibodies from the 8th day. The results of the precipitation-in-agar-gel tests are in marked contrast to the findings in healthy vaccinated and revaccinated individuals, none of whose sera gave a positive result for antibody by this technique. In unvaccinated patients the antibody response was frequently delayed and the titres lower than those attained by the previously vaccinated patients. There was no exact correlation in antibody titres obtained by the four methods of measurement, HI antibody, in particular, reaching in some cases relatively high titres when other tests showed low titres. It is suggested that with the methods and materials used, a positive precipitation test in agar gel, a CF titre of 1/20 or more and an HI titre of 1/80 or higher in a single specimen of serum would be suggestive of recent

smallpox infections. Such a result might be of special value in the retrospective diagnosis of missed cases and in the detection of minimal or subclinical infections.

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Haemorrhagic smallpox

By A. W. DOWNIE, D. S. FEDSON, L. St VINCENT, A. R. RAO and C. H. KEMPE

Department of Pediatrics, University of Colorado Medical Center, and the Infectious Disease Hospital, Tondiarpet, Madras

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The most acutely fatal cases of smallpox in which death occurs within 8 days of the onset of illness, often before focal lesions are present in the skin, have been classified by Dixon (1962) as fulminating. The majority of these patients show petechial haemorrhages in the skin, bleeding into the conjunctiva and from the mouth, nose, rectum, vagina and often frank blood in the urine. These cases have been classified by Rao as haemorrhagic type I (Ramsay & Emond, 1967). A second severe type of infection is manifested by the appearance of a flat, often delayed, focal eruption in the skin associated with haemorrhages at the base of vesicles, but also into apparently normal skin between focal lesions. The majority of these patients die from 8 to 12 days after onset and have been classified by Rao as haemorrhagic type II.

It has been suggested that these severe infections associated with haemorrhage are manifestations of an allergic reaction (Dixon, 1962; Herrlich, Mayr & Mahnel, 1959); approximately 50% have a history of previous vaccination. Defects in the clotting mechanism have been revealed by the studies of Roberts *et al.* (1965) and McKenzie *et al.* (1965). The severity of the virus infection in these patients has been shown by the severe and sometimes prolonged viraemia determined by blood culture (Downie *et al.* 1953; Mitra *et al.* 1966) and by the presence of soluble antigen in the blood of a proportion of them. This antigen has been detectable by complement-fixation tests (Downie *et al.* 1953) or in a few instances by precipitation in agar gel (Paniker & Kalra, 1962).

The present studies were carried out on a series of haemorrhagic smallpox patients seen in the Infectious Disease Hospital in Madras between the years 1963 and 1966. They were designed to determine the presence of virus and virus antigen in the blood and the immune response in these patients as measured by antibody estimations on blood serum. Examination of blood for virus was made immediately after its collection, while tests for the presence of soluble antigen were made in Madras and later repeated in Denver on samples of serum or plasma which had been kept frozen at -20 to -30°C. until 1967. The determinations of antibody by precipitation in agar gel were made in Madras and later repeated in Denver.

Requests for reprints should be addressed to Dr C. H. Kempe, 4200 East Ninth Avenue, Denver, Colorado 80220.

MATERIAL AND METHODS

There were 77 patients included in the study. Of these 37 were classified as type I on clinical grounds by one of us (A. R.) and 40 as type II. There were 39 females and 38 males. Five of the type II patients survived. Of the females 13 were known to be pregnant and 10 of these suffered from type I disease. Of the 77 patients, 20 bore no scars of vaccination. Only three of the patients were under the age of 10 years. Tests for antibody in the serum by three or four techniques were carried out in 47 cases.

Detection and measurement of viraemia

This examination was carried out on 75 of the cases and in one patient on three separate occasions. Blood was collected in most cases into heparin. In 42 instances the blood was centrifuged, the plasma removed, the buffy coat pipetted off and washed once before suspension in a volume of diluent equal to the original blood sample. The leukocytes, whole blood and plasma were tested separately by inoculation of 0·1 ml. quantities on the chorioallantois (CA) of two or three 12-day chick embryos. The eggs were examined 3 days later and the number of lesions noted. Where there was doubt about occasional lesions, the excised chorioallantois was extracted and inoculated into further eggs. There was little difference in the results obtained from the plasma and washed buffy coat, but in three specimens, whole blood and leucocytes yielded a few lesions on the chorioallantois when the plasma was negative. Twelve specimens were tested by inoculation into tissue cultures of Hela cells as well as on chick embryos. On only one of these specimens, which was positive on the CA and negative on tissue culture, did the results differ. In the subsequent figures and tables the degree of viraemia has been recorded in plus signs: + represents 1–10 lesions on the CA; + +, 10–100 lesions; + + +, 100–1000 lesions or semiconfluent takes; and + + + +, confluent takes on the CA.

Detection of antigen in the blood serum or plasma

The sera from many of the cases were tested in Madras for antigen by the precipitation-in-agar-gel technique. The sera were tested undiluted against a vaccinia immune rabbit serum or against a convalescent smallpox serum which gave strong reactions with antigen from smallpox vesicle fluid or from vaccinia-infected rabbit skin. The sera were tested for antigen by the complement-fixation technique only in Denver in 1967. Between the tests for antigen in Madras and the tests in Denver the sera had been stored frozen but had been heated three times at 58° C. to render them non-infectious before being shipped from Madras to Liverpool and again from Liverpool to Denver. This heating affected the results of precipitation tests for antigen; for 16 sera showed antigen by precipitation in Madras, and only two gave positive reactions for antigen by precipitation in Denver. On the other hand, all sera which showed antigen by precipitation in Madras showed good titres for antigen when tested by complement fixation in Denver. Dumbell & Nizamuddin (1959) remarked that heating at 60° C. greatly lessened the activity of crust extracts when tested for antigen by precipitation in agar gel.

Because of the apparent discrepancy between the result of precipitation tests for antigen in Madras and in Denver, experiments were made by adding rabbit vaccinial antigen to a normal human serum and testing the mixture for antigen by both precipitation and complement-fixation tests before and after heating for 15 and for 30 min. at 59°C. The unheated antigen gave positive precipitation reactions up to dilutions of 1/16, whereas the heated antigen gave weak positive reactions only at a dilution of 1/2. On the other hand, the heated antigens had undiminished complement-fixing activity (Table 1). It would appear, therefore,

Table 1. *Complement-fixing activity of antigen-in-serum, heated for* 15 *and* 30 *min. at* 59°C.

Antigen in serum	Antigen dilutions							Antigen controls
	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/100
Heated 15 min., 59°C.	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	–	–
Heated 30 min., 59°C.	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ +	–	–
Unheated	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	–	–
Antiserum control, 1/80	–	.	.	.	.	.	.	

+ + + + = Complete fixation; + + +, + + and + = diminishing degrees of fixation; – = no fixation of complement.

that the combination of heated antigen plus antibody may fail to precipitate in agar gel, but complement-fixing activity is unimpaired. It may be noted that heating of sera to 59°C. has no deleterious effect on precipitating or CF antibody. In the results shown below those of precipitation tests for antigen were obtained in Madras, while the results of tests for antigen by complement fixation are those obtained in Denver. In these CF tests sera were tested for antigen in dilutions from 1/10 upwards against an antivaccinial rabbit serum used at optional dilution (1/80).

Tests for antibody

The techniques used for antibody estimations were those described in the previous paper (Downie, St Vincent, Rao & Kempe, 1969).

RESULTS

The severity of infection as reflected by the presence of virus or virus antigen in blood

The findings in relation to the degree of viraemia on different days after onset are shown in Fig. 1. The type I cases showed the heavy viraemia in the early days of illness, but in a few of the type II cases the viraemia was still pronounced before death. Of the unvaccinated patients, four, examined on the 7th, 8th, 9th and 11th days, showed no viraemia by the techniques used and of the remaining 16 shown in Fig. 1, nine had a + + + + viraemia. Of the five type II patients who survived three had + viraemia on the 5th, 6th and 7th days and two were negative

on the 7th and 9th days. Only after the 8th day of illness (type II cases) were there more negative than positive blood cultures (5 to 4).

Of the 16 patients who showed antigen in serum by the precipitation-in-agar-gel test all had viraemia of + + + + degree (Table 2); all were type I cases. One was

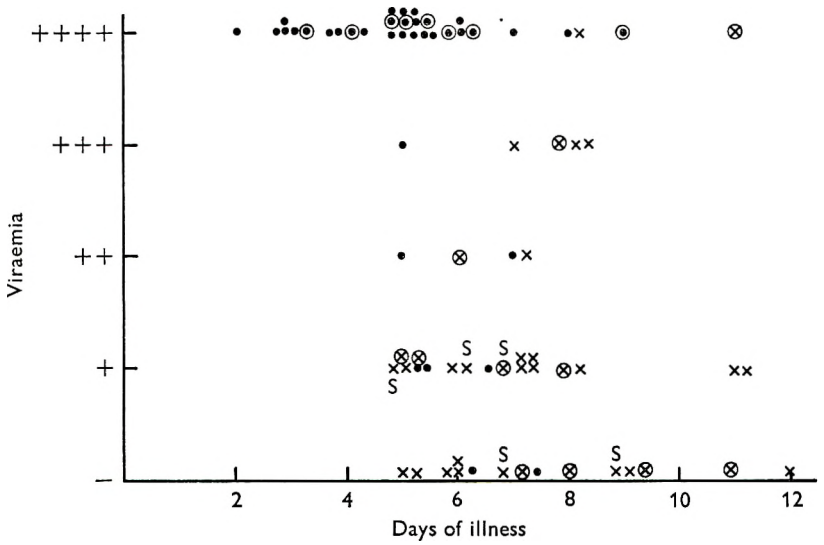

Fig. 1. Viraemia in haemorrhagic smallpox patients. ●, Type I; ×, type II; ⊙, ⊗, unvaccinated, type I or type II; S, survived.

Table 2. *Relationship of viraemia to antigen in blood serum detected by precipitation in agar gel*

	Precipitinogen	
Blood culture	–	+
–	9	0
+	11	0
+ +	2	0
+ + +	2	0
+ + + +	4	16
Totals	28	16

Table 3. *Comparison of results of tests for antigen in serum by precipitation in agar gel and by complement fixation*

	Precipitinogen	
Complement-fixation titre	–	+
< 10	10	0
10	3	0
20	6	2
40	4	5
80	0	5
160	0	3
Totals	23	15

positive on the 7th day, the others on the 2nd to 6th day of illness. The results of tests for antigen in the blood by complement fixation are shown in Fig. 2. The high titres of antigen were shown in specimens examined up to the 6th day of illness. Of the 38 sera examined for antigen both by precipitation and CF techniques, 15 were positive by precipitation and 28 by CF (Table 3). All eight sera giving a titre of 1/80 or 1/160 by CF, five of nine giving a titre of 1/40 and two of eight giving a titre of 1/20 were positive by the precipitation tests. The CF technique

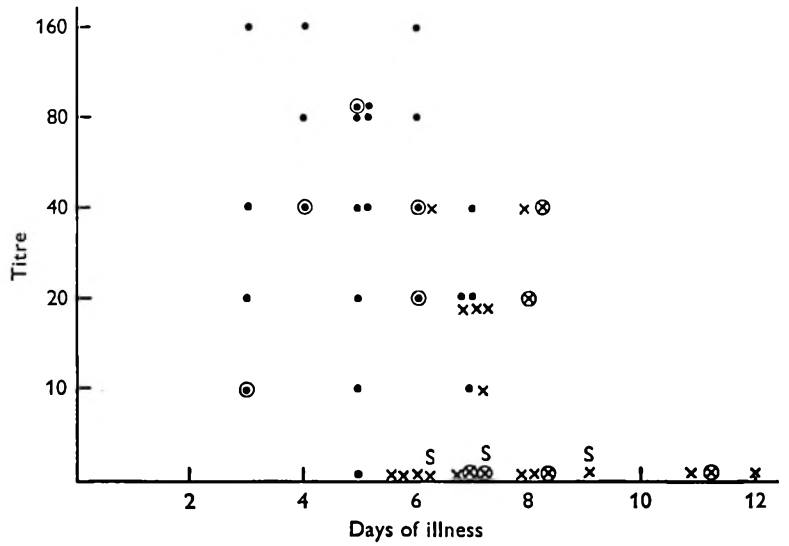

Fig. 2. Antigen in serum of haemorrhagic smallpox patients. •, Type I; ×, type II; ⊙, ⊗, unvaccinated, type I or type II; S, survived.

Table 4. *Relationship of viraemia to presence of antigen in serum measured by complement-fixation technique*

Blood culture	CF antigen titre < 10	10	20	40	80	160	Totals
−	7	1	−	−	1	−	9
+	4	2	3	1	−	−	10
+ +	−	1	2	−	−	−	3
+ + +	2	−	−	2	−	−	4
+ + + +	−	1	3	6	5	3	18
Totals	13	5	8	9	6	3	44

is obviously a more sensitive test for detecting antigen in blood than is the precipitation-in-agar-gel test. In general, the amount of antigen detected in the blood (CF technique) was proportional to the degree of viraemia (Table 4), although there were two patients with + + + viraemia in whom antigen was not detected. One patient, who had an antigen titre in his blood of 1/80 by CF test, had no virus in his serum, but whole blood was not cultured from this patient.

These findings are set out in relation to the two types of haemorrhagic smallpox in Table 5. The results in the two type II cases showing a + + + + viraemia were obtained on the 8th and 11th days of illness, the day of death in each case. It can be seen from Table 5 that the greater severity of infection in type I patients is reflected in the greater degree of viraemia and amount of soluble antigen in the blood. Examinations were made earlier in type I patients as they did not live so long, but the difference is apparent if those examined on the 6th and 7th days are compared (see Figs. 1 and 2, and Table 6).

Table 5. *Virus and virus antigen in blood of types I and II haemorrhagic smallpox patients*

	Degree of viraemia					Antigen in blood by: Precipitation		Antigen in blood by: Complement fixation	
	+ + + +	+ + +	+ +	+	−	+	−	+	−
Type I	30	0	2	3	2	16	6	23	1
Type II	2	4	3	14	13	0	23	9	14

Antibody studies in haemorrhagic smallpox

These were carried out in only 47 patients and the results are shown in Table 6. The results from type I and type II patients are listed separately in order of the days of illness on which specimens were collected.

Precipitins

Only five gave a precipitation test in agar gel for antibody when tested with a vaccinial or smallpox antigen (nos. 26, 31, 42, 43 and 46). All were type II cases. In four of the five, antibody was detectable by CF test and all five had a neutralizing titre of 1 in 90, or over. Virus was present in small amount in the blood of two (nos. 31 and 42, Table 6).

Haemagglutinin inhibiting antibody

The results of these tests are shown in Fig. 3 and Table 6. The results in unvaccinated patients tended to be lower than the others. But the titre of HI antibodies were not appreciably less than those in non-haemorrhagic cases (compare Fig. 3, and fig. 2 in the previous paper (Downie, St Vincent, Goldstein, Rao & Kempe, 1969)).

CF antibody

Only six patients showed antibody in serum by this test. All were type II cases and all had neutralizing titres of 1/68 or over. In one of these patients (no. 31, Table 6) both antigen and antibody were detected in the blood by the CF technique.

Table 6. *Virus, antigen and antibody in blood of types I and II haemorrhagic smallpox patients*

Case no.	Age and sex	Vaccination history	Day of collection	Virus in blood	Antigens in blood		Antibody in serum				Day of death
					Ppt.	CF	Ppt.	CF	HI	Neut.	
				Haemorrhagic smallpox type I							
1	40 F	+	3	+ + + +	+	160	–	–	10	< 10	3
2	30 F	+	3	+ + + +	+	40	–	–	160	< 20	4
3	20 FP*	–	3	+ + + +	–	10	–	–	< 10	12	4
4	36 F	+	3	+ + + +	+	20	–	–	20	< 30	4
5	22 FP	+	4	+ + + +	+	80	–	–	20	< 10	4
6	25 F	–	4	+ + + +	+	40	–	–	NT	< 10	4
7	22 F	+	4	+ + + +	+	160	–	–	20	35	4
8	26 M	+	5	+ + + +	+	80	–	–	NT	< 10	5
9	30 FP	+	5	+ + + +	+	40	–	–	40	< 20	5
10	25 F	+	5	+ + +	NT	–	–	–	20	10	5
11	22 M	+	5	+ + + +	+	20	–	–	80	40	6
12	18 FP	+	5	+ + + +	+	80	–	–	40	< 10	6
13	7 M	+	5	+ + + +	+	80	–	–	20	< 20	6
14	25 FP	+	5	+	NT	10	–	–	NT	30	6
15	14 F	+	5	+ + + +	–	40	–	–	40	< 10	7
16	28 F	–	5	+ + + +	+	80	–	–	20	< 10	11
17	30 FP	+	6	+ + + +	+	160	–	–	40	< 20	6
18	24 M	–	6	+ + + +	+	40	–	–	40	< 10	6
19	30 FP	+	6	– †	NT	80	–	–	40	< 10	7
20	55 M	–	6	+ + + +	–	20	–	–	10	< 10	8
21	28 FP	+	7	+ +	–	20	–	–	80	125	7
22	29 F	+	7	–	–	10	–	–	80	20	7
23	25 M	+	7	+ + + +	+	40	–	–	40	< 10	8
24	30 M	+	7	+	–	20	–	–	40	47	8
				Haemorrhagic smallpox type II							
25	37 FP	+	6	+	–	40	–	–	20	32	7
26	50 F	+	6	NT	NT	–	+	40	160	240	8
27	53 M	+	6	–	–	–	–	–	80	42	10
28	25 M	+	6	–	–	–	–	–	40	< 10	11
29	25 F	+	6	+	–	–	–	10	80	500	Sur.
30	30 M	+	7	+ +	–	20	–	–	80	20	7
31	30 F	+	7	+	NT	20	+	40	160	200	8
32	26 FP	+	7	+	–	10	–	–	20	< 10	9
33	28 M	+	7	NT	NT	20	–	–	80	< 10	9
34	22 M	+	7	NT	–	–	–	–	40	35	16
35	17 M	–	7	–	–	–	–	–	20	< 10	16
36	20 M	–	7	+	–	–	–	–	20	18	Sur.
37	25 F	–	8	+ + +	–	40	–	–	40	< 10	8
38	23 M	+	8	+ + +	–	40	–	–	40	< 10	8
39	20 F	–	8	–	NT	–	–	–	40	< 10	9
40	35 FP	+	8	+ + +	NT	–	–	10	80	68	9
41	25 M	–	8	+	–	20	–	–	NT	< 10	10
42	41 M	+	8	+	NT	–	+	160	80	800	11
43	32 F	+	9	–	–	–	+	–	20	90	Sur.
44	55 F	–	11	–	–	–	–	–	160	90	13
45	70 M	+	11	+	–	–	–	–	10	< 30	19
46	25 F	+	12	–	–	–	+	160	160	110	17
47	30 M	?		NT	–	20	–	–	80	80	?

Ppt. = precipitation test; CF = complement-fixation test; HI = haemagglutinin inhibition test; neut. = neutralization test; NT = not tested; Sur = survived.

* P after F = pregnant.

† Only blood serum tested for virus.

Neutralizing antibody

The titres of neutralizing antibody are shown in Fig. 4 and Table 6. They are obviously low in comparison with those found for non-haemorrhagic cases (fig. 4 of the previous paper).

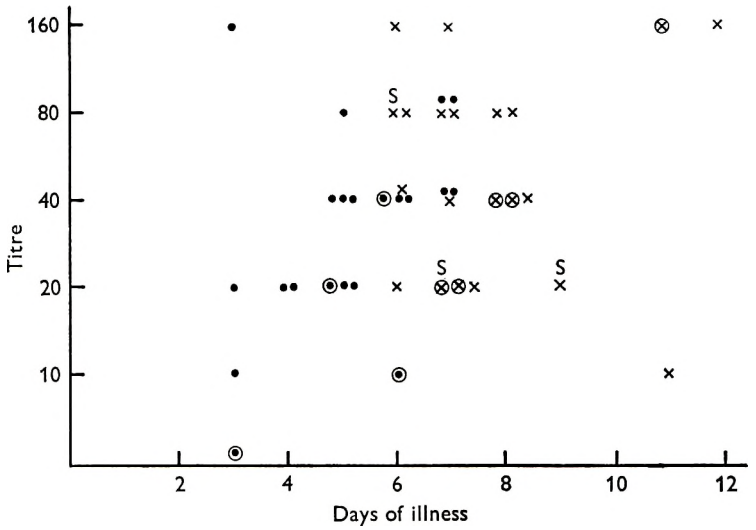

Fig. 3. Haemagglutinin-inhibiting antibody in serum of haemorrhagic smallpox patients. •, Type I; ×, type II; ⊙, ⊗, unvaccinated, type I or type II; S, survived.

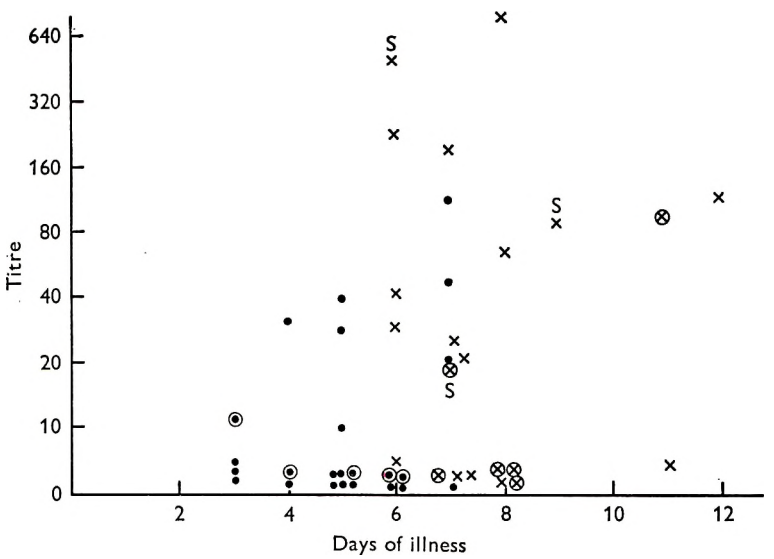

Fig. 4. Neutralizing antibody in serum of haemorrhagic smallpox patients. •, Type I; ×, type II; ⊙, ⊗, unvaccinated, type I or type II; S, survived.

In general, the type II patients showed higher antibody titres than the type I patients. This appears to be so when the comparison is restricted to results on sera collected on the 6th and 7th days of illness. The five patients who recovered were type II infections.

DISCUSSION

The haemorrhagic cases of smallpox differ from the other clinical types in that virus is found in the blood of the haemorrhagic cases in greater amounts and later in the disease (Downie *et al.* 1953; Mitra *et al.* 1966). This has been confirmed in the present study, although examination for virus was made in a comparatively small number of the non-haemorrhagic patients. Virus was found in small amounts between the 4th and 7th days of illness in the blood of two nonhaemorrhagic cases and three type II haemorrhagic cases who recovered. The finding of small amounts of virus in the blood after the second day of illness does not necessarily indicate a fatal illness as suggested by Downie *et al.* (1953). In none of the non-haemorrhagic cases of smallpox was soluble antigen found in blood whereas it was present in most of the type I and in more than a third of the type II haemorrhagic patients. These findings show that particularly in type I haemorrhagic smallpox there is an overwhelming proliferation of virus in the tissues with overflow of virus into the circulation and the appearance there of soluble viral antigens often in high concentration. The situation is perhaps similar to that in acute meningococcal septicaemia which may present a similar clinical picture. Many of the patients die before an antibody response might be expected in an ordinary case, but in those who survive beyond the sixth day of illness, antibody response is poor or absent (Table 6).

The haemorrhages into the skin and mucous membranes which are a feature of the most severe forms of smallpox probably result from the severity of the generalized infection. Histological evidence indicates that before skin lesions appear there is infection of capillaries in the dermis as indicated by swelling of the vascular endothelium and an infiltrate of mononuclear cells around the vessels. Indeed growth of virus in the endothelial lining of these vessels may contribute to the prolongation of viraemia in severe cases. The damage to the walls of capillaries may be severe enough to permit the extravasation of blood and may trigger disseminated intravascular clotting as in other viral diseases characterized by haemorrhages into the skin (McKay & Margaretten, 1967). It has been suggested that the defects in the clotting mechanism found in haemorrhagic smallpox are a consequence of disseminated intravascular clotting (Roberts *et al.* 1965; McKenzie *et al.* 1965). These defects may contribute to the bleeding tendency and be amenable to therapy with heparin to interrupt the intravascular clotting.

Herrlich *et al.* (1959),* because of the early appearance of antibody in eight of ten cases of primary haemorrhagic smallpox and the occurrence of this form of the disease in vaccinated persons, suggested that allergy might play a part in its causation. The antibodies were demonstrated mostly by HI tests and estimation of neutralizing antibody titres was not made. Our own experience recorded above and that of Sarkar, Chatterjee & Mitra (1967) indicates that the overall antibody response is poor in haemorrhagic cases. It is true that haemorrhagic infections are seen frequently in patients who show scars from previous vaccination, but the

* Herrlich no longer believes that allergy is an important factor in the genesis of haemorrhagic smallpox (personal communication).

extensive data from Madras indicate that the incidence of the severest forms of smallpox is considerably less among vaccinated than among unvaccinated adult patients (Report, 1968), and haemorrhagic smallpox is much more frequently seen in adults than in children. The severity of the generalized viral infection in haemorrhagic smallpox indicates an unusual lack of resistance on the part of the patient, irrespective of the history of previous vaccinations, but the reason for this susceptibility is unknown. The pregnant woman is five to ten times more prone to develop haemorrhagic smallpox than males or non-pregnant females of the same age group (Rao, Prahlad, Swaminathan & Lakshmi, 1963), and we have previously suggested that a high level of circulating steroids may be responsible for the severity of smallpox in the pregnant woman.

Sarkar & Mitra (1967) have suggested that strains of virus isolated from haemorrhagic cases are of enhanced virulence as compared with strains from milder clinical cases of variola major. Epidemiological evidence does not support this view, for contacts of severe cases may suffer from mild clinical illness and haemorrhagic smallpox patients may derive their infection from mild cases. The occurrence of acute fulminating smallpox appears to be dependent on the unusual susceptibility or lack of resistance in the host rather than on enhanced virulence of the infecting strain.

The observations recorded above indicate that in the acute haemorrhagic smallpox case, the culture of smallpox virus from the blood or the demonstration of antigen by the precipitation-in-agar-gel test or the more sensitive CF test may provide confirmation of the diagnosis in the absence of a focal eruption. These examinations may not be so useful in late haemorrhagic cases but in such patients virus or virus antigen can be readily demonstrated in the focal skin lesions. The demonstration of antigen in the blood carries a bad prognosis for we have found it only in patients who succumb to their disease.

SUMMARY

In practically all acute fulminating smallpox infections—haemorrhagic type I cases—there is severe viraemia with 10^4 or more infective particles of virus per ml. of blood. In most of these patients soluble antigen can be demonstrated in serum by precipitation in agar gel tests, or by the complement-fixation technique. In late haemorrhagic cases (type II) the degree of viraemia is less and soluble antigen is less often demonstrated in the blood. Five of forty type II patients recovered. The majority of the 77 patients studied were adults and bore scars of previous vaccination. Thirteen were pregnant women and 10 of these suffered from type I infections.

The antibody response in patients who survived 6 days or longer as determined by the estimation of precipitins, CF antibodies and neutralizing antibodies in serum, was considerably less than that seen in non-haemorrhagic smallpox patients.

In acute fulminating smallpox infections, the finding of virus or soluble antigen in the blood is of value in establishing the diagnosis. Soluble antigen is usually found

in the blood of patients suffering from severe viraemia and with the methods used has been demonstrated only in patients who are to die of their disease. Haemorrhagic smallpox represents a generalized virus infection of unusual severity in patients who show little resistance to their infection. The cause of this unusual susceptibility is unknown but there is little evidence that specific allergy to the virus is a feature of this form of the disease.

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Conjunctivitis and subclinical infection in smallpox

By C. H. KEMPE, F. DEKKING, L. St VINCENT, A. R. RAO and A. W. DOWNIE

Department of Pediatrics, University of Colorado Medical Center, and the Infectious Disease Hospital, Tondiarpet, Madras

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Of the milder manifestations of smallpox infection, variola sine eruptione has long been recognized. This is seen in vaccinated contacts and serological evidence of the variolous nature of these cases has been obtained (Downie & McCarthy, 1958). Occasionally virus has been recovered from throat swabs or throat washings from such cases (Verlinde & Van Tongeren, 1952; Bingel & Kruse, 1959; Marennikova, Gurvich & Yamasheva, 1963). Conjunctivitis occurs in a proportion of cases of ordinary smallpox and has recently been recognized by us as the only clinical manifestation of smallpox infection (Dekking, Rao, St Vincent & Kempe, 1967). A peculiar form of pneumonitis without any skin eruption, probably allergic in nature, has been described in well vaccinated contacts (Evans & Foreman, 1963), and serological evidence of subclinical infection in contacts of variola minor patients has recently been obtained by Salles-Gomes, Angulo, Menezes & Zamith, (1965).

The present paper is concerned with the study of conjunctivitis in smallpox patients and conjunctivitis in contacts without other clinical evidence of smallpox. In addition, antibody studies have been made in close family contacts who had no evident clinical illness. The significance of antibody titres as evidence of subclinical infection in these contacts has been assessed in the light of our findings in vaccinated or revaccinated persons and in cases of smallpox.

MATERIAL AND METHODS

Most smallpox patients admitted to the Infectious Disease Hospital in Madras were accompanied by a member of the family or a relative who remained with them during their hospital stay. These family members were obviously in close contact with the patient they were attending and in a heavily infected environment, often for many days. They were revaccinated on the day of admission or next day, and were under close surveillance during their hospital stay.

Of these contacts 21 developed conjunctivitis but no rash or other signs of smallpox infection. From these 21, swabs of conjunctival exudate were examined for the presence of smallpox virus and the sera from 12 were examined for antibody. All these patients bore scars of previous vaccination with the exception of patient no. 3 in Table 1.

Requests for reprints should be addressed to Dr C. H. Kempe, 4200 East Ninth Avenue, Denver, Colorado 80220.

From 30 further contacts, who had no signs or symptoms of illness during their hospital stay and had been vaccinated earlier in life, blood was collected usually on the 1st or 2nd day after admission; a later sample was collected for serological examination from only four of these.

Four additional contacts developed typical smallpox, 3, 5, 10 and 10 days after admission to hospital. These were all adult females; three had been vaccinated earlier in life but had not been revaccinated before admission to hospital. The fourth had not been vaccinated before admission. Antibody titrations were made on the sera of these contact cases; the results are included in the second paper of this series (Downie *et al.* 1969) and are not further considered here.

In addition to the observations on the hospital contacts the results of virological examination of conjunctivitis seen in 84 cases of smallpox are recorded in this paper.

From patients showing conjunctivitis material was collected on cotton-wool swabs which were rubbed gently over the inner surface of the eyelids and the conjunctival sac. Each swab was then extracted in 1 ml. of Hanks's solution to which broth had been added to make 10%. This extract was used to inoculate the chorioallantois of 11- to 13-day chick embryos. Negative and doubtful membranes were subcultured to further chick embryos. Examination for antibodies was made as described.

RESULTS

Conjunctivitis in smallpox patients

The results of examination for virus in relation to the stage of disease are shown in Fig. 1. Of those patients from whose conjunctivas virus was recovered late in the disease, the conjunctivitis had been present not later than 15 days from the onset of illness. In a few cases the conjunctivitis occurred at the onset of fever and before the appearance of the focal eruption. In some patients from whom virus was not recovered, conjunctivitis occurred late in the course of the disease, although in others conjunctivitis, apparently not variolous in nature, was seen early in the illness.

Conjunctivitis in contacts who did not develop smallpox

Variola virus was recovered in culture of the conjunctival exudate from 12 of the 21 cases, but in only five of these was serum examined for antibody. The results of these tests for antibody are shown in Table 1. In case no. 3 serum was collected 9 days before the onset of conjunctivitis and antibody had not developed at this time. In the other four the antibody tests, made 6-14 days after the onset of conjunctivitis, showed the kind of antibody titres obtained in typical cases of smallpox, and in the three patients from whom a second sample of serum was taken there was an obvious rise in antibody following the conjunctivitis.

Smallpox virus was not recovered from the conjunctival exudate of nine contacts who developed conjunctivitis after admission. Antibody findings in seven of these are shown in Table 2. In the first patient there was no significant rise in antibody following the conjunctivitis but the results of precipitation, complement-

fixation and neutralization tests indicate that this contact had suffered either clinical or subclinical infection with smallpox virus before admission to hospital. Contact no. 4 showed a marked rise in antibody to levels typical of those seen in response to smallpox infection. Examination of conjunctival exudate on three

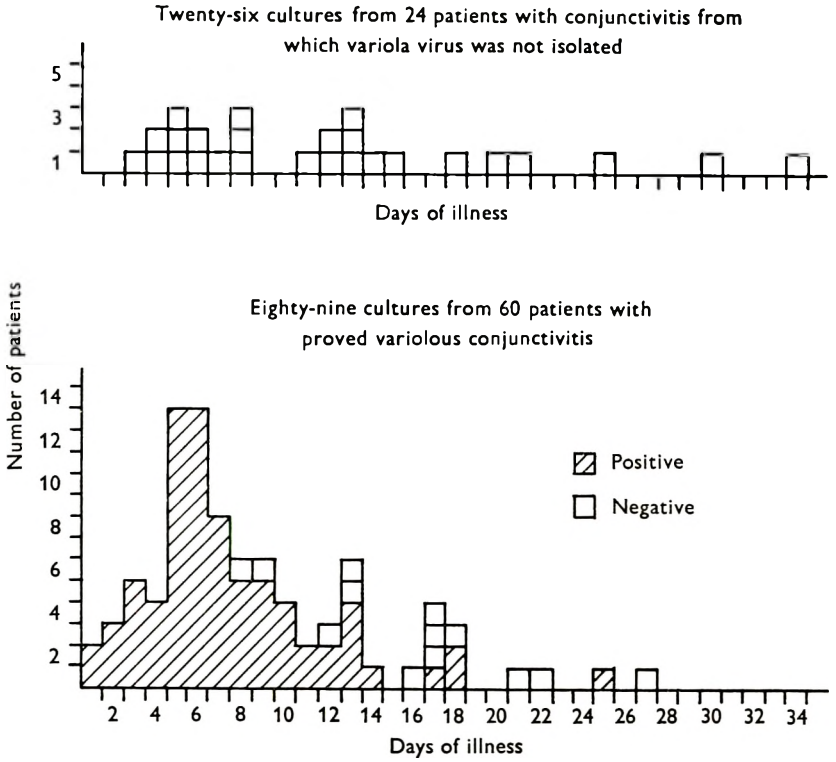

Fig. 1. Tests for virus in the conjunctival exudate of 84 smallpox patients showing clinical evidence of conjunctivitis. ▨, Positive; □, negative.

Table 1. *Antibody titres in five of twelve contacts who developed variolous conjunctivitis, but no other clinical evidence of smallpox*

Contact no.	Precipitation	CF titre	HI titre	Neutralization titre	Date of serum sample in relation to date of Conjunctivitis (days)	Date of serum sample in relation to date of Revaccination (days)
1	–	–	10	80	– 5	– 1
	+	–	80	500	13	17S*
2	+	20	40	300	14	20F*
3	–	–	20	9	– 9	2S
4	–	–	20	30	4	7S
	+	160	80	5000	8	11S
5	–	–	40	20	3	7F
	+	320	80	1000	6	10F

* S = vaccination on admission to hospital successful; F = vaccination on admission to hospital, no major reaction.

separate occasions failed to recover variola virus and it seems likely that this contact suffered subclinical infection with smallpox virus after admission to hospital. The remaining contacts in this group showed no significant increase in antibody after their conjunctivitis nor were their antibody levels suggestive of recent smallpox infection.

Table 2. *Antibody titres in seven of nine contacts who developed conjunctivitis from which virus was not isolated*

Contact no.	Precipitation	CF titre	HI titre	Neutralization titre	Date of serum sample in relation to date of Conjunctivitis (days)	Date of serum sample in relation to date of Revaccination (days)
1	+	640	40	700	−11	0F
	+	320	20	600	10	21F
2	−	−	10	60	−10	0F
	−	10	20	140	3	13F
3	−	−	10	100	−3	0S
4	−	−	10	100	−10	0F
	+	160	80	2000	16	26F
5	−	−	10	120	−4	−1F
	−	−	10	160	20	23F
6	−	−	10	95	−9	−8F
	−	−	<10	95	6	7F
7	−	−	<10	100	−21	−20*
	−	−	20	100	7	8*

In the last column, F and S have the same significance as in Table 1.
* Result of revaccination not recorded.

Antibody studies in contacts who suffered no illness while in hospital

Of thirty contacts in this category, tests were made on sera collected on the 2nd day after admission and, in four of them, 16–31 days later. None of these four sera showed a rise in titre; precipitation and CF tests were negative and neutralizing titres were relatively low. From the remaining 26 contacts serum was collected only on the 1st to 3rd day after admission except in one contact bled on the 29th day. Antibody levels in 18 were such as might be expected in persons with a previous history of vaccination—all gave negative precipitation tests, all except one were negative for CF antibody and all had neutralizing titres less than 1/350. In eight the findings were suggestive of recent smallpox infection (Table 3). The sera showed precipitation in agar gel with a vaccinia or variola antigen and other antibody titres consistent with smallpox infection. None of these patients gave a history of smallpox nor did any exhibit evidence of scarring. The serological findings, however, suggest that these patients had recently suffered from smallpox infection, either minimal or subclinical through contact with the disease before admission to hospital, or in no. 8, Table 3, possibly after admission to hospital.

Table 3. *Antibody titres in eight of thirty contacts who developed no illness*

Contact no.	Precipitation	CF titre	HI titre	Neutralization titre	Days after revaccination
1	+	20	40	200	4S
2	+	80	80	500	1F
3	+	80	40	500	1F
4	+	40	10	< 100	0F
5	+	160	80	700	1F
6	+	160	80	2000	1F
7	+	160	20	4000	1S
8	+	< 10	40	200	29F

In the last column, F and S have the same significance as in Table 1.

DISCUSSION

The results of virological examination of conjunctival exudate from smallpox patients showing conjunctivitis indicate that, while conjunctivitis developing in smallpox convalescents is not usually caused by variola virus, this may also apply to a small proportion of patients in whom conjunctivitis occurs in the first 8 days of illness (12 of 63 in Fig. 1). The occasional appearance of variolous conjunctivitis at or before the onset of fever suggests that the conjunctiva may have been the portal of entry of the virus in these patients. As noted by us earlier (Dekking *et al.* 1967) conjunctivitis occurring in immunized individuals may be the only clinical manifestation of smallpox infection.

Evidence of latent infection in contacts of variola minor in a hospital ward outbreak was obtained by Salles-Gomes *et al.* (1965) by serum antibody studies. Most of these latent infections occurred in vaccinated contacts, although occasionally they were seen in individuals who had not been vaccinated nor had suffered previously from variola. In variola major, because of the greater virulence of the virus, subclinical infection would seem unlikely to occur in persons not previously immunized by vaccination or smallpox infection. One possible instance of such an occurrence has been recorded by Verlinde & Van Tongeren (1952). In the present work evidence of latent infection was obtained only in previously vaccinated persons. Although there was no history of recent smallpox in those studied, it is impossible on the information at our disposal to rule out recent occurrence of minimal clinical infection such as variola sine eruptione. More extensive studies are required in this field.

SUMMARY

Attempts were made to demonstrate variola virus in the conjunctival exudate of 84 smallpox patients who developed conjunctivitis in the acute stage of the illness or during convalescence. Variola virus was isolated from 60 but not from the remaining 24. Of the 64 from whom virus was isolated the conjunctivitis developed from the onset up to the 15th day of illness. From conjunctivitis developing later virus was not recovered. In some patients who developed conjunctivitis early in the disease we failed to recover virus from the conjunctival exudate.

Of 55 close family contacts who stayed in hospital with smallpox patients four developed smallpox. In 21, conjunctivitis but no other illness developed. From 12 of these, variola virus was recovered from the conjunctival exudate and four of these 12, who were further investigated, showed after the appearance of conjunctivitis antibody titres similar to those seen in typical smallpox cases. From nine of the contacts who developed conjunctivitis virus was not recovered. One of these had antibody titres in serum collected before the onset of conjunctivitis which indicated recent smallpox infection. In another there was a marked antibody rise during her hospital stay although examination of conjunctival exudate on three separate occasions failed to yield variola virus. Twenty-six family contacts who developed no illness in hospital had antibody determinations made on sera collected soon after admission to hospital. In eight of these antibody titres were such as to indicate recent smallpox infection although there were no signs, in the form of scarring, or history of recent smallpox infection. These findings have been discussed in relation to the occurrence of minimal and subclinical infection in close family contacts of smallpox patients.

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Variability in the characteristics of pocks produced on the chick chorioallantois by white pock mutants of cowpox and other poxviruses

By DERRICK BAXBY

Department of Bacteriology, Liverpool University, P.O.Box 147, *Liverpool, England*

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The 'pocks' produced on the chorioallantoic membrane (CAM) of the chick embryo by the variola-vaccinia group of viruses are usually sufficiently characteristic to make presumptive identification of these viruses very reliable. As well as being of use in confirming clinical diagnosis the pock character is used by those interested in the characteristics and inter-relationships of these viruses. Thus pock character has been used to study spontaneous mutants of cowpox (Haddock, 1952), rabbitpox (Fenner, 1958) and also in genetic recombination (Fenner & Comben, 1958), genetic hybridization (Woodroofe & Fenner, 1960) and non-genetic reactivation (Fenner & Woodroofe, 1960).

Stability of pock character is essential for the true interpretation of such studies and the results obtained by various workers appear to show such stability.

However, the work described here shows that some poxvirus pock mutants when grown on the CAM can 'mimic' those viruses from which they were derived, and also shows that environmental factors are important in determining pock character.

MATERIALS AND METHODS

Virus strains

Brighton strain of cowpox. Most of the work was done with the white pock mutant of this strain of cowpox. Suspensions with various passage histories on the CAM were available, including a suspension provided by Professor K. McCarthy which was made in 1956 and which had been stored at $-20°$C. since then. Dr C. R. Madeley kindly supplied a white pock mutant isolated on the rabbit skin by Professor K. R. Dumbell and passaged subsequently only on the rabbit skin.

Control experiments were done with parental red cowpox virus adapted to either CAM or rabbit skin.

Other strains of white cowpox. Professor Dumbell kindly made available the following strains of white cowpox. Austria, Larkin, Juffermans, Carmarthen, Maund and Ruthin.

Other poxviruses. Some experiments were done with white pock mutants isolated from the Utrecht strain of rabbitpox, Levaditi and ISM strains of vaccinia, Evans (British) and Wyeth (American) commercial vaccine strains, and the Merck strain of monkeypox. The Lister strain of vaccinia, Mill Hill strain of ectromelia, Harvey strain of variola and Butler strain of alastrim were also used.

Fertile hens' eggs

Fertile hens' eggs of various breeds were obtained from commercial suppliers. They were prepared for inoculation as described by McCarthy & Dumbell (1961). 12-day-old embryos from a particular breed of White Leghorn fowls (WL 4) were used unless otherwise stated. Virus dilutions were made in 10% nutrient broth saline and 0·1 ml. volumes were inoculated 1 hr. after the CAM had been prepared.

Incubation

The incubators used were controlled by 'Accuron' thermostats and heaters. Constant recording of temperature was made by 'Grant' recorders. Thermostats and recorders were accurate within $\pm 0{\cdot}1^{\circ}$C.

General virological techniques

Techniques for measuring the virus content of single pocks, measuring pock diameters and for excision, passage and histology of single pocks were essentially those described by Fenner (1958).

Gel diffusion

Extracts of infected tissue were tested by gel diffusion for presence of 'd' antigen using reference materials already described (Baxby & Rondle, 1968). Antigen 'd' is present in red cowpox-infected tissues but not in white cowpox-infected tissues (Rondle & Dumbell, 1962).

RESULTS

When referring to cowpox viruses and the pocks they produce the following terms will be used.

Parental red cowpox virus (PCV). This produces haemorrhagic, ulcerated lesions (PCV pocks) on the CAM (Downie, 1939), and also produces a white pock mutant which is genetically stable (Downie & Haddock, 1952).

White cowpox virus (WCV). This is the white pock mutant. It is shown in this paper to produce two types of pock, one white (WCV pock) and one indistinguishable in appearance from PCV pocks, although it has the genotype of WCV. This second type of pock is described as an atypical WCV pock (AWCV pock).

White cowpox virus

Attempts to show whether WCV pocks and AWCV pocks yield different viruses

These investigations were prompted by the presence of a high proportion (*ca.* 40%) of lesions resembling parental red cowpox pocks on CAM inoculated with the white pock mutant of Brighton cowpox. The unexpected presence of these 'atypical' pocks was thought to be due to accidental presence of PCV in the WCV stock, but various attempts to separate the two types failed. Passage of either single AWCV or single WCV pocks from these apparently mixed preparations through 10 single pock passages gave suspensions which both produced the same proportions of AWCV pocks. Ten serial passages of confluently infected CAM gave

a similar result (38 % AWCV pocks). After nine serial passages through RK 13 cells, which are 10 times more sensitive to PCV than to WCV (Baxby & Rondle, 1967) suspensions produced a similar proportion of AWCV pocks (36 %) when inoculated on CAM. Finally a new line of WCV was established by pock picking from the parental red strain. After 12 serial single pock passages it produced 38 % AWCV pocks, although parental red cowpox virus could no longer be recovered after the 2nd passage.

Of the various explanations possible, the one favoured was that white cowpox could produce two types of pock, one white and another indistinguishable from the parental type.

Rabbit inoculation

When inoculated on the rabbit skin various preparations of white cowpox gave no evidence of lesions characteristic of parental red cowpox. This suggested that the white cowpox suspensions were free of red cowpox. It also suggested that the factors responsible for production of 'atypical' white cowpox lesions on the CAM were not effective in the rabbit skin.

Gel diffusion

Further evidence that white cowpox virus preparations were uncontaminated with parental red cowpox strains was obtained by the failure of infected tissues to produce antigen 'd' and of infected rabbits to produce antibody to this antigen.

Characteristics of lesions produced by parental red cowpox and white cowpox viruses on the CAM

Fenner (1958) compared the characters of various poxviruses. Of the characters he used the only ones applicable to single pocks are the appearance, size and amount of virus extractable from single pocks.

Appearance of pocks

Macroscopically the atypical white cowpox lesions were indistinguishable from those produced by parental red cowpox. Stained sections of PCV pocks showed the characteristic inclusions and extensive haemorrhage first described by Downie (1939) (Pl. 1 A). Large cytoplasmic inclusions were present throughout the ectoderm and endoderm and could often be seen in the walls of blood vessels. WCV pocks showed no haemorrhage but were characterized by marked leucocyte infiltration and endodermal proliferation. Inclusions were smaller and were usually limited to cells at the edge of the pock (Haddock, 1952) (Pl. 1 C). AWCV pocks produced by white cowpox virus were indistinguishable from parental red cowpox (Pl. 1 B), haemorrhage being extensive and inclusions large and widespread.

Size of pock

As shown in Table 1 pocks produced by PCV were slightly larger than the AWCV pocks. However, the variation in size of both types was so great that they could not be distinguished with any certainty.

Amount of virus extracted from pocks

Fenner (1958) showed that about 10 times more virus could be extracted from disrupted pocks of PCV than from WCV. This was confirmed for PCV and WCV pocks. However, the same amount of virus could be extracted from both PCV pocks and the AWCV pocks (Table 1).

Factors affecting production of atypical pocks by white cowpox virus

The character of a pock is undoubtedly determined by the interplay of many complex physiological factors which are difficult to control in such a self-contained host as the chick embryo. Hence the effects of changing only simple environmental factors on the characteristics of the pocks were tested.

Table 1. *Size and virus content of pocks produced on chorioallantoic membrane by parental red cowpox and its white pock mutant*

	Pock size			Virus content		
	No. tested	Diam. (mm)*	S.D.† (mm)	No. tested	Log titre*	Log S.D.†
White cowpox virus pock	59	1·5	± 0·4	13	5·8	± 0·4
Atypical white cowpox virus	52	1·4	± 0·4	15	6·8	± 0·36
Parental red cowpox	84	1·7	± 0·5	10	6·8	± 0·38

* Pocks tested after 72 hr. incubation.
† S.D. = standard deviation.

Table 2. *Passage histories and strains of white cowpox in which atypical pocks have been seen*

Strains	Passage history before test*	Atypical pocks(%)	Strains	Passage history before test*	Atypical pocks(%)
Brighton	R 6	48	Austria	E 2	36
Brighton	E 40 RK 9†	36	Larkin	E 4	39
Brighton	E 20	38	Ruthin	E 18	43
Brighton	E 25‡	39	Juffermans	E 8	43
Brighton	E 40	42	Carmarthen	E 8	49
Brighton	E 50	38	Maund	E 11	36
Brighton	E 40 W 10§	38			
Brighton	E 40 R 10‖	41			
Brighton	Red/W 12¶	38			

* R = passaged on rabbit skin, E = on CAM.
† E 40 RK 9 = 40 confluent CAM passages then 9 passages in RK 13 cells.
‡ This suspension was made in 1956 and stored at −20° C., until tested in 1969.
§ E 40 W 10 = 40 confluent CAM passages then 10 single white pock passages.
‖ E 40 R 10 = 40 confluent CAM passages then 10 single atypical pock passages.
¶ Red/W 12 = New white cowpox strain after 12 single pock passages.
All suspensions were tested on White Leghorn (type 4) embryos at 35° C. (see Table 3).

Passage history of virus

As indicated previously serial passage of various suspensions did not alter the proportion of red pocks, suggesting that the phenomenon was independent of passage history. These figures are shown in Table 2. It is interesting that atypical pocks were produced both by a suspension of WCV which had been prepared in 1956 and stored at $-20°$C. since then, and also by the white pock mutant isolated on the rabbit skin on its first passage on the CAM.

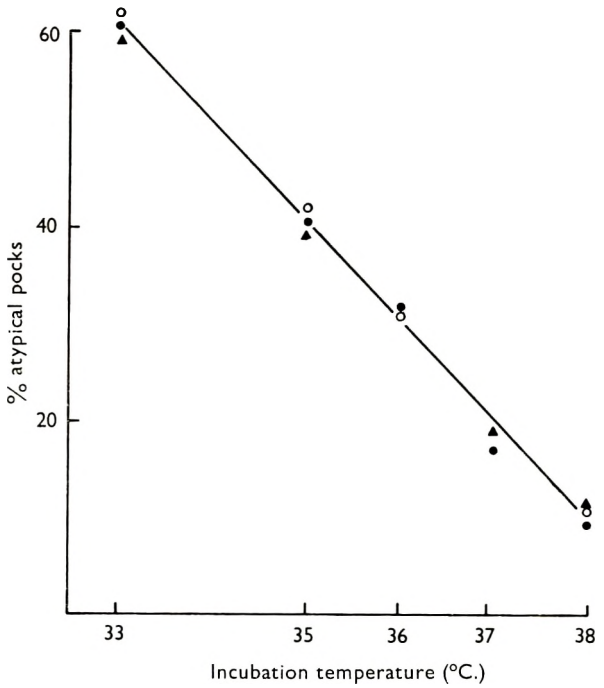

Fig. 1. Effect of incubation temperature on production of atypical pocks by white cowpox. ●, ○, ▲, three separate experiments.

Virus strains

The original observations on Brighton white cowpox were also repeated with all of six other strains (Table 2).

Inoculation technique

Most of the variables tested by Westwood, Phipps & Boulter (1957) in their study of poxvirus titration techniques were tested for their effect on pock character of white cowpox. The only factor found to alter the proportion of atypical pocks was the age of the embryos. Embryos 10, 11 and 14, 15 days old produced only half as many AWCV pocks as embryos 12 and 13 days old.

Dose response

With various poxviruses it has not been uncommon to find a certain degree of haemorrhage on CAM bearing confluent infections. With CAM bearing semi-

confluent and confluent infection with white cowpox virus the degree of haemorrhage was so marked that many membranes were indistinguishable from those infected with parental red cowpox. On CAM inoculated with up to *ca.* 150 pocks/membrane the percentage of atypical pocks remained constant at about 36–42 %.

Incubation temperature

The effect of incubation temperature on the pock character of white cowpox virus is shown in Fig. 1. The proportion of AWCV pocks was reduced by *ca.* 10 % for each 1°C. rise in temperature over the range 33–38°C. Above 38°C. atypical pocks were infrequent and Pl. 2 B shows a membrane incubated at 39°C. which

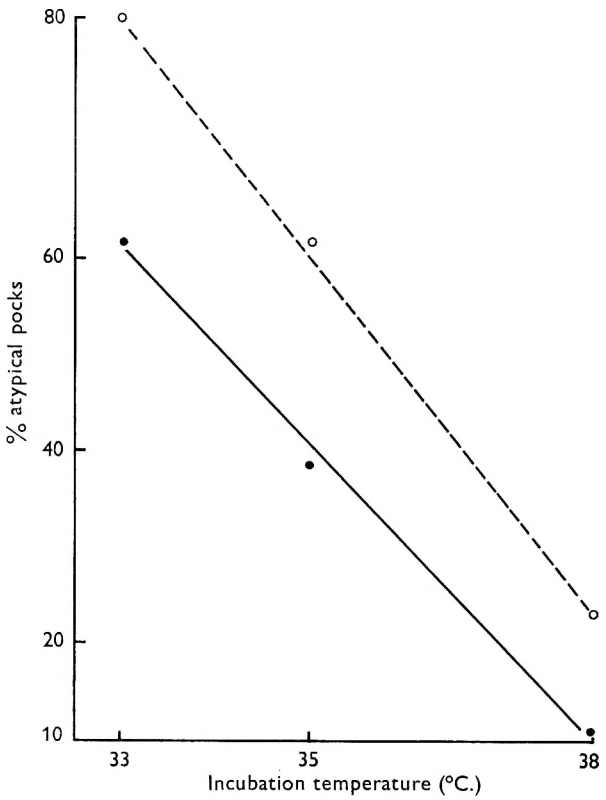

Fig. 2. Effect of position of chorioallantoic membrane during incubation on production of atypical pocks by white cowpox virus. ●——●, Embryos incubated with CAM lowered; ○- - -○, embryos incubated with CAM raised up against vitelline membrane 4 hr. after infection.

gives the 'classical' appearance of white cowpox. Plate 2 C shows a membrane similarly inoculated but incubated at 33°C., showing a very high proportion of atypical pocks. Plate 2 A shows parental red cowpox virus pocks on a membrane incubated at 35°C for comparison.

Position of CAM during incubation

Initially it was noticed that atypical pocks were often more frequent round the periphery of the inoculated area and that such pocks were often on a part of the CAM which had risen back up against the vitelline membrane. Subsequently the outline of the inoculated area was marked on the shell and counts from membranes which had risen were rejected. Figure 2 shows the effect of incubating embryos with the CAM replaced in its original position by suction after incubation; the proportion of atypical pocks is considerably increased. Controls showed that the effect was caused by the actual position of the CAM during incubation rather than the trauma involved in manipulation.

Table 3. *Production of atypical white cowpox lesions on the chorioallantoic membrane of chick embryos from different sources*

Embryo*	Source. County and Dealer	Percentage atypical pocks at 33°	35°	38°	Slope of graph (%)†	Efficiency of plating‡	Mortality %§
WL 1	Kent (A)	33	18	3	7	0·7	30
WL 2	Cheshire (B)	41	27	0·7	8	0·8	30
WL 3	Cheshire (C)	52	32	4	10	1·4	80
WL 4	Glos. (D)	61	42	9	10	1·0	100
WR	Cheshire (B)	50	25	4	9	1·3	60
WC	Cheshire (B)	30	19	2	5	0·7	20
BPR	Cheshire (E)	36	18	0·2	8	1·0	0
RIR	Cheshire (B)	44	25	3	10	1·0	50
RIR × LS	Kent (A)	66	43	4	10	0·8	100
RIR × LS 2	Cheshire (F)	47	30	10	9	0·8	60
WL × RIR	Cambs (G)	59	40	6	10	1·2	90

* WL = White Leghorn; WR = White Rock; WC = White Cornish; BPR = Buff Plymouth Rock; RIR = Rhode Island red; LS = Light Sussex.

† Increase in white pocks per 1°C. rise in temperature.

‡ Ratio of infective titre in WL 4 embryos to titre in embryos tested.

§ Percentage of embryos killed in 3 days by 10^3 infective doses.

Breed of embryo

Tests on embryos from hens of different breeds are shown in Table 3. Although the percentage of atypical pocks produced at a given temperature in various breeds sometimes differed, in all breeds a 1°C. rise in incubation temperature gave *ca.* 8% decrease in atypical pocks. Although extensive tests of embryo mortality were not made there was some correlation between the proportion of atypical pocks and embryo mortality. Embryos of a breed which produced a high proportion of atypical pocks tended to be killed by virus doses which did not kill embryos producing fewer atypical pocks. The extremes were best demonstrated with a dose of *ca.* 10^3 infectious units. This killed 90–100% of White Leghorn (type 4), Rhode Island Red × Light Sussex and White Leghorn × Rhode Island Red, but killed only 0–20% of Buff Plymouth Rock and White Cornish embryos. The differences in mortality were not due to differences in efficiency of plating

(Table 3), but could be due to the more invasive nature of the atypical lesion and the greater amount of virus it contained.

The slight differences that were detected in embryos of different breeds were reproducible using different batches of such embryos. However, it was found that embryos of the same breed from one supplier did not necessarily give the same result as embryos of the same named breed from another supplier. Also, different results were obtained from different named breeds from the same supplier, which had received the same treatment as regards housing, feeding and vaccination. Hence with the evidence available it is difficult to say whether the slight differences are truly genetic, or due to some obscure environmental factor.

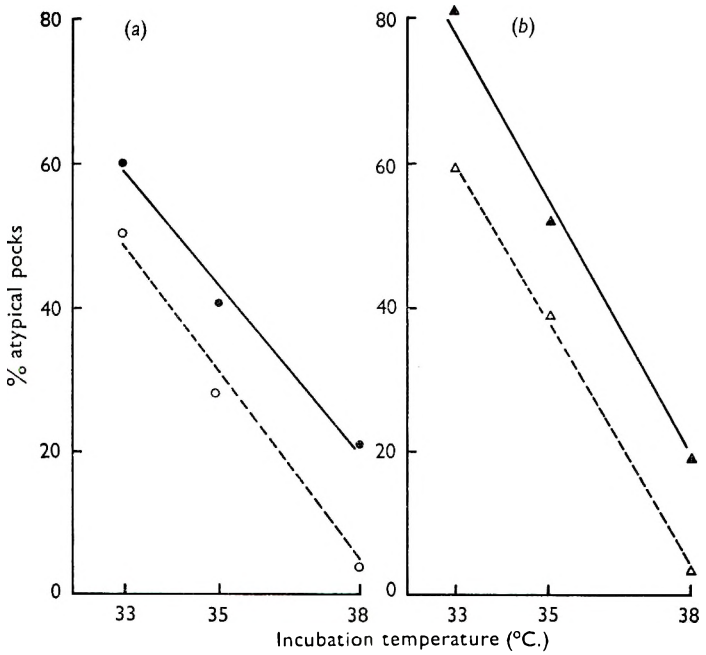

Fig. 3. Effect of incubation temperature on production of atypical pocks by (A) white pock mutant of rabbitpox virus (Utrecht), and (B) white pock mutant of vaccinia (Levaditi) in White Leghorn (type 4) and Buff Plymouth Rock embryos. Rabbitpox in WL4 (●——●), and BPR (○- - -○). Vaccinia in WL4 (▲——▲), and BPR (△- - -△).

Other poxviruses

Parental red cowpox

As white cowpox produces pocks indistinguishable from those of parental red cowpox, some of the red pocks on CAM inoculated with parental red cowpox should be of white cowpox genotype. This was tested by estimating the proportion of white pocks produced by parental red cowpox at different temperatures. At 33°C. this was 1·2 %, at 35°C., 4·8 %, and at 38°C., 15 %. However, at 38°C. some pocks which contained parental red cowpox were classed as white and so it seems that elevated temperatures can also affect the pock character of the parental virus to some extent. Cloning experiments indicated that only *ca.* two-thirds of the white pocks produced by parental red cowpox at 38°C. were white pock mutants.

White variants of rabbitpox, monkeypox and vaccinia

The pocks produced by rabbitpox virus, the Merck strain of monkeypox and various strains of vaccinia are naturally red owing to haemorrhage, although this is not usually as marked as it is with parental red cowpox. These viruses also produce white pock mutants, and some experiments were done with white pock mutants isolated from rabbitpox (Utrecht), monkeypox (Merck) and the Levaditi, ISM, Evans and Wyeth strains of vaccinia. All such variants produced some pocks resembling those produced by the parent virus. The effect of altering incubation temperature on the pock character of rabbitpox (Utrecht) and vaccinia (Levaditi) is shown in Fig. 3, whilst Pl. 2 D and E illustrates pocks produced by the Levaditi mutant at 39 and 33°C. respectively.

Lister strain of vaccinia, and variola, alastrim and ectromelia

The Lister strain of vaccinia has always been recorded as producing white pocks, and suspensions did so under all conditions tested.

In view of the variability of almost all the other poxviruses tested it was reassuring to find that the pocks produced by variola and alastrim appeared typical on the membranes of all the embryos tested. Whilst embryos of different breeds were available the opportunity was taken to test the ceiling temperatures of variola and alastrim in them. At 38·25°C., the temperature recommended by Dumbell, Bedson & Rossier (1961) for distinguishing between the two viruses, variola virus produced pocks in all breeds of embryo, alastrim in none.

Pocks produced by the Mill Hill strain of ectromelia remained constant under all conditions tested.

DISCUSSION

The pock character of white mutants has been influenced by incubation temperature, position of CAM during incubation and source and age of the chick embryo. What we do not know is the precise physiological mechanism being affected by these environmental changes. The fact that there is a particular age of embryo which produces a high proportion of atypical pocks suggests the need for a particular developmental stage in the embryo. The effect of changing the position of the CAM may affect gaseous exchange. This, and many other factors, would be influenced by temperature. As white pocks are characterized by leucocyte infiltration, and red pocks by haemorrhage and damage to blood vessels, it is possible that the environmental changes are affecting the haematopoietic and vascular systems.

These results emphasize the way in which virus and host *together* are responsible for determining pock character. Thus, parental red cowpox produces very haemorrhagic lesions on the CAM and the rabbit skin, whereas white cowpox can produce red lesions on the CAM in some circumstances, but not on the rabbit skin. Although with white cowpox the same stimulus was given to both hosts, the response by different hosts varied.

The practical significance of these results for those working with poxviruses is that pock character is not as reliable for use in the identification of certain pox-

viruses as is generally thought. Fortunately the pock character and ceiling temperatures of variola and alastrim appear to be stable and so no confusion is likely to be caused in the laboratory diagnosis of smallpox, and confusion between the other viruses and their white pock mutants is unlikely to have serious consequences in human or veterinary medicine. However, the variability in pock character described here is likely to cause confusion and to increase greatly the technical difficulties involved in doing genetic and other work in which pock character has been used in presumptive identification of poxviruses. It would be most useful if tissue culture plaque assays were available which distinguished between parental and mutant types, but McClain's results (1965) indicate that perhaps too many factors need careful control to make such methods reliable routinely. The most reliable method for distinguishing between white pock mutants and their parental types is still intradermal rabbit inoculation (Haddock, 1952; Fenner, 1958).

The fact that the phenomenon described here has not been reported before may be explained by failure of previous workers to recognize it, or because it is of recent origin brought about by changes in the virus or chick embryo. Such evidence as is available favours the last alternative. White pock mutants of cowpox have been studied extensively since their initial isolation in 1952, and nothing unusual was seen in their pock character as late as 1966 (Baxby & Rondle, 1967). In particular, attempts have been made to obtain parental red cowpox by recombination between strains of white cowpox (Bedson & Dumbell, 1964; Fenner & Greenland, 1964) and in these circumstances production of red pocks would surely have been noticed and investigated. These factors, plus the fact that atypical pocks have now been seen elsewhere (C. R. Madeley, 1969, personal communication), leads one to suggest that the phenomenon has not been missed previously but is of recent origin. That it is not due to any change in the virus is indicated by the fact that virus suspensions made in 1956 and stored at $-20°$C. until 1969 behaved in the same way as suspensions which have been passaged continually. Hence it is possible that the phenomenon may be due to some recent changes in the chick embryo.

SUMMARY

White pock mutants of cowpox virus produce pocks in the CAM which 'mimic' those produced by the parent virus, in macroscopic and microscopic appearance, and in the amount of virus they contain. The proportion of such 'atypical' pocks was influenced by the age and source of embryo, incubation temperature and position of the CAM during incubation. Similar results were obtained with white pock mutants isolated from strains of vaccinia, rabbitpox and monkeypox. The appearance of pocks produced by variola, alastrim, ectromelia and vaccinia (Lister) was unaffected by changes in environmental conditions.

I would like to thank Professor K. McCarthy, Professor K. R. Dumbell, Dr C. H. Taylor-Robison and Dr C. R. Madeley for their interest and for kindly providing virus strains, and Miss M. Seyfang for technical assistance.

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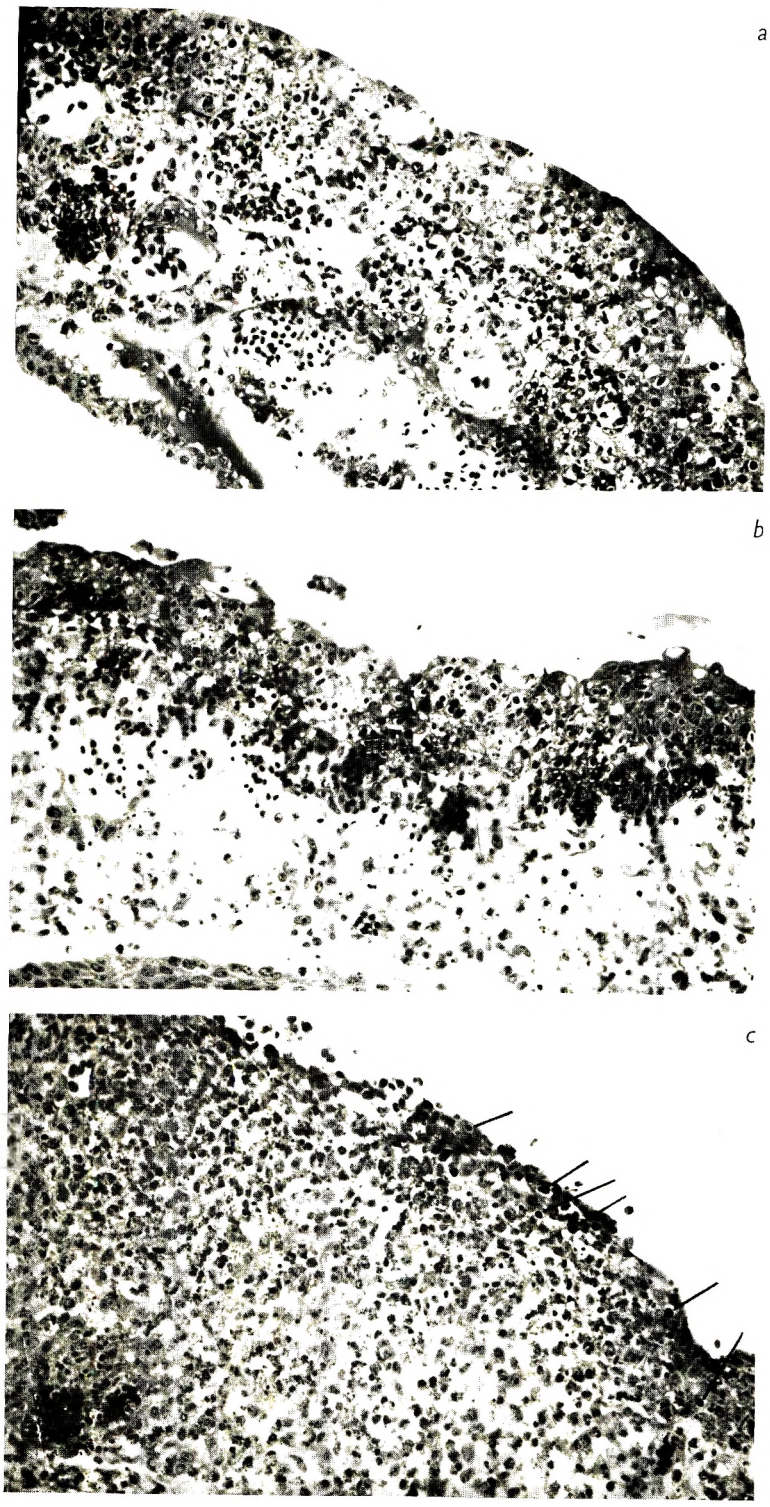
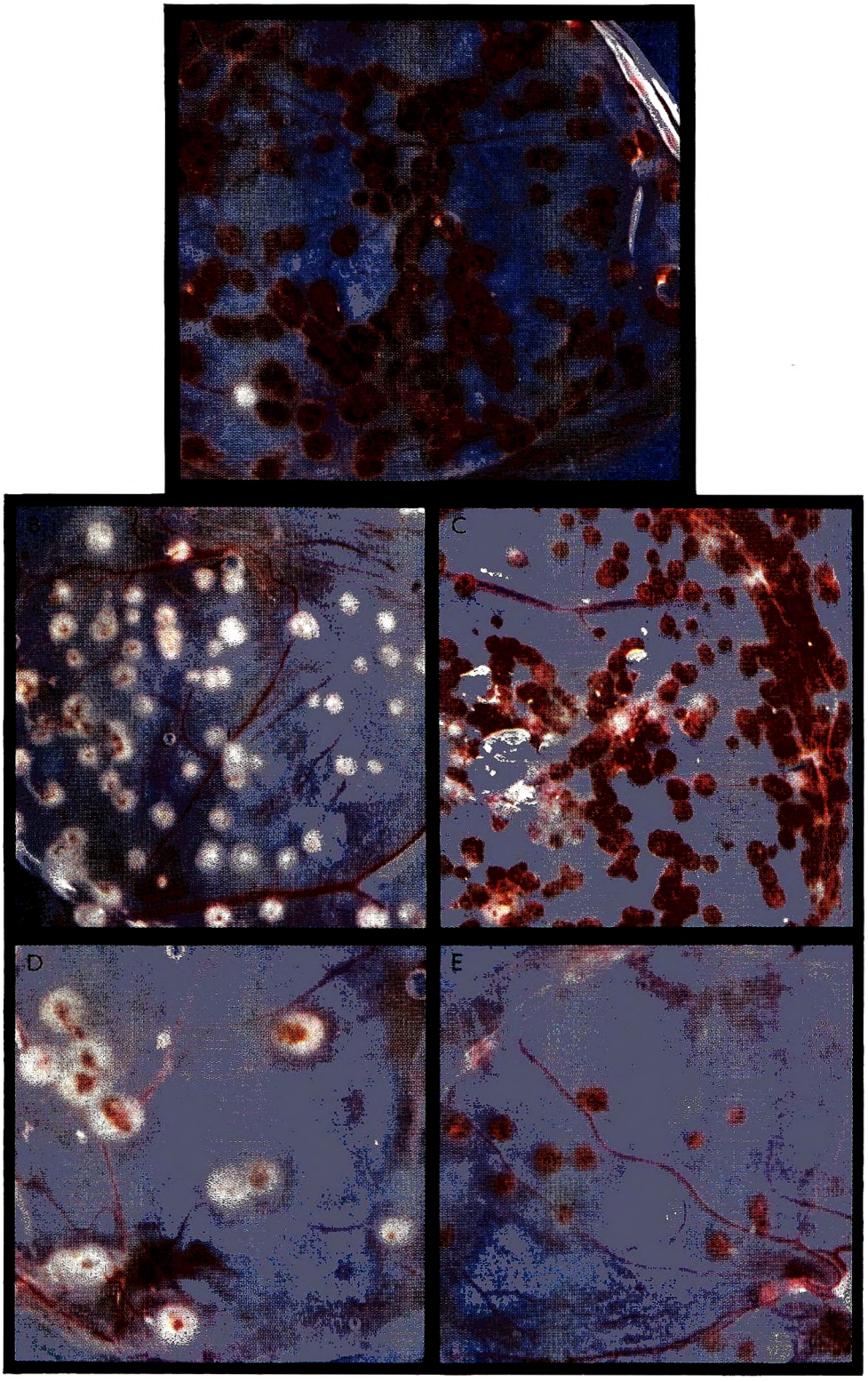


DERRICK BAXBY

(*Facing p.* 646)



DERRICK BAXBY

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EXPLANATION OF PLATES

Plate 1

Sections of single pocks taken from CAM of White Leghorn embryos after 72 hr. incubation at 35° C. Stained with haematoxylin and eosin, × 260.

A. Parental red cowpox.

B. Atypical white cowpox.

C. White cowpox. Inclusions arrowed.

Plate 2

CAM of White Leghorn embryos 72 hr. after inoculation with the stated virus, × 2. Photographed on Kodachrome 11 A using a blue tile as background.

A. Parental red cowpox incubated at 35° C. Note one white mutant pock.

B. White cowpox incubated at 39° C., showing classical appearance of white cowpox.

C. White cowpox incubated at 33° C., showing a very high proportion of atypical pocks.

D. White pock mutant of Levaditi vaccinia incubated at 39° C., showing typical white, ulcerated pocks.

E. White pock mutant of Levatidi vaccinia incubated at 33° C., showing atypical pocks.

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With 1 *plate*
Printed in Great Britain

Studies of the effectiveness of an isolation ward

By R. E. O. WILLIAMS and LYNN HARDING
Wright-Fleming Institute, St Mary's Hospital Medical School, London, W.2

(*Received* 3 *May*, 1969)

Isolation wards have been provided in great variety in general hospitals in the last few years, but there have been relatively few reports of any attempts to measure the effectiveness of the isolation provided. In 1965, a 14-room isolation ward was constructed at St Mary's Hospital in what had previously served as bedrooms for domestic staff and, for structural reasons, each room was provided with an individual ventilation plant. Since this provides a system that is both simple and economical in building costs, it seemed desirable to carry out some bacteriological tests to discover whether it was effective.

WARD LAY-OUT AND ORGANIZATION

The isolation ward

As will be seen from figure 1, the 14 bedrooms open off one long corridor, the service rooms in the centre dividing the patient rooms into two groups. The ward is situated on the fifth floor of the hospital and, being above the level of most of the surrounding buildings, is exposed to moderate wind pressures.

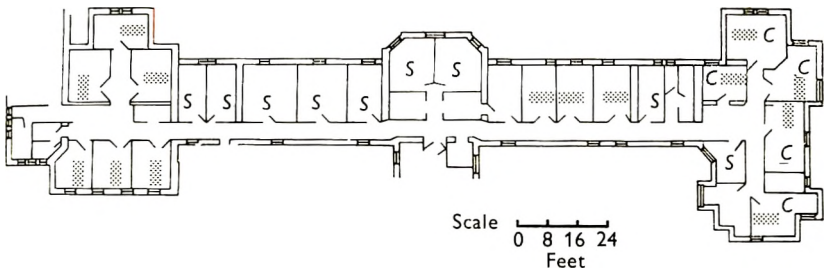


Fig. 1. Plan of the isolation ward. Bedrooms marked *C* have a ducted cool air supply as well as the individual room ventilator. *S* = service rooms, including offices and kitchen.

The ventilation system. Warmed air, passed through a Vokes automatic roll filter, is supplied under slight pressure to the corridor. In the corridor wall of each bedroom is fitted an individual ventilator unit (Pl. 1A, B), comprising a fan with a capacity of 150–180 ft^3/min., a two-stage 3 kW. heater controlled by a room thermostat, and a washable nylon filter with an efficiency rating of 90 % against particles down to 5 μ. All the servicing of the unit is carried out from the corridor side, and the filter is readily removable for cleaning; in practice this has been found necessary every 3–4 months.

The ventilator units provide about six air changes per hour with a slight positive pressure within the bedrooms. Ordinarily the air is discharged through a gap left at the top or bottom of the window; it would certainly have been preferable to fit non-return louvres since, on windy days, there can be a definite inward air current. When the door is opened the room air discharges into the corridor, which must thus become contaminated. Contaminated air should not, however, enter the bedrooms since the input is taken through the filter and there should be no flow from corridor to room through the doorway. It was thought that this system had an advantage over the supply of air under pressure to the corridor with infiltration to the bedrooms because it would have been difficult to retain an adequate positive pressure in a corridor with windows down one side, and because the corridor air must always be at risk of contamination from patients and staff using it. Individual room ventilating units were much simpler and cheaper to install than a ducted air supply system to all rooms. The rooms at the south-west corner of the ward (marked *C* in Fig. 1) are, however, provided with a supplementary supply of cooled and filtered fresh air through ducts.

The patients. The ward is used mainly for patients with open septic lesions; occasionally patients with generalized infection are admitted, as are a few who are in need of protective isolation because of immunosuppressive or cytotoxic treatment. This last use is, in principle, undesirable, but is preferable to the nursing of such patients in open wards that have no separate isolation rooms.

Nursing organization and technique. The nursing complement of the ward numbers 14, including one ward sister, two or three staff nurses and one trained State-enrolled nurse. Each nurse looks after patients in several different rooms. When on duty all the nursing and domestic staff wear plastic aprons, which are wiped down with 2·5% Roccal after the performance of any treatment or after close contact with a patient's bed. Disposable plastic gloves are worn during the performance of wound dressings and other treatments. Soiled dressings are enclosed in plastic bags before removal from the bedroom for destruction. Masks are not ordinarily worn while on duty in the ward. The staff wear easily cleaned shoes that are not worn elsewhere; plastic overshoes are provided for visitors and there are disinfectant-soaked mats at the entry to the ward corridors, though, in the light of more recent investigations (Subcommittee on Aseptic Methods in Operating Theatres, Report, 1968), this is probably not of great value.

Cleaning techniques. The ward was carefully designed to facilitate cleaning; most of the equipment needed is kept in a locker in each bedroom.

Bed linen is changed daily or more often, and all the blankets and pillows from a bed are changed and sterilized after each patient's discharge. All linen is enclosed in a plastic bag, which is firmly closed, before being removed from the bedroom. Mattresses, which have plastic covers, are washed *in situ*.

METHODS

Bacteriological methods

Nasal swabs were taken from all patients, generally within 24 hr. of admission and again on one day in each week. These were plated on blood agar and examined for coagulase-positive staphylococci (*Staphylococcus aureus*) after 18 hr. incubation at 37° C. Coagulase-negative strains were not studied further and the word 'Staphylococcus', when used in this paper, refers to *S. aureus*. All strains of *S. aureus* were submitted to phage typing.

Nasal swabs from the nurses and other ward staff were examined in the same way once weekly.

Records were obtained from the hospital diagnostic laboratory of all infected lesions; staphylococci isolated from such lesions are phage typed as a routine.

The staphylococcal content of the air was determined by the exposure of culture plates with an inside diameter of 13·8 cm. for 12 or 24 hr. The plates were ordinarily placed on a window ledge or shelf, or on top of the ventilator unit, about 1–1·5 m. above the floor.

During the greater part of the investigation the air was sampled only when one of the patients in the ward was thought to be an active disperser of staphylococci; in these circumstances plates were exposed for 12 hr. in the disperser's room, the corridor, and a number of the nearby rooms. During a 6-week period early in 1968, plates were exposed for 24 hr. on 5 days a week in each bedroom, at the two ends of the corridor and in the service areas of the ward. During the first period, the air was sampled on a culture medium containing tryptone, yeast extract and serum with phenolphthalein phosphate as indicator (TY medium of Harding & Williams, 1969), and the plates were incubated aerobically at 37° C. overnight; colonies of *S. aureus*, detected after exposure to ammonia vapour, were subcultured for coagulase testing and for phage typing. During the second period the culture medium had in addition 1 % glucose and the plates were incubated anaerobically at 41° C. for 24 hr. (medium TYG of Harding & Williams, 1969). During the first period a maximum of about five colonies were chosen at random for phage typing; during the second period up to eight colonies were selected from plates with this number or more.

Air-flow determinations

Smoke tests to demonstrate the direction of the air flow through the doors of the bedrooms were carried out once a week during the period November 1966 to April 1967. Titanium tetrachloride was used as a source of the smoke (Williams, Blowers, Garrod & Shooter, 1966).

RESULTS

Pattern of air flow

The direction of air flow through the partly opened doorways of the bedrooms was determined on 20 days between November 1966 and April 1967. A total of 264 tests were made. In 171 (65 %) the air-flow was outward, from room to corridor, as planned. In 61 (23 %) the flow was indeterminate or different at the top

and bottom of the doorway. In only 32 (12%) was there a clear inward flow from corridor to bedroom. One room showed inward flow on five of 20 occasions and two others on four of 19 and 20 occasions respectively. On only one day was there an inward flow in as many as four roms. It has to be stressed that, for these tests, the room doors were partly open; they were ordinarily kept shut all the time except when someone was entering or leaving the room, so that even if the test showed corridor-to-room flow, this can only have happened intermittently.

Table 1. *Numbers of colonies of* Staphylococcus aureus *from ward air*

	No. of colonies per 24 hr. plate (0·36 m.² hr.)							
	0	1–	5–	11–	21–	51–	101+	Total
Occupied patient rooms								
No. with 0 type	98	—	—	—	—	—	—	98
No. with 1 type	—	77	11	13	10	18	20	149
No. with 2 types	—	24	15	3	8	1	4	55
No. with 3 types	—	0	3	3	1	1	0	8
No. with 4 types	—	0	0	0	0	0	1	1
Total	98	101	29	19	19	20	25	311
Service areas								
No. with 0 type	84	—	—	—	—	—	—	84
No. with 1 type	—	84	10	5	1	1	0	101
No. with 2 types	—	37	12	4	1	1	0	55
No. with 3 types	—	8	12	3	0	0	0	23
No. with 4 types	—	3	6	2	0	0	0	11
No. with 5 types	—	—	5	1	0	0	0	6
No. with 6 types	—	—	1	0	0	0	0	1
Total	84	132	46	15	2	2	0	281
Empty bedrooms								
Total	73	14	0	0	0	0	0	87
Grand total	255	247	75	34	21	22	25	679

Staphylococci in the ward air

The results from the first part of the study, in which the air was sampled rather sporadically, were analysed separately from those of the short period of comprehensive sampling, but differences between the two periods were very small and it will suffice to combine them for presentation.

A total of 311 plates were exposed in bedrooms occupied by patients (Table 1); only 98 (31%) yielded no colonies of *S. aureus*; 101 (33%) had 1–4 colonies and 25 (8%) had more than 100, the highest count being about 800. The median count, determined graphically, was 4·6 colonies of *S. aureus* per plate exposed for 24 hr., or 13 col./m.² hr. In the service areas approximately the same proportion of plates yielded no *S. aureus* but on average the counts were lower than in the bedrooms, and no plate yielded more than 100 colonies; the median count was 2·5 col. per 24 hr. plate (7 col./m.² hr.).

Only one of the plates from occupied bedrooms yielded more than three different phage types of staphylococcus and on 149 (70%) of the 213 positive plates all the

colonies examined were of one type. In contrast, in the service areas only 52 % of the positive plates yielded a single type and 9 % of the plates yielded colonies of four or more distinct types. The rarity with which we found multiple types on the plates with very high staphylococcal counts is doubtless partly an artifact, deriving from the fact that we never picked more than 8 colonies for typing.

In considering the sources of the staphylococci recovered from the air, the different types isolated were scrutinized separately. Thus if a culture plate yielded 10 colonies of *S. aureus* and, of the 5 typed, 4 proved to be of one type and one of another, the results were treated as if we had had one air sample with 8 colonies and a second sample with 2. On this basis the 424 positive plates shown in Table 1 yielded the 667 'strains' considered in Table 2.

Table 2. *Numbers and apparent sources of* Staphylococcus aureus *from ward air*

	No. of single strains				
		On plates with (colonies/24 hr. or 0·36 m.² hr.)*			
	Total	1–	6–	21–	101+
Occupied bedrooms, total	291	183	47	37	24
S. aureus of type carried					
By patient in room	127	36	31	37	23
By patient in other room	99	88	10	0	1
By patient in other room or staff	1	1	0	0	0
By staff, not patient	22	20	2	0	0
Source not found	38	38	4	0	0
Service areas, total	362	327	31	4	0
S. aureus of type carried					
By patient in ward	195	163	28	4	0
By patient or staff	8	8	0	0	0
By staff, not patient	35	33	2	0	0
Source not found	124	123	1	0	0
Empty bedrooms, total	14	14	0	0	0
S. aureus of type carried by patient in ward	11	11	0	0	0

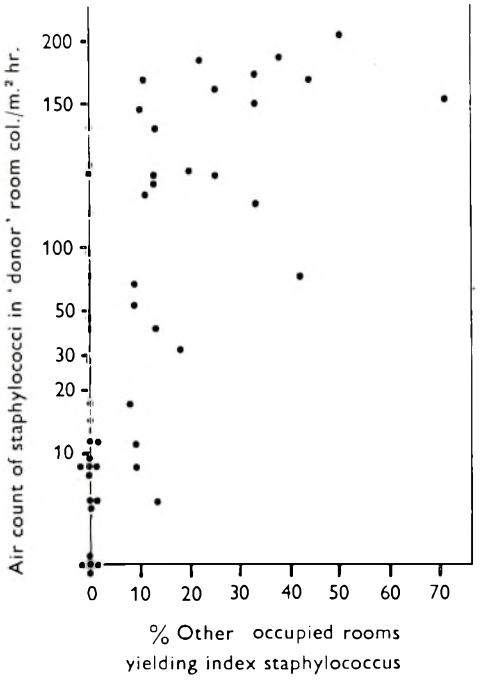
* About half the plates were exposed for only 12 hr.; the counts on these plates have been doubled for entry into this Table.

In all but one of the cases where the number of colonies found on plates exposed in bedrooms was more than 20, the staphylococci were of the same type as that harboured by the patient in the room. The one example of major cross-contamination was observed on the first day of the study and was quite possibly due to retrograde spread of air, from a neighbouring room in which there was a patient who was a very active disperser, along the cool-air ventilation duct. Subsequently a filter was installed in the duct to prevent this occurrence.

With low counts, the staphylococci seemed very frequently to have been derived from a patient in another room. Similarly, staphylococci on plates exposed in service areas were, in half the cases, of a type harboured by one of the patients

present in the ward at the time. Strains of types carried by members of the staff were found both in bedrooms and in service areas but not very frequently, nor ever in large numbers.

During the study there were 15 patients in the ward who at some time dispersed staphylococci to give air counts in their rooms in excess of 20 col. per plate in 24 hr.; it is these patients who were responsible for most of the spread to other rooms (Figs. 2, 3).



Fig, 2

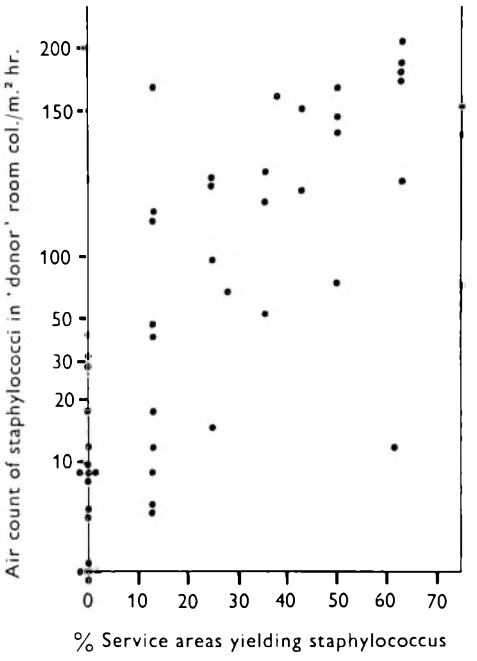


Fig. 3

Fig. 2. Spread of staphylococci from one bedroom to another.
Fig. 3. Spread of staphylococci from bedrooms to service areas.

In Fig. 4 an attempt is made to measure the degree of protection against airborne staphylococci provided by the ward design. Line *A* gives the cumulative distribution of staphylococcal air counts generated by carriers within their bedrooms. Line *B* gives the distribution of counts from other bedrooms for staphylococci of the types being dispersed by the carriers. The counts in the recipient rooms appear to be about 1/500th of those in the donor rooms. This is much less than was found in a partially subdivided ward in this hospital (Williams, 1967).

In an attempt to track the mode of spread from one patient room to another, we compared the frequency of cross-contamination of air from rooms occupied by carriers who shed *S. aureus* into the air of their own rooms to other rooms at the two ends of the corridor (cf. Fig. 1). For this analysis, which was based entirely on the second period of air sampling (cf. p. 651), we excluded all occasions when the same phage type of staphylococcus was known to be harboured by more than one patient. Since there were commonly two or more dispersers, of different types,

present at one time the 'number of plates examined' quoted in Table 3 includes, as separate 'plates', the successive examinations of the same plate for different types of staphylococci. Cross-contamination was certainly most frequent in nearby rooms, and these observations would be compatible with the idea that it was leakage of air from the corridor that accounted for the staphylococci found in the air of the bedrooms. Presumably, however, the opportunities for transfer by personnel would also be greater between nearby than between distant rooms.

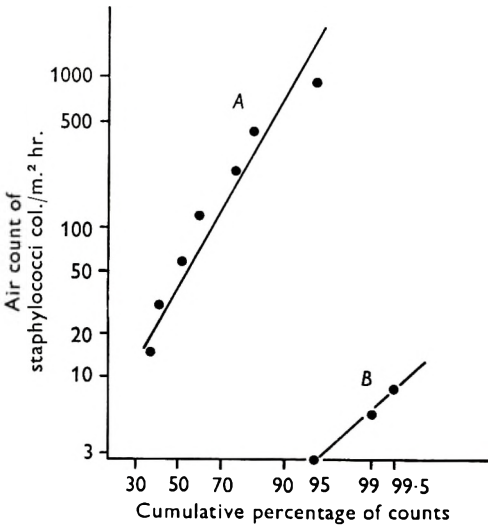


Fig. 4. Comparison of air counts in dispersers' rooms (upper line) with count for same staphylococci in other rooms (lower line).

Table 3. *Spread from rooms occupied by* Staphylococcus aureus *shedders*

	Plates examined	Percentage yielding *S. aureus* of patient's type
Occupied bedrooms		
At the same end of corridor	106	29·3
At other end of corridor	129	17·1
Corridor or 'sluice'		
At the same end of corridor	46	74·0
At other end of corridor	47	25·6

* For this table, 'shedders' were defined as patients who were nasal carriers of *S. aureus*, and whose staphylococci were found in the air of their own room and at least one other room or ward area.

Acquisition of Staphylococcus aureus *by patients*

There was no change in the mean nasal carrier rate for *S. aureus* with increasing length of stay in the ward (Table 4), such as has been observed in open surgical wards (e.g. Williams *et al.* 1959). Unfortunately the staphylococci were not all tested for antibiotic sensitivity so we cannot judge whether there was an increase

in the carrier rate for resistant strains (cf. Lidwell, Polakoff, Jevons & Parker, 1966).

Thirteen patients appeared to acquire staphylococci in the nose during their stay in the ward; two patients seemed to acquire two strains each and one acquired three strains so that the total number of apparent acquisitions was 17. In 11 cases of the 17 a staphylococcus was found in the nose of a patient who had two previous swabs that yielded either no staphylococci, or cocci of a different phage type ('probable acquisitions'); in the rest there was only one negative or different culture ('possible acquisitions'). In all 13 cases for which the sensitivity results were available the new strain was sensitive to tetracycline.

Table 4. *Nasal carrier rates for* Staphylococcus aureus

Week of swabbing	No. patients examined	Percentage carrying *S. aureus*
1	197	38·1
2	117	33·3
3	90	32·2
4	60	31·7
5	41	41·5
6	30	36·7
7	24	33·3
8	20	35·0
9, 10	27	37·0

The 17 apparent acquisitions were observed among the 409 second or subsequent examinations of the patients; they represent a rate of 4·2 per 100 patient weeks at risk. The 11 more certain acquisitions represent a rate of 2·7 per 100 patient weeks.

Five of the 11 probable acquisitions were with staphylococci of a phage type known to be carried by another patient present in the ward and one was of a type found in the ward air but not in any person; for the six possible acquisitions the corresponding figures were 1 and 1. No source was known for eight acquisitions, and one of the 'probably acquired' strains was untypable. Thus acquisitions from recognized sources totalled 6, a rate of 1·3 per 100 patient weeks at risk, and acquisitions with no recognized source were 11, or 2·8 per 100 patient weeks.

There were five patients who appeared to acquire *S. aureus* in a wound during their stay in the ward and a further five patients who possibly acquired *S. aureus* in a wound. The staphylococci from two of the former were of a type not known to have been present in the ward previously; those from the remaining three were of the same type as had been present in the patient's own nose for some time previously. Two of the last three patients were active dispersers of their staphylococci; the third was one of the patients who had acquired the staphylococcus in her nose during her stay in the ward. One of the five patients for whom the evidence of ward-acquired infection was scanty was infected with a staphylococcus that was probably the same type as a strain carried by a ward orderly. No carriers were found as possible sources for two strains and the remaining two were lost before they could be typed.

DISCUSSION

The main object of providing separate rooms for isolation nursing is to reduce the risk of airborne transfer of infection from one room to another, though doubtless this form of isolation also reduces the risk of transfer on fomites and probably has an important psychological effect in reminding staff of the perennial need for strict aseptic precautions. The study reported here was limited to the spread of staphylococci because there is evidence that these bacteria are conveyed between patients by the air, at least to set up nasal carriage, and because the necessary bacteriological techniques have been well developed.

The air sampling revealed low counts of *Staphylococcus aureus* in the patients' rooms: the median was about 4·6 per plate in 24 hr. or about 13 col./m.^{2} hr. This is not very different from the figure observed in a subdivided surgical ward in this hospital (Williams, 1967) but is lower than the figure from open surgical wards, where counts of 40 col./m.^{2} hr. have been observed. The mean nasal carrier rate among the patients was no lower than observed in previous studies so that lack of sources for dispersal cannot be the explanation of the low air counts.

There was some spread of staphylococci from one room to another, and it is presumed that this was by way of the air, although it is impossible to exclude that some may have been transferred on the person of members of the staff moving from room to room. Nevertheless the average count of staphylococci in the air of 'recipient' rooms seemed to be only about 1/500th of that in the room containing the patient dispersing the cocci.

Lidwell *et al.* (1966) have examined the relationship between the number of staphylococci inhaled and the acquisition of the nasal carrier state and have shown that the slope of the dose-response curve is very low, that is that a very substantial reduction in the dose inhaled is needed to effect much reduction in the acquisition rate. Our estimates of acquisition in the nose were not entirely satisfactory, but they indicate a rate between 2·7 and 4·2 per 100 patient weeks at risk, which is substantially less than the rates of about 9 per 100 patient weeks observed in a series of medical wards (Public Health Laboratory Service, Report, 1965) and in the wards at the Queen Elizabeth II Hospital, Welwyn Garden City (Lidwell *et al.*, in preparation) and may be compared with the rate of 3·4 per 100 patient weeks recorded in a subdivided surgical ward by Lidwell *et al.* (1966), the 5·9 per 100 patient weeks observed in a subdivided ward at St Mary's Hospital (Williams, 1967) or the 6·3 per 100 patient weeks observed by Parker, John, Emond & Machacek (1965) in a fully subdivided isolation hospital ward. The fact that the staphylococci that were acquired were all, in so far as they were tested, sensitive to tetracycline, suggests that a large proportion of the acquisitions may have been spurious in the sense used by Parker *et al.*

SUMMARY

Studies were made of a 14-room isolation ward in which the bedrooms were provided with individual ventilation units to provide a slight positive pressure within the rooms. Airborne staphylococci were detected with sedimentation plates.

The median count of *Staphylococcus aureus* in the occupied bedrooms was 4·6 col. per 14 cm. plate exposed for 24 hr. or 13 col./m.^{2} hr. When the air count was high virtually all the staphylococci proved to be of the phage type harboured by the patient in the room; with low counts a substantial proportion were apparently derived from patients in other rooms.

The apparent rate of acquisition of *S. aureus* in the nose of the patients was between 2·7 and 4·2 per 100 patient weeks at risk. This is substantially lower than has commonly been observed in open wards.

We are very grateful to Miss M. A. Adams, Sister in charge of the Almroth-Wright isolation Ward, and all her staff for all their help and co-operation during this study, which was supported by funds provided to St Mary's Hospital by the Ministry of Health for clinical research.

The reconstruction of the ward was designed by Mr A. Stableford, Group Engineer to St Mary's Hospital; the ventilation units were designed and supplied by White Bays and White Ltd., London.

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EXPLANATION OF PLATE

A. The ventilation unit, bedroom side.

B. The ventilation unit, corridor side.

A

B

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A comparative study of methods for the diagnosis of respiratory virus infections in childhood

By PATRICIA M. STURDY, JOYCE McQUILLIN and P. S. GARDNER

Department of Virology, The Royal Victoria Infirmary and University of Newcastle upon Tyne

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In the last 20 years much progress has been made in the development of techniques for the isolation of a wide range of viruses from patients with respiratory disease (Rowe *et al.* 1953; Chanock, 1956; Chanock & Finberg, 1957; Beem *et al.* 1960; Tyrrell *et al.* 1960; Tyrrell & Bynoe, 1965). By 1965 techniques had become sufficiently advanced for a viral cause to be established in 62% of children with bronchiolitis and 42% with pneumonia (Elderkin, Gardner, Turk & White, 1965). The routine method for the investigation of children with respiratory disease has been the collection of material from the upper respiratory tract by cough and nasal (CN) swabs (Andrew & Gardner, 1963; Holzel *et al.* 1963).

In 1939, Auger devised a method for aspirating secretion from the naso-pharynx using a catheter attached to a syringe. He considered that this was an improved method for the isolation of pneumococci from children with pneumonia, although he made no direct comparison with other isolation procedures. Similar methods for obtaining secretions were later used by virologists for the isolation of parainfluenza viruses from children with croup (Morgan *et al.* 1956; Beale, McLeod, Stackiw & Rhodes, 1958; McLean *et al.* 1961); secretions are abundant and easily obtainable in this condition.

Recently, a number of workers have used nasopharyngeal secretions for rapid diagnosis. Doane *et al.* (1967) examined preparations of nasopharyngeal secretions from patients with tracheitis by electron microscopy and observed large numbers of parainfluenza virions. McQuillin & Gardner (1968) and Gardner & McQuillin (1968) examined cellular deposits from nasopharyngeal secretions by an indirect immunofluorescent technique for the rapid diagnosis of respiratory syncytial virus (RSV) infections in infants, and found many infected cells present. The large numbers of parainfluenza viruses visible by electron microscopy in nasopharyngeal secretion and of exfoliated cells exhibiting specific fluorescence for RSV suggest that this material is heavily infected. Therefore, nasopharyngeal secretions should prove to be an equal or more fruitful source of virus than swabs from the upper respiratory tract. Such aspirates may reflect more closely the pathogens present in the lower respiratory tract in bronchitis, bronchiolitis and pneumonia.

In this laboratory, nasopharyngeal (NP) secretions were taken from children mainly of 1 year of age or less who were suffering from acute lower respiratory disease. This material, examined by the fluorescent antibody technique to demon-

strate the presence of RSV, was also inoculated on tissue culture to isolate RSV or other viruses which might be associated with the illness. CN swabs, collected together in a bottle of Hanks's medium, were taken from each patient and cultured in the same manner as the NP secretions. The aim of this work was to ascertain whether NP secretions were a better source of virus than CN swabs and whether an immunofluorescent antibody technique, applied directly to the cells of the nasopharynx, compared favourably with RSV isolations in tissue culture; it was also an opportunity to assess the place of NP secretions in diagnostic virology.

METHODS AND MATERIALS

Collection of specimens

CN swabs were collected together in the same 5 ml. bottle of Hanks's medium, containing 0·2 % bovine albumin, penicillin 500 units/ml., streptomycin 250 μg./ml. and mycostatin 50 μg./ml. CN swabs were always taken before the nasopharyngeal aspirates, so that the results from CN swabs would be comparable with those obtained in previous years.

NP secretions were obtained in the following way: A polythene feeding tube (size 8) was attached to a sterile plastic mucus extractor, and this in turn was connected to a suction pump, which provided a maximum negative pressure of 26 lb./in.^{2} The feeding tube was passed through the nose into the region of the nasopharynx and when the suction was applied, the aspirate was trapped in the mucus extractor. This procedure was repeated through the other nostril. The suction pump was operating both when the tube was placed into the nasopharynx, and when it was removed from there. The mucus extractor containing the NP secretion, and the CN swab specimen in transport medium, were both carried from the wards to the laboratory in a tray of melting ice.

Preparation of specimens

The NP secretion was centrifuged in the mucus extractor at 1000 rev./min. for 10 min. at 4° C., to deposit the cells. The top of the extractor was removed with a hot wire loop, and the supernatant was separated for culture. The cellular deposit, after washing, was transferred to slides for examination by the immunofluorescent technique as described by McQuillin & Gardner (1968). A suspension of supernatant material, between 5 and 10 %, was made in the Hanks's transport medium previously described.

Isolation and identification of viruses

The suspension of NP secretion and Hanks's medium containing the CN swabs were each inoculated in 0·2 ml. volumes into tissue culture tubes; the cell lines used were Bristol HeLa, HEp 2, rhesus monkey kidney and a human diploid cell line (W.I. 38). Technical procedures have been described elsewhere (Gardner & McQuillin, 1968). The CN swab specimens and suspensions of NP secretions were always inoculated on the same cell lines at the same time. Moreover, to ensure

consistency of results, each CN specimen and NP secretion was inoculated on the same batch of tubes of each particular cell line. All tubes were examined daily for cytopathic effect; W.I. 38 were kept for 10 days, monkey kidney 21 days and HeLa and HEp 2 cells were kept a total of 28 days. Should passage of the HeLa and HEp 2 cultures be required near the 28th day, then the passage tubes were kept a further 10 days.

Respiratory syncytial virus. When a giant cell degeneration compatible with that caused by RSV was observed on any of the cell lines, the agent was identified by both a neutralization test and the immunofluorescent technique. At a later stage in the investigation, when the specificity of the fluorescent antibody technique had been established, the neutralization test was discontinued.

Parainfluenza viruses. Parainfluenza viruses were isolated on rhesus monkey kidney cells. Haemadsorption tests were carried out on all monkey kidney tubes at 10 and 21 days after inoculation. Parainfluenza viruses were identified by the haemadsorption neutralization test, using parainfluenza types I, II and III antisera.

Influenza viruses. Influenza viruses were also isolated on rhesus monkey kidney cells. A complement-fixation test using influenza A, B, C and mumps antisera was performed on tissue culture fluid of haemadsorption agents which were not identified as parainfluenza viruses.

Adenoviruses. When the cytopathic effect characteristic of adenovirus degeneration was observed in tissue culture tubes, a complement-fixation test was carried out on the antigen present, using a standard positive adenovirus antiserum. The virus was then typed by a neutralization test.

Herpesvirus hominis. When a round cell degeneration occurred in tissue culture suggestive of herpesvirus hominis, the virus was identified by both a neutralization test and fluorescent antibody technique.

Rhinoviruses. Agents producing islands of round cell degeneration on W.I. 38 or monkey kidney cells were tested for their acid stability. Acid-labile viruses were then classified as M- or H-strain rhinoviruses.

Picornaviruses excluding rhinoviruses. Agents causing cytopathic changes on tissue culture suggestive of enterovirus infection were identified by a neutralization test.

Fluorescent antibody methods for the identification of viruses

The fluorescent antibody technique used in this study to identify RSV in exfoliated cells of the nasopharynx and to confirm strains of RSV and herpesvirus hominis, have been described elsewhere (McQuillin & Gardner, 1968; Gardner & McQuillin, 1968; Gardner, McQuillin, Black & Richardson, 1968).

The study groups

Study group A

NP secretions and CN swabs were collected from 111 children who were admitted to hospital in Newcastle upon Tyne with acute lower respiratory infections during 1968 (Table 1). One hundred and six children included in this series were less than

1 year of age, the oldest of the remainder being 2. NP secretions were more easily obtained from children in this age group, and this was the only selective process used in the study.

Study group B

The results for the whole of 1967 and 1968 were compared at two ages, under 1 year and over 1 year. In 1967, 99 children less than 1 year and 82 children over 1 were investigated; in 1968, the numbers were 161 and 119 respectively. Children in 1967 had only CN swabs taken but in 1968 two-thirds of the children under 1 year had NP secretions taken as well. This afforded another means of comparing the two types of specimens.

Table 1. *The clinical categories and viruses isolated from nasopharyngeal secretions and cough/nasal swabs of* 111 *children—Study group A*

	Clinical category				
	Pneumonia	Bronchiolitis	Bronchitis	Croup	Total
Children examined	18	78	12	3	111
Children infected with virus	15 (83)	68 (87)	6 (50)	1	90 (81)
Viruses isolated					
Adenovirus	2	1	—	—	3
Parainfluenza	2*	4	1	—	7
Influenza B	—	1	—	—	1
Rhinovirus	2	—	—	—	2
Echovirus	—	2	—	—	2
RSV	9 (50)	60 (77)†	5 (42)	1	75 (68)
RSV diagnosed by fluorescent antibody method	8	58	4	1	71

Figures in parentheses are percentages of the total children in each group.

* Adenovirus type 1, herpesvirus hominis and an H-strain rhinovirus were also isolated from one of these patients.

† A poliovirus was also isolated from two of these patients, and a Coxsackievirus A 9 from a third.

RESULTS

The viruses associated with acute lower respiratory disease in 111 *children from whom nasopharyngeal secretions and cough/nasal swabs were taken—Study group A*

Table 1 summarizes the viruses isolated from children with acute lower respiratory disease, and the clinical categories with which they were associated. When more than one virus was isolated from a child, the one considered to be the most likely aetiological agent was included in the table and others were recorded in the footnotes. A child with pneumonia was simultaneously infected with four viruses; this was her third admission to hospital with acute lower respiratory disease, her previous admissions having been attributed to RSV and parainfluenza virus

type III. This unusual sequence of events was probably related to her hypogammaglobulinaemia. Viruses were associated with 81 % of all lower respiratory infections; the individual figures were 87 % for bronchiolitis and 83 % for pneumonia.

If CN swabs alone had been used a viral cause would have been established in only 68 % of patients (76 out of 111) as compared with 79 % (88 out of 111) when NP secretions alone from the same patients were examined. Croup was associated with older children but few NP secretions were taken from patients in this clinical category.

Table 2. *Comparison of methods for the diagnosis of virus infections—Study group A*

Viruses	Viruses isolated from nasopharyngeal secretions	Viruses isolated from cough/nasal swabs	Positive secretions by fluorescent antibody technique	Total no. of viruses isolated from patients
RSV	74	64	71	75
Parainfluenza type I	1	1	—	1
II	1	1	—	1
III	5	5	—	5
Adenovirus type 1	1	1	—	1*
2	—	1	—	1
4	1	—	—	1
5	1	—	—	1
Rhinovirus M-strain	2	2	—	2
H-strain	1	—	—	1*
Influenza B virus	1	—	—	1
Herpesvirus hominis	—	1	—	1*
Echovirus type 12	1	1	—	1
19	1	1	—	1
Coxsackie A virus type 9	1	—	—	1*
Poliovirus	2	2	—	2*
Total no. of viruses	93	80	71	96

* These viruses occurred in multiple infections.

Respiratory syncytial virus

RSV was the virus most commonly associated with all forms of acute lower respiratory disease in this age group, infecting 67·5 % of all these patients, including 77 % of those with bronchiolitis and 50 % of those with pneumonia. These results were based solely on the isolation of viruses; serological results were not included. Seventy-one of the 75 RSV infections (95 %) were diagnosed on the patient's day of admission by the fluorescent antibody technique. All positive fluorescent antibody results were later confirmed by isolation of the virus.

Table 2 shows that of the 75 isolations of RSV, 74 were made from NP secretions and 64 from CN swabs. There is a significant difference in the isolation rates for the two types of specimens ($\chi^2 = 7{\cdot}003$, $P < 0{\cdot}01$).

RSV was isolated on Bristol HeLa, HEp 2, rhesus monkey kidney and occasionally W.I. 38 cells. Table 3 shows the number of days necessary to isolate RSV on

Table 3. *The time taken for the isolation of RSV on Bristol HeLa and HEp 2 cells—Study group A*

Time of isolation (days)	Nos. of isolations on Bristol HeLa cells inoculated with NP secretion	Bristol HeLa cells inoculated with CN swabs	HEp 2 cells inoculated with NP secretion	HEp 2 cells inoculated with CN swabs
0–5	1	0	1	0
6–10	28	10	26	8
11–15	22	22	24	19
16–20	11	16	11	16
21–25	3	10	4	4
26–30	2	4	3	2
31–35	0	0	1	2
Total isolations	67	62	70	51
Percentage positive by tenth day	43	16	38·5	15·5

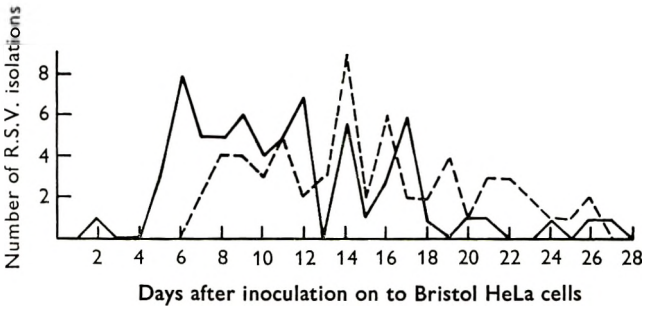

Fig. 1. The isolation of RSV on Bristol HeLa cells. Comparison between the number of days taken to isolate RSV on Bristol HeLa cells from nasopharyngeal secretion and cough/nasal swabs. —, Represents tissue cultures inoculated with nasopharyngeal secretion; - - -, represents tissue cultures inoculated with cough/nasal swabs.

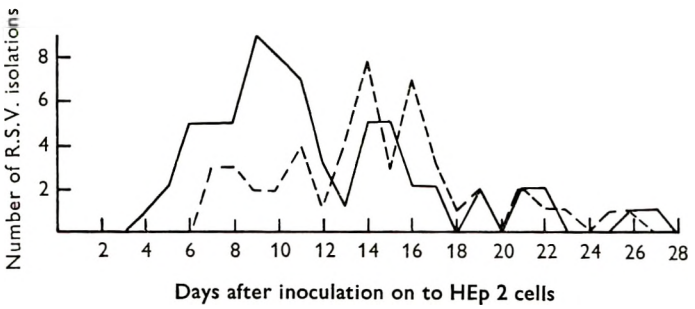

Fig 2. The isolation of RSV on HEp 2 cells. Comparison between the number of days taken to isolate RSV on HEp 2 cells from nasopharyngeal secretions and cough/nasal swabs. —, Represents tissue cultures inoculated with nasopharyngeal secretion; - - -, represents tissue cultures inoculated with cough/nasal swabs.

HEp 2 and Bristol HeLa cells when inoculated with either NP secretion or CN swab. Forty-three per cent of isolations on Bristol HeLa cells occurred within 10 days of inoculation with NP secretion, in contrast to 16% from CN swabs. Similar figures were obtained on HEp 2 cells. Virus was isolated from two NP secretions on the second and fourth day after inoculation. Figures 1 and 2 show these differences in graphic form.

Parainfluenza viruses

A total of seven parainfluenza viruses were recovered from the 111 children examined (Tables 1 and 2). All the parainfluenza viruses were isolated on rhesus monkey kidney cells from both CN swabs and NP secretion. Two parainfluenza viruses type III were recovered from NP secretions on HEp 2 and Bristol HeLa cells, but not from the CN swabs. The child infected with a parainfluenza virus type 1 was also shown to be infected with an adenovirus type 1, an H-strain rhinovirus and herpesvirus hominis.

Adenoviruses

Adenoviruses of types 1, 2, 4 and 5 were recovered from four patients. Types 1 and 2 were recovered from CN swabs and types 1, 4 and 5 were isolated from NP secretions (Table 2).

Influenza B virus

An influenza B virus was isolated from a patient with bronchiolitis. The virus was grown on rhesus monkey kidney cells inoculated with the material from the NP secretion; the virus was not isolated from the CN swabs taken at the same time (Table 2).

Picornaviruses

Rhinoviruses. The two M-strain rhinoviruses were isolated on rhesus monkey kidney cells and W.I. 38 cells within 5 days of inoculation from both types of specimens.

The H-strain rhinovirus, isolated from the child with the multiple virus infection, was only recovered from the NP secretion on W.I. 38 cells. Herpesvirus hominis was isolated from the parallel CN swab on W.I. 38 cells.

Enteroviruses. Two echoviruses were isolated from children with bronchiolitis from both CN swabs and NP secretion. Two further children with bronchiolitis from whom RSV was isolated were also infected with two types of poliovirus, almost certainly vaccine strains. Coxsackievirus A 9 was recovered from the NP secretions of another child with bronchiolitis; this virus was not isolated from the parallel CN swab. RSV was also isolated from this child.

Comparison of virus isolations in 1967 *with those in* 1968—*Study group B*

From January to December 1967, the majority of children under the age of 1 year admitted to hospital with acute lower respiratory infection were investigated only by CN swabs, but from January to December 1968 the majority also had

NP secretions taken. Table 4 shows all the viruses which were encountered in acute lower respiratory tract infections during these 2 years and also compares the percentage virus isolations using two different methods of collecting specimens. In 1967, only 53 (54 %) viruses were associated with 99 illnesses when CN swabs were used but in 1968, 123 (76 %) were found in 161 illnesses when NP secretions were first introduced for virus isolation. There is a significant statistical difference in the virus isolation rate for the 2 years ($\chi^2 = 14{\cdot}44$, $P < 0{\cdot}0005$). RSV shows this increased isolation rate well, but though the number of other viruses isolated is small, there is a significant increase in isolation rate of those which were considered pathogenic, viz. influenza, parainfluenza and rhinoviruses.

Table 4. *A comparison of virus isolations from children under the age of* 1 *year with lower respiratory disease, for the years* 1967 *and* 1968—*Study group B*

Clinical category	No. in each category	Viruses: RSV	Para-influenza	Adeno-virus	Rhino-virus	Influenza	Entero-virus	Total	%
Pneumonia	45*	13	1	3	0	0	1	18	40
	28*	13	1	0	2	2	0	18	64
Bronchiolitis	37	24	0	0	0	0	2	26	70
	91	76	4	1	0	1	1	83	91
Bronchitis	14	6	1	1	0	0	0	8	54
	32	10	1	1	1	0	0	13	41
Croup	3	1	0	0	0	0	0	1	33
	10	4	3	0	1	0	1	9	90
Total 1967	99	44	2	4	0	0	3	53	54
Total 1968	161	103	9	2	4	3	2	123	76

* For each clinical category the upper line gives the figures for 1967, and the lower those for 1968.

Few NP secretions could be taken from children over the age of 1 year, so that the isolation rates for 1967 and 1968 represented the efficiency of CN swabs at this age level. In 1967, 19 of 82 children older than 1 year showed evidence of a virus aetiology (23 %) and the figure for 1968 was 121 children investigated, with 33 isolations (27 %). There is no significant difference in the virus isolation rates for the 2 years; this contrasts sharply with the improvement in isolation rates in children under the age of 1 year, when the taking of NP secretions was introduced as a method for virus isolation.

DISCUSSION

Over the past year, 111 secretions were obtained from children mainly under the age of one year, admitted to hospital with acute lower respiratory disease (Study group A). Secretions were selected as it had been shown previously that this type of specimen was more suitable than throat swabs for direct examination by the fluorescent antibody technique for the rapid diagnosis of RSV infection. In this

series all patients had both NP secretions and CN swabs taken; these were used to isolate any viruses which may have been present in the respiratory tract of the patients and to confirm that those secretions which gave positive fluorescence with RSV antiserum, were due to RSV infection. This afforded a means of comparing the suitability of NP secretions with CN swabs for the isolation of viruses.

The collection of NP secretions from children depends upon two factors, the patient's co-operation and the amount of secretion present in the nasopharynx. These difficulties are not normally encountered in the taking of CN swabs. In the age group studied, aspiration of secretions did not present many problems and secretions were abundant in the majority of patients with respiratory disease.

The investigation demonstrated an increase in total virus isolations, and RSV in particular, from NP secretions as compared with CN swabs (Table 2). The introduction of NP secretions in addition to CN swabs for the isolation of viruses resulted in a considerable increase in our knowledge of the viral causes of both bronchiolitis and pneumonia as compared with our experience and the experience of others over previous years (Chanock *et al.* 1961; Holzel *et al.* 1963, 1965; Elderkin *et al.* 1965; Holdaway, Romer & Gardner, 1967). Evidence for this was gained from Study group B by comparing the total virus isolations for the year 1967 with those for 1968 (Table 4). There was a significant increase in the virus isolations from children under 1 year of age in 1968 in comparison with 1967, a year when only CN swabs were used. There was no significant increase in 1968 in virus isolations from children over 1 year of age, from whom only CN swabs were taken.

When NP secretions and CN swabs from children with RSV infections were inoculated on HEp 2 and Bristol HeLa cells, the virus was recovered much more rapidly from the tissue cultures inoculated with NP secretions (Figs. 1 and 2, Table 3). This was an additional reason for advocating the use of NP secretions whenever possible. More than one cell line was always inoculated for the isolation of RSV, since the presence of inhibitory substances and the variation in the sensitivity of cells are both factors influencing its growth in tissue culture (Jordan, 1962). The most efficient method for the isolation of RSV was by the inoculation of NP secretions on HEp 2 cells, when 70 viruses out of a possible 75 were isolated. However, the number of positive results obtained by the direct examination of NP secretions by the fluorescent antibody technique was marginally greater than this and 95% of those with RSV infections were diagnosed on the day of their admission to hospital. In the future, there would appear to be justification for discontinuing the culture of RSV from secretions which are positive by the fluorescent antibody technique.

There are limitations in the application of the fluorescent antibody technique to NP secretions. The occasional false negative result or results showing only scanty positive cells may be due to the stage in the disease when the specimen was examined, difficulty in obtaining secretions, or the amount of mucus present, preventing effective staining, but continued culture of the occasional doubtful specimen should not present a problem.

Twenty-one viruses other than RSV were isolated from patients in this investiga-

tion. This small number makes similar comparisons impracticable. Beale *et al.* (1958) found that NP secretions were suitable for the isolation of parainfluenza viruses, and McLean *et al.* (1961), also using this material, recovered parainfluenza viruses from 52% of children with acute laryngotracheobronchitis. None of these workers, however, compared NP secretions with CN swabs for suitability as material for the isolation of parainfluenza viruses. In this series, all the parainfluenza virus isolations were made from both kinds of specimens on monkey kidney cells. No comparison could be made between the numbers of days required for the isolation of these viruses from the two types of specimens, since the haemadsorption test was only carried out on the tenth and twenty-first day after inoculation. However, it was found that two parainfluenza type III viruses grew on Britsol HeLa and HEp 2 cells, as well as on rhesus monkey kidney cells when the specimen used was the NP secretion; the virus did not grow on HeLa or HEp 2 cells inoculated with the parallel CN swabs. This suggested that those NP secretions contained more infective virus than the CN swabs. The large numbers of parainfluenza viruses found in NP secretions by Doane *et al.* (1967), suggest that it may be possible to apply a fluorescent antibody technique for the examination of secretions for the rapid diagnosis of parainfluenza virus infections.

Of the four adenoviruses isolated, three were non-epidemic types (types 1, 2 and 5) which are of unproven pathogenic significance (Gardner, 1968). Table 2 shows that types 1, 4 and 5 were isolated from NP secretions and types 1 and 2 from CN swabs. This is too small a difference on which to comment, but it is noteworthy that adenovirus type 4, the only epidemic strain isolated, came from a NP secretion.

The single isolation of influenza B virus was from a child with severe bronchiolitis; the virus was recovered from NP secretion, but not from the CN swab.

Rhinoviruses have been known for sometime to infect the upper respiratory tract (Tyrrell *et al.* 1960). Though Portnoy, Eckert & Salvatore (1965) suggested rhinoviruses were of no aetiological significance in lower respiratory disease of childhood, Hilleman, Reilly, Stokes & Hamparian (1963) found that 'coryza viruses' (rhinoviruses) were associated with lower respiratory tract infection in a third of a small number of children investigated, and Stott *et al.* (1967) found rhinoviruses in 7% of children with acute lower respiratory infections. In this series, three rhinoviruses were isolated from NP secretions, but only two from CN swabs (Table 2). All these three children were suffering from pneumonia, though in one child with a multiple virus infection the rhinovirus was unlikely to be the aetiological agent. This suggests that in this age group, rhinoviruses are not an important cause of lower respiratory infection but if the viruses in the nasopharynx reflect the viruses present in the lung, then rhinoviruses may be the pathogens involved in sporadic cases.

The single isolation of herpesvirus hominis was from the CN swab but not from the NP secretion.

The significance of enteroviruses is difficult to judge; they often occurred as dual infections with RSV and could be isolated easily from both types of specimen.

NP secretions occasionally had toxic effects on tissue culture, which resulted in

partial or total destruction of the cell sheet. It was found that if these tubes were passaged, particularly within the first few days after inoculation, then the isolation of RSV was considerably delayed and in one instance when the specimen was passaged twice within the first 5 days, isolation failed completely. CN swabs rarely produced toxic effects in tissue cultures; bacterial contamination only occasionally occurred with either specimen. The direct examination of NP secretions by the fluorescent antibody technique could be an additional safeguard for obtaining a positive result, should toxicity or contamination prevent the effective routine culture of specimens.

Our results have shown that NP secretions are a better source of virus and, in addition, more suitable specimens for rapid diagnostic techniques than CN swabs (McQuillin & Gardner, 1968). When all viruses are considered together, the results obtained in this investigation suggest that the examination of NP secretions reflects more accurately the aetiology of acute lower respiratory disease.

SUMMARY

Nasopharyngeal secretions and cough/nasal swabs were taken from 111 children admitted to hospital in Newcastle upon Tyne with acute lower respiratory disease. A comparison was made between nasopharyngeal secretions and cough/nasal swabs as material for isolation of viruses in tissue culture. These results were, in turn, compared with those obtained by applying a fluorescent antibody technique to the exfoliated cells in the nasopharyngeal secretions for the rapid diagnosis of respiratory syncytial virus infection.

More viruses were isolated in tissue culture from nasopharyngeal secretion than from cough/nasal swabs. Further evidence for the superiority of nasopharyngeal secretions was obtained by comparing the virus isolations in the laboratory in 1967 with those in 1968. Respiratory syncytial virus was not only isolated more often but more quickly in tissue culture inoculated with nasopharyngeal secretions.

The fluorescent antibody technique not only provided a diagnosis on the patient's day of admission in 95 % of those infected with respiratory syncytial virus but also proved to be as sensitive as the culture of nasopharyngeal secretions and considerably more sensitive than the culture of cough/nasal swabs for the diagnosis of respiratory syncytial virus infection.

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Airborne excretion of foot-and-mouth disease virus

By R. F. SELLERS and J. PARKER
Animal Virus Research Institute, Pirbright, Surrey

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Foot-and-mouth disease (FMD) virus may spread in a number of ways: by direct contact between infected and susceptible animals, by animal products such as meat and milk, by mechanical transfer on people, non-susceptible animals, birds, vehicles and fomites, and by the airborne route. The initial pattern of outbreaks at the beginning of the epidemic in the West Midlands of England in 1967 suggested that spread was airborne. The meteorological evidence for this and for past epidemics in Great Britain has been investigated by L. P. Smith, P. B. Wright and M. Hugh-Jones (personal communications, 1968–9) and by Hurst (1968). Henderson (1969) has also studied the spread of disease in the Worcestershire area and attributed much of it to wind carriage. Methods and results of aerosol sampling of virus and infected cattle were reported by Thorne & Burrows (1960) and by Hyslop (1965). In this paper the results are given of the measurement of virus in aerosols produced by cattle, sheep and pigs infected with FMD virus.

MATERIALS AND METHODS

Animals

The cattle were 18-month-old Devon steers about 400 kg. in weight. Crossbred sheep were about 30 kg. in weight and Large White pigs 30–40 kg. They were housed (two cattle, eight sheep or eight pigs per box) in looseboxes 3·65 m. × 3·35 m. × 3·05 m.

Virus

Four strains of FMD virus were used: 0_1 Lombardy, 0_1 Swiss 1/66, 0_1 BFS 1860 and 0_2 Brescia. They had been passaged in cattle or pigs or given one passage in IB-RS-2 tissue cultures (de Castro, 1964).

Infection of animals

Cattle were inoculated intradermally at four sites on the tongue, sheep on the coronary band of one foot and pigs on the bulbs of the heel of one foot with 10^4 to 10^5 ID 50 of virus. The animals were observed daily and the extent of lesions noted. In one experiment with the multistage impinger pigs were exposed to infection by being placed in the same box as inoculated pigs and subsequently removed.

Air sampling

The air in the box was sampled once or twice daily by drawing through a large-volume sampler (Litton Model M (Modified), Litton Systems Inc., Minneapolis, Minnesota, U.S.A.). The sampler was run for 1 hr. at 1000 l./min. with a collecting

fluid of phosphate buffered saline (PBS) containing 5 % inactivated ox serum which had been screened for the absence of substances inhibitory to FMD virus. Between runs the sampler was disinfected and cleaned by pumping through 0·2 % citric acid in distilled water, followed by detergent, distilled water and PBS. At the time of collection, the air inlet and outlet in the box were blocked. Air in the central area of the isolation unit was also sampled.

In experiments to determine particle size, a multistage liquid impinger (May, 1966) was run for 45 min. at 55 l./min. The sampling fluid was PBS and the apparatus was sterilized by autoclaving between samplings.

Temperature and relative humidity were recorded during sampling.

Virus assay

Samples were assayed by inoculation of unweaned mice (Skinner, 1951) and calf thyroid tissue culture tubes (Snowdon, 1966). Specificity of reaction was checked by complement fixation and further passage.

RESULTS

Conditions of operation of large-volume air sampler

To test whether loss of virus titre occurred during sampling, virus at varying concentrations was added to the sampling fluid and circulated in the large-volume sampler for an hour at a relative humidity of 55 % and a temperature of 21° C. About 25 % of the volume of fluid was lost through evaporation but no fall in virus titre was noted.

Four ml. of collecting fluid was taken every 15 min. up to 60 min. during sampling in a loosebox. With a high concentration of virus in the box maximum titre was found after 45 min., with a low concentration at 60 min.

Air in infected looseboxes was sampled with or without the electrostatic precipitator. No significant difference was found in the amount of virus collected.

Sampling of infected animals

The results of sampling the air of looseboxes containing cattle, sheep or pigs infected with strains of 0_1 and 0_2 virus are shown in Tables 1–3. The maximum amount of virus recovered per animal per sampling period was the same for cattle and sheep but was about 30-fold higher for pigs. Airborne excretion from pigs lasted 5 days and totalled about 10^6 ID 50 of virus per animal, whereas from sheep and cattle the total virus excretion per animal was about 3×10^4 ID 50 over 4 days. The time of maximum recovery from sheep took place before lesions were visible; in the majority of sheep, lesions were not visible or were difficult to find. Maximum recovery from cattle and pigs was found immediately after generalization from the site of inoculation had occurred but before vesicles had ruptured.

The temperature in the looseboxes varied from 8·5 to 18·5° C. and the relative humidity from 72 to 100 %.

Air was also sampled in the central area of the isolation unit outside the loosebox. When pigs were infected, titres of $10^{4\cdot7}$ ID 50 per sample were found over a period of 2 days.

Table 1. *Extent of lesions and recovery of virus in the large-volume sampler from infected cattle*

Hours post-infection	Extent of lesions	No. of collections	Virus recovery: Range	Virus recovery: Mean	Virus recovery: Mean per animal
17	Unruptured vesicles on tongue	4	2·8–2·9*	2·85	2·55
22		4	2·8–3·2	3·0	2·7
41	Unruptured vesicles on tongue.	4	3·1–4·0	3·5	3·2
46	Vesicles developing on lips and feet	4	2·7–3·0	2·9	2·6
65	Ruptured vesicles on tongue and mouth, vesicles on feet	4	2·7–3·0	2·8	2·5
89	Ruptured vesicles on feet	4	< 1·9–2·8	< 2·3	—
113	Tongue healing. Ruptured vesicles on feet	4	< 1·9	< 1·9	—

* Total virus (log ID 50) recovered over 60 min. at 1000 l./min.

Table 2. *Extent of lesions and recovery of virus in the large-volume sampler from infected sheep*

Hours post-infection	Extent of lesions	No. of collections	Virus recovery: Range	Virus recovery: Mean	Virus recovery: Mean per animal
17	Pain in feet	3	3·65–4·5*	4·1	3·2
41	Swelling and discharge in inoculated foot	3	2·8–3·6	3·3	2·4
65	Lesions in uninoculated feet and in mouth (30 % of animals)	3	2·7–2·8	2·75	1·85
89	Feet healing, lameness	2	< 1·9–2·4	< 2·15	—
113	No change	2	< 1·9	< 1·9	—

* Total virus (log ID 50) recovered over 60 min. at 1000 l./min.

Table 3. *Extent of lesions and recovery of virus in the large-volume sampler from infected pigs*

Hours post-infection	Extent of lesions	No. of collections	Virus recovery: Range	Virus recovery: Mean	Virus recovery: Mean per animal
17	Vesicles in 25 % of sites inoculated	4	< 1·9	< 1·9	—
41	Vesicles at sites inoculated, vesicles on other feet	4	5·2–5·9*	5·6	4·7
65	Primary vesicles ruptured. Vesicles on feet, tongue, snout, mouth	4	4·8–5·5	5·2	4·3
89	Ruptured vesicles	4	3·6–4·2	3·9	3·0
113	No change	4	3·0–4·4	3·9	3·0
137	No change	4	2·7–2·9	2·8	1·9

* Total virus (log ID 50) recovered over 60 min. at 1000 l./min.

Sampling with a multistage liquid impinger

The air in looseboxes containing infected pigs was sampled with a multistage liquid impinger and the results of one experiment are shown in Table 4. By analysis of variance the results are significant at the 5% level. On average, 65% of virus was recovered in the first stage, 24% in the second and 11% in the third; in a second experiment the percentages were 71, 19 and 10. The amount of virus recovered by the multistage impinger was of the same order as that recovered in the large-volume sampler, when allowance was made for rate and time of sampling.

Table 4. *Recovery of virus in stages of multistage liquid impinger from infected pigs*

	Hours post-infection							
	17	22	41	46	65	70	89	94
Stage 1	< 1·2	2·1*	3·95	3·1	3·3	3·2	3·3	2·8
Stage 2	< 1·2	< 1·2	3·3	3·0	2·8	3·1	2·6	2·2
Stage 3	< 1·2	< 1·2	3·35	2·2	2·6	2·4	2·55	1·85

* Total virus (log ID 50) recovered over 45 min. at 55 l./min.

DISCUSSION

The amount of virus recovered from the air of boxes containing infected cattle was similar to that found by Hyslop (1965), if allowance is made for the different rate of sampling. However, with the strains of 0_1 and 0_2 used, virus was not found after the 4th day whereas Hyslop (1965) described virus recovery up to the 14th day after infection with a strain of SAT 1. Kiryukhin & Pasechnikov (1966) recovered 6·3 to 630 ID 50 per litre of exhaled air from calves infected with type 0 virus. This is from 40- to 4000-fold higher than the maximum we obtained, but these authors collected from a mask attached to the animal. In the experiments described above, pigs excreted the greatest amount of virus over the longest period. With both pigs and cattle infected by injection maximum excretion was found when lesions at secondary sites were just visible. In pigs infected by contact maximum excretion was found at the same time as lesions were observed. With sheep maximum excretion occurred before even experienced observers had seen clear signs of disease and in the majority of sheep lesions did not develop, although virus was recovered in pharyngeal samples 7 and 14 days after infection.

The source of virus is uncertain. It is unlikely to be solely from rupture of lesions on the tongue and feet, since at the time of maximum collection lesions were not ruptured in cattle and pigs and had not yet been observed in sheep. Nor is it likely to be from excessive salivation, since this sign was not observed in the cattle until the 3rd day after infection. Virus was found in the pharynx (Burrows, 1968*a*; Sellers, Burrows, Mann & Dawe, 1968) and nasal mucous membrane (Korn, 1957) during the incubation period and it is probable that the virus, recovered as aerosol, came from these sites in the upper respiratory tract. As far as the lower part of the respiratory tract is concerned, Eskildsen (1969) described recovery of virus

from consolidated areas of the lung after infection by the intratracheal route and development of lesions. Other possible sources of virus are the skin of the animals and the dust and faeces in the box. Maximum titres of pig and sheep faeces ($10^{2.9}$ and $10^{2.7}$ ID 50 per g.) were found on the 2nd or later days after infection, and in cattle, titres of $10^{4.9}$ ID 50 per g. or greater were found from the 2nd to 5th day of infection. From these findings one would expect that if faeces were the source of airborne virus, cattle would excrete the greatest amount, but this was not the case. However, spreading of slurry of cattle faeces could set up an aerosol.

Unpublished experiments (G. J. Harper, J. N. Wilson & R. F. Sellers, 1968) showed that FMD virus survival in an aerosol depended on a high relative humidity, loss of infectivity occurring rapidly at a relative humidity of less than 70%. Provided this condition is satisfied and provided that inactivation from other causes does not take place, the concentration and dosage of virus downwind from a source can be calculated from Pasquill's (1961) formula. At a wind speed of 5 m./sec., a lateral spread of 10° and a vertical spread from 10 to 100 m. depending on distance, 100 pigs excreting $10^{2.9}$ ID 50 per pig per min. could give rise to a concentration of 5 ID 50 per 10^3 l. of air at 100 m., 5 per 10^4 l. at 1 km., 1 per 10^5 l. at 10 km., 1 per 10^6 l. at 50 km. and 5 per 10^7 l. at 100 km. One hundred cows or sheep would give rise to about thirty times less. If the upper limit of the range is taken, these concentrations could be doubled. It is not known how efficient the large-volume sampler is. With a larger model Gerone *et al.* (1966) state that recovery of generated aerosols of Coxsackie A 21 virus varied from 1 to 20%. If the same efficiency applies to the smaller sampler, the concentrations can be raised a further 5- to 100-fold.

The results with the multistage liquid impinger indicated that 65–71% of virus recovery was correlated with particles greater than 6 μ, 19–24% between 3 and 6 μ, and 10–11% less than 3 μ (May, 1966). Provided that these sizes are not changed during transport in air, on analogy with man these particles might be expected to infect the upper respiratory tract, bronchi and alveoli respectively. The minimum infecting dose is not known; Eskildsen (1969) reported that 10–100 mouse ID 50 were sufficient to set up infection when inoculated by the intratracheal route and Sutmöller, McVicar & Cottral (1968) infected six out of six animals by injection of tonsillar sinuses and two out of six by intranasal inoculation with 10^2 p.f.u.

The volume of tidal air breathed varies among animals sampling the aerosol. At rest a 400 kg. Holstein cow would sample 85 l./min., a 25 kg. pig 9·27 l. (Brody, 1945) and a sheep about 5 l. (Amoroso, Bell & Rosenberg, 1951). Over a period of 50 hr. 100 cattle would sample $2{\cdot}5 \times 10^7$ l., while 100 sheep or pigs would sample 1·5 to $2{\cdot}8 \times 10^6$ l. This would be sufficient to sample the concentration of virus specified at 100 km. over this period. Within species the tidal air volume in adults is greater than in young animals. One would therefore expect that, given the same concentration of virus in the air, the larger animal would be infected first. In an experiment (Burrows, 1968*a*) where cattle, sheep and pigs were placed in the same unit as infected animals, 50% of cattle had virus in the pharynx at 72 hr., 50% of sheep at 129 hr. and 50% of pigs at 130 hr. If in the field under conditions of

airborne spread animals are exposed to the same concentration of virus, it would be expected that larger herds or flocks would be infected first, that larger animals such as cattle would be infected before pigs or sheep, and adults before calves, piglets or lambs. However, after infection, lesions are observed earlier in cattle than in sheep or pigs (Tables 1–3, and Burrows, 1968*a*) and lesions in sheep may be difficult to detect (Results, and Burrows, 1968*b*); thus the picture would not be clear unless pharyngeal samples were taken for examination for virus. But if in the field lesions are observed in pigs and sheep at the same time as cattle, it is probable that pigs or sheep were infected first and the disease may then have spread to the cattle. If only cattle are affected and pigs or sheep are also on the same farm, it does not necessarily follow that the cattle were infected first, since pharyngeal sampling might establish that virus was also present in the pigs or sheep. Indeed, differential rate of infection together with the varying rate of lesion development may explain certain anomalies in the field, such as cattle showing lesions but not calves, and sheep but not lambs.

Cattle are generally regarded as the most important animals in the dissemination of foot-and-mouth disease because of the nature and extent of lesions and their high virus content and because of the high virus content in milk and faeces. However, in airborne transmission of virus the role of pigs and sheep is emphasized. Sheep act as maintenance hosts, pigs as amplifiers and cattle as indicators.

SUMMARY

A large-volume sampler was used to recover virus excreted as aerosol by cattle, sheep and pigs infected with foot-and-mouth disease. Pigs were found to excrete virus to a maximum of $10^{4.7}$ ID 50 per animal per hour and sheep and cattle to a maximum of $10^{3.2}$ ID 50. Excretion from pigs totalled 10^6 ID 50 per animal over 5 days and from cattle and sheep 3×10^4 ID 50 per animal over 4 days. Maximum recovery occurred 41 hr. after infection in pigs and cattle when lesions had generalized and 17 hr. after infection in sheep before lesions had been observed. Sampling in a multistage liquid impinger showed that 65–71 % of virus was excreted as an aerosol of size $> 6\ \mu$, 19–24 % 3–6 μ and 10–11 % less than 3 μ. The site of production of virus excreted as aerosol is suggested to be the upper respiratory tract. Under conditions of relative humidity greater than 70 % and at low temperatures, survival of virus to a distance of 100 km. is likely to occur and because of the minute respiratory volume the aerosol would be sampled more efficiently by cattle than pigs or sheep and by large animals than by small. These findings are discussed in relation to spread of virus in the field.

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Some factors affecting the viability of freeze-thawed T 4 bacteriophage

II. The influence of certain electrolytes on the degree of inactivation

By P. R. M. STEELE, J. D. DAVIES and R. I. N. GREAVES
University Department of Pathology, Cambridge, England

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Steele, Davies & Greaves (1969) reported that when preparations of T 4 bacteriophage, suspended in phosphate-buffered salt solutions, were subjected to freezing and thawing, the resulting loss of viability was due to two different mechanisms. First, T 4 phage which had been frozen to temperatures *above* the eutectic temperature of the suspending medium were inactivated if the frozen samples were thawed rapidly. This inactivation did not occur when the frozen samples were thawed slowly, and it appeared to be due to 'osmotic shock', i.e. rapid dilution of a concentrated suspending medium (Anderson, 1953; Leibo & Mazur, 1966). Secondly, when osmotic damage was avoided by slow thawing there was inactivation of T 4 phage samples which had been cooled to temperatures *below* the eutectic temperature of the suspending medium. This relationship between eutectic temperature and freeze-thawing damage suggested that damage was due to the removal of the last traces of 'unbound' water as ice.

However, it was later observed that when the phosphate buffer was absent from the suspending medium, considerable inactivation of frozen-thawed T 4 phage occurred above the eutectic temperature, even when frozen samples were thawed slowly (i.e. avoiding osmotic shock). The extent of this inactivation appeared to be dependent on the type of ions in the suspending medium. The purpose of the present study was to investigate these observations, especially with regard to the accepted theories of injury to cells during freeze-thawing.

MATERIALS AND METHODS

Host bacteria

The host organism *Escherichia coli* B was grown in nutrient broth (Hartley's tryptic digest broth, pH 7·4) at 37° C. in 6 ml. volumes for titre determination, or in 500 ml. volumes of the defined salt medium of Adams (Adams, 1959), with aeration, for phage preparation. Stocks were maintained on 1·5% nutrient agar plates.

Bacteriophage

The T 4 phage and T 4 Bo osmotic shock-resistant phage (a gift from Dr S. P. Leibo) were prepared from 500 ml. lysed cultures of *E. coli* B. The methods used for phage preparation, purification and titre determination were the same as those described previously (Steele *et al.* 1969).

Experimental procedure

An experimental stock suspension of the T 4 or T 4 Bo phage at a concentration of 2×10^9 p.f.u./ml. in phosphate buffer (or 2×10^{11} p.f.u./ml. when very low experimental survival was anticipated) was diluted 1000-fold into the experimental suspending medium. Samples of 0·1 ml. were cooled at 1° C./min. (unless otherwise stated). At −5° C. ice formation was induced by touching the surface of each sample with a fine wire cooled in liquid nitrogen. Samples were thawed slowly by placing them in a thick block of polystyrene maintained at 4° C. The samples were diluted 10-fold, 100-fold or 1000-fold with phosphate buffer prior to being assayed.

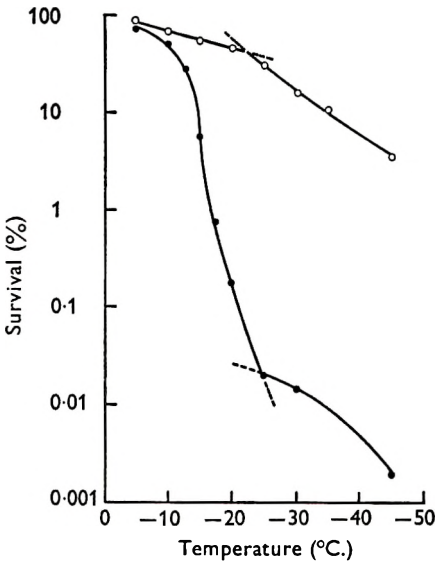

Fig. 1

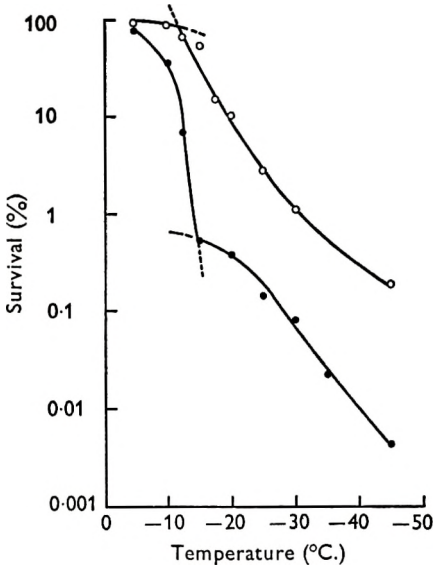

Fig. 2

Fig. 1. The survival of frozen-thawed T4 phage suspended in either 0·1 molal NaCl (○), or 0·1 molal NaBr (●). Samples were cooled at 1° C./min. to the indicated temperatures and then thawed slowly.

Fig. 2. The survival of frozen-thawed T4 phage suspended in either 0·1 molal KCl (○), or 0·1 molal KBr (●). Samples were cooled at 1° C./min. to the indicated temperatures and then thawed slowly.

RESULTS AND DISCUSSION

Inactivating effect of neutral salts

Samples of the T 4 phage suspended in 0·1 molal solutions of NaCl, NaBr, KCl and KBr were cooled to temperatures from 0° to −45° C. and then thawed slowly. The resulting percentage viabilities are shown in Figs. 1 and 2. There was a marked alteration of slope in each inactivation curve which occurred very close to the eutectic temperature of the respective salt (NaCl −21·5° C., NaBr −28° C., KCl −11° C., KBr −13° C.). Inactivation above the eutectic temperature was much greater in the presence of Br^- compared to Cl^-. Below the eutectic tempera-

ture the rate of inactivation was slightly greater with potassium salts than with sodium salts, presumably because of the higher eutectic temperatures of the potassium salts.

Since the extent of inactivation above the eutectic temperature was clearly dependent on the species of ions in the suspending medium, the inactivating effect of other neutral salts above their eutectic temperatures was investigated (Fig. 3). The effectiveness of different anions in causing inactivation of frozen-thawed T 4 phage increased in the order $Cl^- < Br^- < I^-$. Lithium salts caused more inactivation than the corresponding sodium or potassium salt, but the differences were less than those between the effects of different anions.

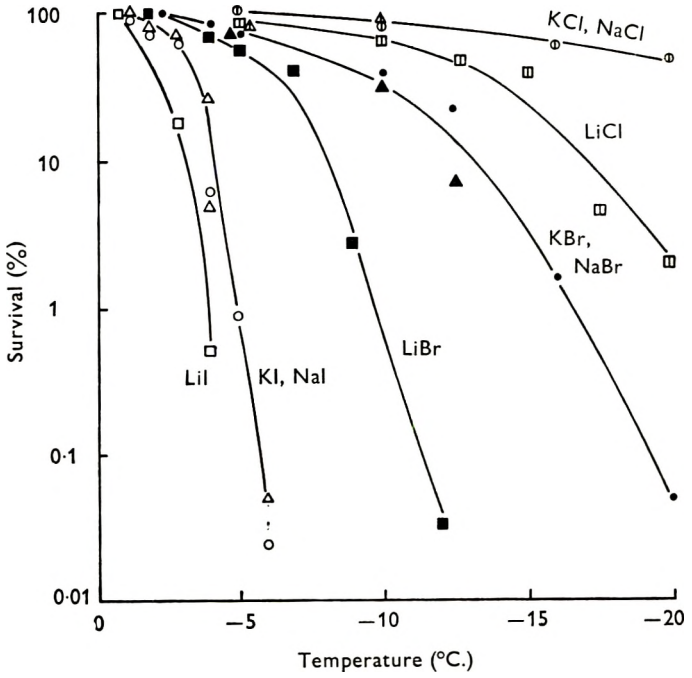

Fig. 3. Samples of the T4 phage, suspended in 0·1 molal solutions of various neutral salts, were cooled at 1° C./min. to the indicated temperatures and thawed slowly. ⌽, NaCl; ●, NaBr; ○, NaI; ⧋, KCl; ▲, KBr; △, KI; ▥, LiCl; ■, LiBr; □, LiI.

When a salt solution is frozen, the formation of ice causes the salt to become increasingly concentrated in the unfrozen aqueous phase. At any particular sub-zero temperature the molal concentration of the unfrozen solution is fixed and independent of the initial salt concentration. The volume of unfrozen solution however is directly proportional to the initial salt concentration. The influence of the volume of unfrozen solution on the survival of frozen-thawed T 4 phage was investigated by freeze-thawing samples of T 4 phage in NaBr solutions of different initial concentrations (0·1, 0·5 and 1·0 molal). It was found that the different initial concentrations of NaBr had no effect on the subsequent survival of frozen-thawed T 4 phage.

Effect of phosphate buffer

It was reported in a previous communication (Steele *et al.* 1969) that T 4 phage, suspended in phosphate-buffered salt solutions, were not inactivated by freeze-thawing at temperatures above the eutectic temperature, so long as osmotic shock was avoided by slow thawing. This protective effect of phosphates was investigated by freeze-thawing T 4 phage and T 4 Bo phage suspended in mixtures of phosphates and NaBr or KBr (Table 1). The addition of mixtures of phosphates gave good protection in all cases. Added K_2HPO_4 gave almost complete protection, whereas added KH_2PO_4 or NaH_2PO_4 raised survival to only 50%. Added Na_2HPO_4 was

Table 1. *Percentage survival of T 4 or T 4 Bo phage in sodium or potassium bromide with the addition of various phosphates*

(The suspensions were cooled at 1° C./min. to the indicated temperatures and thawed slowly.)

Added phosphate, 0·1 molal	T4 phage in 0·1 molal NaBr, −17·5° C.	T4 phage in 0·1 molal KBr, −12·5° C.	T4 Bo phage in 0·1 molal NaBr, −17·5° C.
None	0·8	9·3	0·9
KH_2PO_4–Na_2HPO_4	98	102	93
KH_2PO_4–K_2HPO_4	88	98	85
NaH_2PO_4–Na_2HPO_4	77	98	101
KH_2PO_4	51	51	43
K_2HPO_4	93	102	102
NaH_2PO_4	52	49	42
Na_2HPO_4	64	65	81

not as effective as K_2HPO_4, but this is undoubtedly a reflexion of its high eutectic temperature (−0·5° C.). It is very interesting that dihydrogen phosphates were only half as effective as K_2HPO_4. It might at first appear that the difference was due to the pH of the solution. However, added KH_2PO_4–Na_2HPO_4 was almost completely protective and yet produces an acidic eutectic mixture (Van den Berg, 1959).

In other experiments it was found that $NH_4.OCOCH_3$ and $(NH_4)_2SO_4$ were also very effective in prevention of inactivation of frozen-thawed T 4 phage.

Inactivation in the absence of ice

The relative effectiveness of different salts in inactivating or protecting T 4 phage during freeze-thawing is of the same order as has been reported for their effects on the conformational stability of a large variety of macromolecules (Whitaker & Tapel, 1962; Von Hippel & Wong, 1964; Jencks, 1965; Bello, 1966). Although the mechanism whereby concentrated solutions of different salts exert their characteristic effects on macromolecules is not yet known, their action may be mediated, at least in part, through salt-induced changes in the structure of water (Bello, Riese & Vinograd, 1956; Simpson & Kauzmann, 1953; Von Hippel & Wong, 1964; Jencks, 1965).

It would not be correct, however, to state that T 4 phage are inactivated during freeze-thawing solely owing to the great increase in the concentration of salt in the unfrozen aqueous phase, because in this simple explanation a notable discrepancy is overlooked. It is known, from studies of the effect of osmotic shock on T 4 phage at room temperature, that if osmotic shock is avoided by slow dilution of the suspending medium T 4 phage are not inactivated by exposure to 3 molal solutions of NaCl, NaBr or LiCl (Anderson, 1953; Leibo & Mazur, 1966). During freezing such salts reach a concentration of 3 molal at approximately $-12°$ C., at which

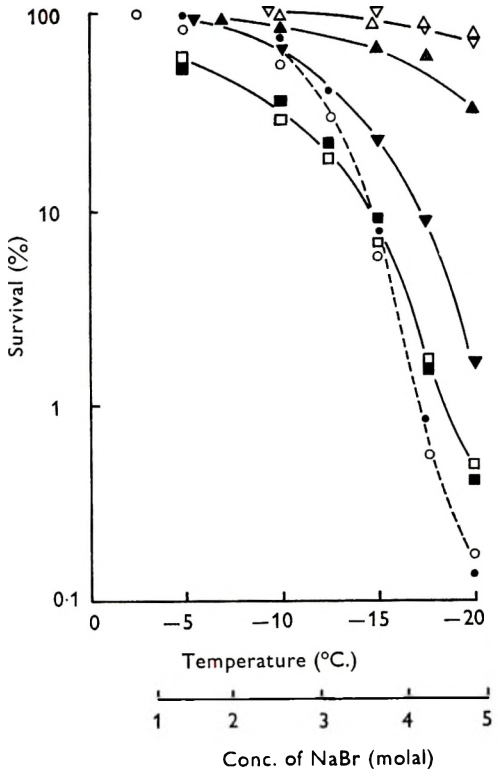

Fig. 4

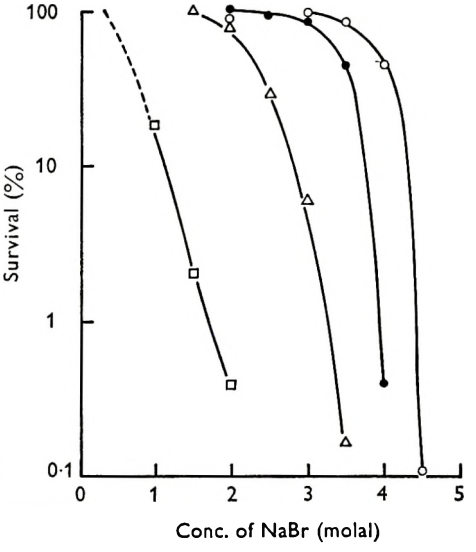

Fig. 5

Fig. 4. Inactivation of phage in the absence of ice. Samples of the T4 and T4Bo phages, suspended in NaBr solutions of different initial molalities, were cooled at 1° C./min. to the indicated temperatures and rewarmed slowly. These samples were not seeded with a cold wire and remained unfrozen throughout the experiment. The dotted line shows the control inactivation curve for samples of T4 and T4Bo phages, suspended in 0·1 molal NaBr, which were cooled with freezing and thawed, as in the normal experimental procedure. The lower scale shows the molal concentration of NaBr in the unfrozen aqueous phase in equilibrium with ice at subzero temperatures. ▽, 1·0 molal NaBr (T4 phage); △, 1·0 molal NaBr (T4Bo phage); ▲, 2·0 molal NaBr (T4Bo phage); ▼, 2·5 molal NaBr (T4Bo phage); □, 3·5 molal NaBr (T4 phage); ■, 3·5 molal NaBr (T4Bo phage); ○, 0·1 molal NaBr (T4 phage, frozen control); ●, 0·1 molal NaBr (T4Bo phage, frozen control).

Fig. 5. The effect of lowered temperature on the degree of inactivation of T4Bo phage. The figure shows the survival of T4Bo phage following 30 min. exposure to unfrozen NaBr solutions of the indicated molalities at 20° C. (○), 0° C. (●), $-10°$ C. (△), and $-20°$ C. (□).

temperature they all produced marked inactivation of T 4 phage and T 4 Bo phage. This inactivation was in no way due to the increase in phage concentration during freezing, since different initial titres of phage in experimental samples were found to have no effect on the subsequent percentage viabilities after freeze-thawing.

In order to investigate this discrepancy, a modified experimental technique was adopted. Samples of T 4 phage and T 4 Bo phage, suspended in concentrated NaBr solutions, were cooled at 1° C./min. to temperatures from $-5°$ to $-20°$ C. and rewarmed slowly. The samples were *not* seeded with a cold wire and remained unfrozen throughout the experiment. The cooled and rewarmed samples of T 4 phage were diluted slowly in a stepwise fashion to avoid osmotic shock during the assay of viability. Samples of the osmotic shock-resistant T 4 Bo phage were diluted directly into phosphate buffer before being assayed. Control inactivation curves were determined for samples of T 4 phage and T 4 Bo phage suspended in 0·1 molal NaBr. These latter samples were seeded at $-5°$ C. as in the normal experimental procedure. The results of these experiments are shown in Fig. 4.

The susceptibilities of the T 4 and T 4 Bo phages were identical, and at any temperature the extent of the inactivation was related to the salt concentration whether ice was present or not. The most interesting observation, however, was that in the absence of ice and change in salt concentration there was still a considerable increase in inactivation as the temperature was lowered. This was not an effect of prolonged exposure of the phage to a concentrated salt solution, since at room temperature a 3·5 molal NaBr solution caused almost no inactivation of phage over a period of several hours.

The experiments indicated that the temperature of the experimental samples was as important as the salt concentration in influencing the degree of inactivation of the T 4 and T 4 Bo phages.

Further experiments were carried out to investigate the effect of temperature. Samples of the T 4 Bo phage were exposed to unfrozen concentrated solutions of NaBr for 30 min. at 20°, 0°, $-10°$ and $-20°$ C. The results are shown in Fig. 5. Clearly the effect of lowering the temperature was to produce a very marked increase in the degree of inactivation of phage exposed to identical salt concentrations.

Storage above the eutectic temperature

In the experiments described so far, samples were cooled to the desired temperature and immediately thawed. A further series of experiments was performed in which frozen samples of the T 4 phage, suspended in various salt solutions, were stored at subzero temperatures for periods of 10 min. to 5 hr. before thawing. In all cases it was observed that inactivation of the phage continued for the first 20–30 min. of storage at a rate comparable to that observed during cooling, but thereafter the inactivation rate decreased by a factor of more than 50-fold. It appeared that a proportion of the phage was in some way protected against the injurious effect of the concentrated salt in the unfrozen aqueous phase. For each suspending medium the fraction of phage which was protected was lower the lower the storage temperature, and at any particular storage temperature was

dependent on the species of ions in the suspending medium, being decreased by different ions in the same order of effectiveness as had been observed for inactivation during freezing and thawing without storage. These observations were investigated further using NaBr solutions as the suspending medium; $-15°$ C. was arbitrarily chosen as the storage temperature.

At first it was thought that the effect might be due to the very low concentration of phosphate buffer in the suspending medium (samples were prepared by a 1000-fold dilution of a stock suspension of T 4 phage, in phosphate buffer, into the experimental suspending medium). Subsequent experiments (Fig. 6) showed,

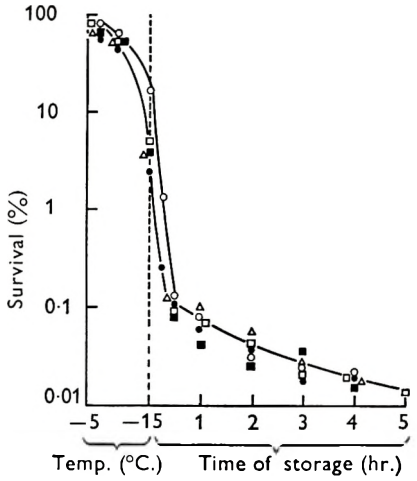

Fig. 6

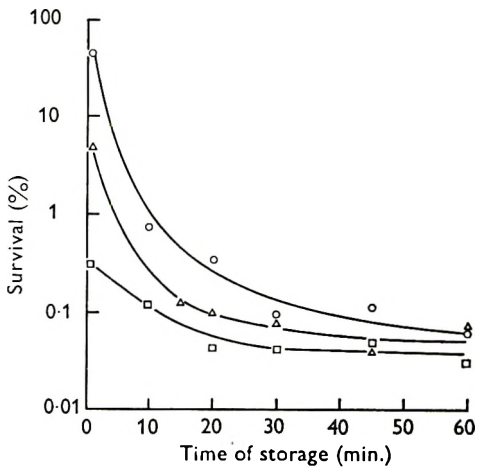

Fig. 7

Fig. 6. The effect of storage on the viability of T 4 phage. Samples of T 4 phage, suspended in 0·1 molal NaBr, were cooled at 1° C./min. to $-15°$ C. and stored at that temperature for the indicated periods, and then thawed slowly. The figure shows the inactivation during both pre-freezing and after storage at $-15°$ C. ○, 100-fold dilution from phosphate buffer; □, 1000-fold dilution from phosphate buffer; ●, 10,000-fold dilution from phosphate buffer; ■, initial T 4 phage titre of 2×10^8 p.f.u./ml.; △, two freeze–storage–thaw cycles.

Fig. 7. The effect of freezing rate. Samples of T 4 phage, suspended in 0·1 molal NaBr, were cooled at 100° C./min. (○), 1° C./min. (△) or 0·3° C./min. (□) to -15 °C. and stored at that temperature for periods of 0–60 min. before thawing slowly.

however, that the same results were obtained whether the experimental samples had been prepared by a 100-fold, 1000-fold or 10,000-fold dilution of the stock suspension. (Stock suspensions with T 4 phage titres of 2×10^8, 2×10^9 and 2×10^{10} p.f.u./ml. respectively were used, so that the initial experimental titre was 2×10^6 p.f.u./ml. in each case.) Moreover, the protected fraction was independent of the initial phage concentration (Fig. 6) and freezing rate (Fig. 7). One set of samples of T 4 phage, suspended in 0·1 molal NaBr, were stored at $-15°$ C. for 60 min. and thawed, and then refrozen and stored at $-15°$ C. a second time. The protected fraction observed during the second period of storage (expressed as percentage of the surviving plaque forming units remaining after the first period of storage) was identical with that observed for a single freeze–storage–thaw cycle (Fig. 6).

Very significant results were obtained when experiments were carried out using different initial concentrations of NaBr in the suspending medium. The initial survival at $-15°$ C. of frozen-thawed T 4 phage had been shown to be independent of the initial NaBr concentration. During storage, however, large differences were observed (Fig. 8). The protected fraction of phage was highest in those samples initially suspended in 0·01 molal NaBr, being about 1 % of the original titre, and was considerably lowered when higher initial concentrations of NaBr were used. There were no protected phage when T 4 or T 4 Bo phage suspended in 3·5 molal NaBr were stored at $-15°$ C. in the absence of ice.

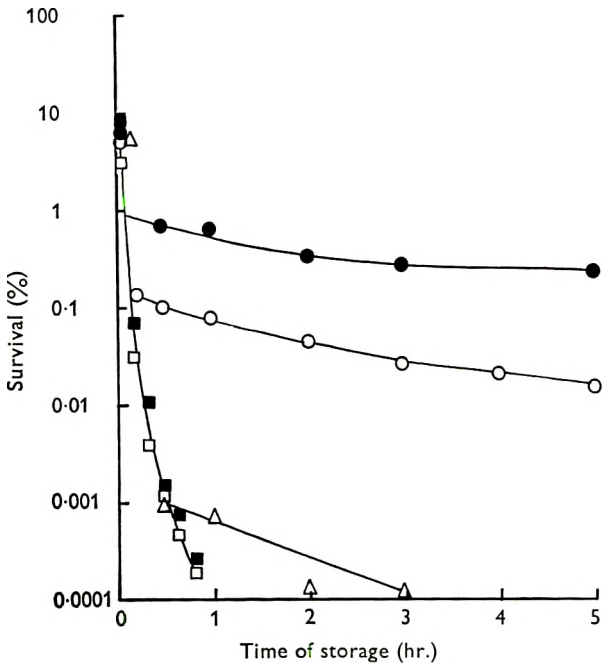

Fig. 8. Effect of initial NaBr concentration. Samples of T 4 and T 4 Bo phages, suspended in NaBr solutions of different initial molalities, were cooled at 1° C./min. to $-15°$ C. and stored at that temperature for the indicated times, and then thawed slowly. ●, 0·01 molal NaBr (T 4 phage); ○, 0·1 molal NaBr (T 4 phage); △, 1·0 molal NaBr (T 4 phage); ■, 3·5 molal NaBr (T 4 phage, unfrozen); □, 3·5 molal NaBr (T 4 Bo phage, unfrozen).

When the initial concentration of salt in the suspending medium is raised, the width of unfrozen channels of liquid between the ice crystals at any subzero temperature is greater (Nei, 1968). The results therefore suggest that during freezing phage particles which are trapped in channels of unfrozen liquid of less than a critical maximum width are in some way protected against inactivation. There is evidence that water in narrow channels may possess a higher degree of structural order than bulk liquid (Bangham & Bangham, 1968; Willis, Rennie, Smart & Pethica, 1969). Perhaps such a 'structuring' of the water protects the phage against inactivation. It is indeed paradoxical to find that ice can act as a protective against freezing injury!

Effect of D_2O

In recent years the work of several investigators, notably Kauzmann (1959), Némethy & Scheraga (1962*a*, *b*), Némethy, Steinberg & Scheraga (1963), and Tanford (1962) and their co-workers, has shown the great importance of hydrophobic bonds in the conformational stability of proteins. Hydrophobic bonds become weaker when the temperature is lowered, and this fact may explain the effect of lowered temperature on the degree of inactivation of T 4 and T 4 Bo phages. Hydrophobic interactions between apolar amino acid side-chains are slightly stronger in D_2O than H_2O (Kresheck, Schneider & Scheraga, 1965), and it has been observed that ribonuclease (Hermans & Scheraga, 1959), gelatin (Harrington & Von Hippel, 1961) and catalase (Guild & Van Tubergen, 1957) are more stable to thermal modification in D_2O than H_2O. Similarly, denaturation of ovalbumin by urea is slower in D_2O than H_2O (Maybury & Katz, 1959).

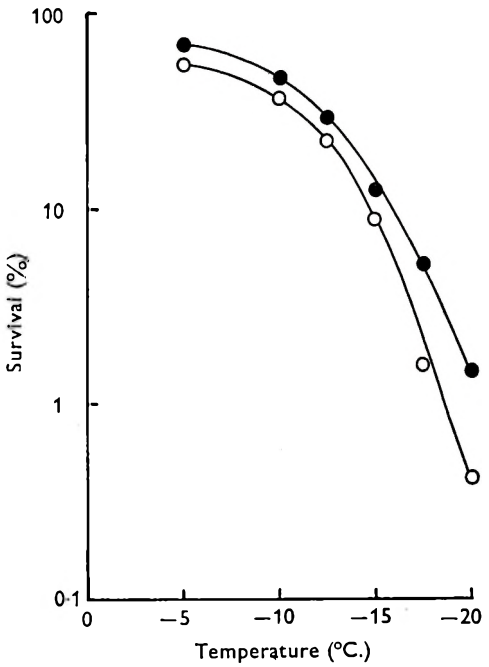

Fig. 9

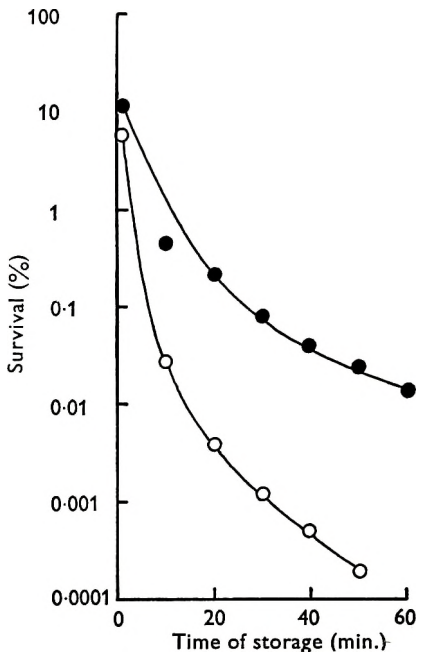

Fig. 10

Fig. 9. Effect of D_2O. Samples of T 4 Bo phage suspended in 3·5 molal NaBr–H_2O (○), or 3·15 molal NaBr–D_2O (●) were cooled at 1° C./min. without freezing to the indicated temperatures and rewarmed slowly.

Fig. 10. Effect of D_2O during storage. Samples of T 4 Bo phage suspended in 3·5 molal NaBr–H_2O (○), or 3·15 molal NaBr–D_2O (●) were cooled at 1° C./min. without freezing to − 15° C. and stored at that temperature for periods of 0–60 min. before rewarming slowly.

In order to investigate a possible stabilizing effect of D_2O on phage, T 4 Bo phage suspended in 3·15 molal NaBr in D_2O (99·7 % D_2O, Koch-Light Labs., England) were cooled to subzero temperatures, without freezing, and rewarmed,

and the results compared with those previously obtained using 3·5 molal NaBr in H_2O (Fig. 9). (Since molality is defined as 'moles of solute per 1000 g. of solvent', the *mole fraction* of NaBr is the same in 3·5 molal NaBr–H_2O as in 3·15 molal NaBr–D_2O, i.e. 0·059.) The percentage viability of T 4 Bo phage cooled and rewarmed in the D_2O solution was clearly greater than in the H_2O solution, the difference being fourfold at $-20°$ C. The difference was considerably magnified during storage at $-15°$ C., reaching a factor of over 100-fold after 50 minutes of storage (Fig. 10).

Protective effect of glycerol

Glycerol has been extensively used as a protective additive against freeze-thawing injury to cells since the discovery of its protective effect by Polge, Smith & Parkes (1949). Lovelock (1953) demonstrated that glycerol lowers the concentration of solutes in equilibrium with ice at any temperature, whilst Rey (1960),

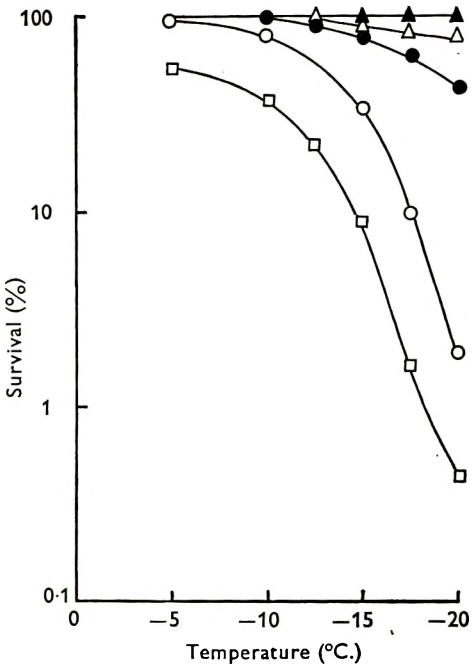

Fig. 11. The protective effect of glycerol in the absence of ice. T 4 Bo phage were suspended in 3·5 molal solutions of NaBr containing 1 % glycerol (○), 5 % glycerol (●), 10 % glycerol (△), 20 % glycerol (▲), or no glycerol (□). Samples were cooled at 1° C./min. without freezing, to the indicated temperatures and rewarmed slowly.

and Greaves & Davies (1965) have shown that the presence of glycerol eliminates salt eutectics during freezing. These two effects of glycerol have usually been regarded as the basis of its protective action against freeze-thawing injury (Smith, 1961; Meryman, 1966). It was therefore of interest to determine the effect of added glycerol on the survival of phage cooled to subzero temperatures in the *absence* of ice.

Samples of T 4 Bo phage were suspended in 3·5 molal NaBr solutions containing 1, 5, 10 and 20 % glycerol. The samples were cooled at 1° C./min., without freezing,

and rewarmed slowly. The resulting viabilities are shown in Fig. 11. The addition of glycerol protected against inactivation although there was no ice present in the experimental samples. At a concentration of 20% the added glycerol was completely protective. An interesting parallel to this result is the observation of Simpson & Kauzmann (1953) that addition of glycerol prevents denaturation of ovalbumin by urea.

SUMMARY

The effects of various salts on the viability of T 4 and T 4 Bo phages during freezing and thawing have been studied. The effectiveness of different salts in causing inactivation or protection of phage during freeze–thawing was of the same order as has been observed for their effects on the conformational stability of macromolecules. There were two important contributory factors causing inactivation of phage during freeze–thawing: (i) concentration of solutes during freezing, and (ii) lowering the temperature, without change in salt concentration.

The viability of phage following storage at subzero temperatures was dependent on the storage temperature, the species of ions in the suspending medium and the initial salt concentration. Viability was greatest when phage had been initially suspended in dilute solutions.

Survival of T 4 Bo phage, following cooling to subzero temperatures and rewarming, was greater in D_2O solutions than in H_2O solutions.

It was found that 20% glycerol completely protected against inactivation of T 4 Bo phage, suspended in 3·5 molal NaBr, which were cooled to subzero temperatures and rewarmed in the absence of ice. Without added glycerol the viability of T 4 Bo phage suspended in 3·5 molal NaBr, which were cooled to $-20°$ C and rewarmed was less than 1%. This protective effect of glycerol is in contradiction to the accepted views of its mode of action in prevention of freezing injury.

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A new nephritogenic streptococcus

By EUGENIA DUCA, Gr. TEODOROVICI, C. RADU, ALLA VÎŢĂ, PAULA TALAŞMAN-NICULESCU, ELISABETA BERNESCU, C. FELDI and V. ROŞCA

Department of Microbiology, Faculty of Medicine and Pharmacy, Iaşi, Romania

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INTRODUCTION

According to our present state of knowledge, only certain types of group A streptococci are usually associated with acute glomerulonephritis as a late non-suppurative complication of sore throat or pyoderma.

We recently had an opportunity of studying a summer outbreak of sore throat in which the infecting organism was a streptococcus of group C, *Streptococcus zooepidemicus*; a third of those infected later developed acute glomerulonephritis. Epidemiological inquiry revealed all the features of a milk-borne epidemic connected with a temporary fault in pasteurization (Teodorovici, 1969).

Since human infections with *S. zooepidemicus* have been rarely, if ever, described, and there is no record of any such infections being complicated with glomerulonephritis, an account of the findings in this outbreak may be of interest to streptococcal workers.

MATERIALS AND METHODS

Clinical material

Between 20 May and 12 July 1968, in the town of P. Neamtz (Romania), 85 cases of sore throat occurred, nearly all of them associated with cervical lymphadenitis and pyrexia. Seventy-four (87 %) of the patients were adults; about a third of them showed acute glomerulonephritis as a late complication. Signs of renal involvement appeared in the second or third week of illness and consisted of oedema of eyelids and feet, oliguria which in a few cases became anuria, haematuria, proteinuria frequently associated with cylindruria, and arterial hypertension.

From two of three patients on whom renal biopsy was performed sufficient material for histological examination was obtained, and typical lesions of subacute glomerulonephritis were observed, consisting of proliferative glomerulitis associated with neutrophil and eosinophil infiltration, thickening of Bowman's capsule and capsular synechia, periglomerular polymorphonuclear and lymphocytic infiltration, and protein exudate in some proximal convoluted tubules.

The clinical, physiological and histological aspects of the outbreak are discussed in detail in a separate paper (Vîţă & Niculescu-Talaşman, 1969).

Because an outbreak of tularaemia with cervical adenitis had occurred in the same region in the previous year (Pencea & Vita, 1968) the early clinical diagnosis

and treatment were at fault in the early cases, and penicillin was used in only a few cases. For the same reason complete bacteriological examinations were requested rather late, 30 days after the first cases occurred, at a time when 15 patients only could still be investigated.

Microbiological investigations

Material for culture

From each patient throat swabs were examined, as this was the probable site of entry; urine and faeces were also cultured as possible routes of excretion. In four patients biopsy specimens from an inflamed cervical lymph node were also cultured.

Methods of culture

Since in the early stages the possible diagnosis might have been any one of a number of bacterial and viral infections, all specimens were inoculated on suitable culture media and into animals. Each throat swab was inoculated on a blood-agar plate, containing 10% defibrinated sheep blood, and a second swab was washed out in 2 ml. of sterile saline which was immediately inoculated subcutaneously into two young Swiss albino mice of 16–18 g. each. Biopsy specimens from lymph nodes were also inoculated intraperitoneally into rabbits and guinea-pigs.

Urine samples were cultured quantitatively on blood-agar plates. Faeces specimens were cultured on Leifson's deoxycholate citrate agar.

Cow's milk samples, after sedimentation, were inoculated on blood-agar plates and also subcutaneously into mice.

All plates were examined after 24 and 48 hr. incubation at 37° C. and the colonies were identified by the usual tests.

Another set of throat swabs and faeces samples were frozen in Hanks's solution containing 1% lactalbumin hydrolysate, penicillin 1000 u./ml. and streptomycin 500 u./ml.; 24 hr. later they were inoculated, after thawing, into HeLa and KB cells and into 10-day-old chick embryos.

Other diagnostic methods

Blood was collected from patients for serological tests. The group-specific precipitating sera for streptococci of groups A, C and G were those obtained from the Institute 'Dr I. Cantacuzino', Bucharest.

Fermentative activity of streptococci was determined in peptone water containing 1% lactose, sorbitol or trehalose, using bromthymol blue as indicator.

Autoserodiagnosis was performed by the ring precipitation test, using antigens prepared from the strain Bi.Gh. This was considered the most representative strain for the outbreak since it was isolated in pure culture from a biopsy specimen from an untreated patient. Two antigens were prepared according to Lancefield's techniques; carbohydrate C, by extracting with N/5 hydrochloric acid for 10 min. at 100° C., without further purification, and 'protein antigen' by extracting with N/20 HCl for 15 min. at 100° C. without further purification. The latter is the technique used for the M protein of group A streptococci (Lancefield, 1928*a*, *b*; 1933).

The antistreptolysin-O titration in patients' sera was performed by the method of Rantz & Randall (1945), using streptolysin-O supplied by the Institute 'Dr I. Cantacuzino', Bucharest.

RESULTS

After 24 hr. incubation the urine and faeces cultures showed nothing significant. From the throat swabs some streptococcus viridans, pneumococci, some Gram-negative diplococci, some yeasts, and two haemolytic streptococci (specimens I.St. and P.Gh.) were isolated (Table 1).

Table 1. *Strains of streptococci isolated from untreated and treated patients, and from convalescents*

Name	Age (yr.)	Sex	Anti-biotics	Specimens TS*	Specimens CL*	Days from onset	Isolated on or in Blood agar	Isolated on or in Mouse	Group	Biochemical type Tr†	Biochemical type So†	Biochemical type La†
Untreated patients												
I.St	56	M	None	+	.	16	+	+	C	−	+	+
P.Gh.	35	M	None	+	.	10	+	+	C	−	+	+
L.V.	39	M	None	+	.	9	−	+	C	−	+	+
M.A.	30	M	None	+	.	12	−	+	C	−	+	+
Bi.Gh.	31	M	None	+	+	7	+‡	+§	C	−	+	+
B.M.	25	F	None	+	.	6	−	+	−	.	.	.
P.M.	8	F	None	.	+	15	+	.	A	+	+	+
V.I.	14	M	None	+	.	8	+	.	G	.	.	.
G.A.	28	F	None	+	.	14	+	.	C	−	+	+
I.E.	36	F	None	+	.	11	+	.	C	−	+	+
D.V.	10	M	None	.	+	23	−	−	−	.	.	.
Treated patients												
Bu.Gh.	44	M	T	.	+	23	+‡	+‖	C	−	+	+
C.V.	31	M	P,T	+	.	19	−	−	−	.	.	.
F.M.	13	M	P,T	+	.	7	−	−	−	.	.	.
R.M.	25	F	P,T	+	.	6	−	−	−	.	.	.
Convalescents												
S.A.	34	M	T	+	.	124	+	.	C	−	+	+
G.M.	26	M	P,T	+	.	120	+	.	C	−	+	+

* TS = throat swab; CL = cervical lymph node.
† Tr = trehalose; So = sorbitol; La = lactose.
‡ Also positive in tissue culture and chick embryo.
§ Also positive in rabbit and guinea-pig.
‖ Also positive in rabbit, negative in guinea-pig.
Antibiotics: T = tetracycline; P = penicillin.

The mice inoculated with the pharyngeal exudate from patient I.St. died in 24 hr. of septicaemia with a capsulated strongly beta-haemolytic streptococcus; 96 hr. later, the mice inoculated with throat swabs L.V. and M.A. died also of septicaemia and intense intravascular haemolysis. In all smears of mouse blood or organs the same capsulated streptococci, often arranged in pairs, were found.

A biopsy specimen from a cervical lymph node of the patient Bi.Gh. was available on the third day of the investigations, and the suspension of this was inoculated intraperitoneally in various animals and on culture media, with the following results: a 3 kg. rabbit died after 24 hr. and his spleen was passed to another rabbit which died after 17 hr. Inoculated mice died after 48 hr. and one of two guinea-pigs after 72 hr. All the dead animals showed intense hyperaemia of the abdominal wall and peritoneum, intense haemolysis and heavy streptococcal septicaemia.

A second lymph-node biopsy was carried out, on patient Bu.Gh. on the 23rd day of illness, while he was being treated with tetracycline and chloramphenicol. Smears showed pus cells and a few streptococci. Both young and adult mice inoculated with this material died after 48 hr. and a rabbit after 96 hr. Inoculated guinea-pigs survived.

Cultures from both these biopsy specimens, on solid and in liquid media and in chick embryos and tissue culture, showed a pure growth of the same beta-haemolytic capsulated streptococcus. Six strains of this streptococcus were isolated from the first ten patients investigated (I.St., P.G., L.V., M.A., Bi.Gh., B.M., Bu.Gh., C.V., F.V., R.M.), a high proportion since most were in the second to the fourth week of illness and four were receiving antibiotic treatment. Three of these, who were receiving penicillin, showed negative cultures.

All attempts to isolate another pathogenic agent were negative, as well as serological investigations for infective mononucleosis, tularaemia, adenovirus and other virus infections.

Streptococci of group A (*Streptococcus pyogenes*), which are responsible for most severe human streptococcal infections, show a low and variable pathogenicity for mice and rabbits, and it was therefore remarkable to isolate six strains from a human epidemic which showed a high initial pathogenicity for these animals, which were strongly haemolytic *in vitro* and *in vivo*, and which predominantly appeared as capsulated diplococci. The explanation was provided by the results of serological grouping of these strains, which all proved to belong to Lancefield's group C.

Group C streptococci occasionally isolated from humans are found as a commensal of pharyngeal and vaginal mucosa, and cause a small proportion of sporadic benign streptococcal infections without delayed non-suppurative complications. Such streptococci belong to the *Str. equisimilis* type, and never appear to cause epidemics. Biochemical typing showed that the strains in this epidemic all fermented sorbitol but not trehalose and therefore belonged to the animal type of group C (*Str. zooepidemicus*). They were all of the lactose-positive subtype. Cottoni & Floch (1939), in their study on the experimental pathogenicity of beta-haemolytic streptococci, pointed out the high virulence of the animal strains for mice and even more for rabbits.

The serological and biochemical typing of these strains thus confirmed the animal origin of this epidemic; such an origin had appeared certain as a result of the epidemiological investigation (Teodorovici, 1969).

During the next few days two more strains of the same group and type were isolated by the local P. Neamtz laboratory (specimens G.A. and I.E.) together

with one strain of group G (V.I.), and one of group A from a lymph node (P.M.). Specimen D.V. was negative (Table 1).

Since *Str. zooepidemicus* has not been previously implicated as the cause of an outbreak with glomerulonephritis as a complication, it is fortunate that we were able to isolate the same agent from uncontaminated specimens, i.e. biopsies of lymph nodes from patients Bi.Gh. and Bu.Gh., thus strengthening the evidence for it being the causal agent. Once this had been established it was possible to prevent further cases of glomerulonephritis by early penicillin treatment of new cases of sore throat; consequently, new attempts to isolate strains of streptococci from patients gave negative results.

Table 2. *Strains of streptococci isolated from throat swabs of carriers at the dairy farm, and from samples of cow's milk*

Name	Age (yr.)	Sex	Isolated on or in: Blood agar	Isolated on or in: Mouse	Group	Biochemical type: Tr*	Biochemical type: So*	Biochemical type: La*
			Carriers					
N.Gh.	22	M	+	.	A	+	−	+
S.M.	45	M	+	.	A	+	−	+
M.C.	29	M	+	.	C	−	+	+
C.I.	27	M	+	.	G	.	.	.
N.I.	34	F	+	.	C	−	+	+
N.L.	39	F	+	.	A	+	−	+
L.M.	23	F	+	.	A	+	−	−
D.M.	41	F	+	.	G	.	.	.
T.C.	22	M	+	.	A	+	−	+
B.V.	29	M	+	.	A	+	−	−
A.V.	37	M	+	.	A	+	−	+
P.A.	40	F	+	.	C	−	+	+
A.V.	25	M	+	.	C	−	+	−
			Cow's milk					
Sample 1	.	.	+	+	C	−	+	+
Sample 2	.	.	+	+	C	−	+	+
Sample 3	.	.	+	+	C	−	+	+

* Tr = trehalose; SO = sorbitol; La = lactose.

Three other strains of *Str. zooepidemicus* were isolated by the local veterinary laboratory from 277 samples of cow's milk from animals with mastitis in one of the great dairy farms supplying the town of P. Neamtz. At the same time, adult workers at this dairy farm were examined in a search for streptococcal carriers. Thirteen carriers were found, and four of these were carrying group C strains, a very high proportion. Of these four, one was of the human type, and the other three were identified as *Str. zooepidemicus* of the lactose-positive subtype similar to the strains isolated from our patients (Table 2). From one of these carriers (P.A.) the same type of streptococcus was isolated four times during 3 months of supervision.

Two further strains of *Str. zooepidemicus* were isolated from convalescent patients (S.A. and G.M.) 4 months after the acute stage of their illness (Table 1). It should be pointed out that two patients show chronic sequelae, nephrotic syndrome and hypertension, 7 months after the acute phase.

Because of the great variety of streptococcal groups and types, we made use of autoserodiagnosis, as a ring precipitation test, as further evidence of the aetiologic role of the agent we had isolated. As shown in Table 3, all the precipitation reactions with patients' sera were positive with both the antigens prepared from the strain Bi.Gh. by the Lancefield techniques.

Antistreptolysin-O (ASO) titres were generally low.

Table 3. *Serological results with sera from untreated and treated patients, and from convalescents*

Name of patient	Streptococcal group	Precipitation with patient's serum using Lancefield's 'C' extract	Precipitation with patient's serum using Lancefield's Protein extract	Antistreptolysin-O titre
		Untreated patients		
I.St.	C	+	+ +	125
P.Gh.	C	+ +	+ + +	125
L.V.	C	+	+	50
M.A.	C	.	.	125
Bi.Gh.	C	+ +	+ + +	100
B.M.	—	±	.	166
P.M.	A	−	−	125
G.A.	C	+ +	−	166
I.E.	C	+ +	+ + +	100
D.V.	—	+	+	50
		Treated patients		
Bu.Gh.	C	+ + +	+ +	333
C.V.	—	.	.	12
F.M.	—	+ + +	+ + +	125
R.M.	—	+ + + +	+ + + +	250
		Convalescents		
S.A.	C	+ + +	+ + + +	120
G.M.	C	+ + +	−	12

DISCUSSION

The evidence for the part played by the group C streptococcus (*Str. zooepidemicus*) of lactose-positive subtype in the outbreak studied was based on the isolation of strains with very similar characteristics from (*a*) two biopsies of inflamed cervical lymph nodes from patients Bi.Gh. and Bu.Gh., (*b*) four out of five throat swabs from untreated patients, (*c*) two convalescent carriers, (*d*) three apparently normal carriers among people in close contact with cows in one dairy farm, (*e*) throat swabs from two other patients, examined by the P. Neamtz laboratory, and (*f*) three samples of cow's milk from the dairy farm in which the carriers under (*d*) worked, isolated by the local veterinary laboratory. All these

strains, isolated from various sources by three different laboratories, belong to the same biochemical type and subtype of group C streptococci, a type which is not usually found in man and which has not yet been shown to cause nephritis in man.

In this outbreak the autoserodiagnostic test proved to be efficient. Despite the fact that the acid extraction techniques used did not furnish pure carbohydrate and protein antigens, we are of the opinion that the positive results obtained with patients' sera were, in this investigation, more valuable than the ASO titration as evidence of infection.

Chronic mastitis in cows is generally produced by *Streptococcus agalactiae* (group B), and the subacute mastitis affecting one-quarter of the udder is often the result of infection with *Str. dysgalactiae*, a non-haemolytic type of group C; other streptococci, such as *Str. pyogenes* (group A) or *Str. uberis* (group D), are seldom encountered. However, Buxton (1949) described two small epizootics of a very severe form of mastitis caused by group C streptococci type *zooepidemicus* subtype lactose-positive, corresponding to the serological type 2 of Bazeley & Battle (1940) which, because of its characteristic appearance as diplococci, was also called 'diplostreptococcus' (Haupt, 1964). The streptococci isolated in the outbreak described here appear to fall in this subtype.

Almost every new epidemic of acute glomerulonephritis reported reveals new types of nephritogenic streptococci of group A (Dillon, Moody, Maxted & Parker, 1967; Dillon, Reeves & Maxted, 1968; Perlman, Herdman, Kleinman & Vernier, 1965; Top, Wannamaker, Maxted & Anthony, 1967). In the outbreak of 1966 in Trinidad, from patients with typical acute glomerulonephritis, in addition to 25 strains of group A, types Trinidad A and B, three strains of group G and one of group C were also isolated; the authors point out that the group G strain possesses the M 12 antigen characteristic of the well known nephritogenic type 12 of group A (Maxted & Potter, 1967; Poon-King *et al.* 1967). It is clear that the list of streptococcal types in group A is not yet closed, and the same is probably true of the nephritogenic streptococci. The search for a nephritogenic factor could probably bring new light into this field.

SUMMARY

A local milk-borne outbreak of 85 cases of sore throat with cervical lymphadenitis, a third of whom later developed acute glomerulonephritis as a complication, is described. Renal involvement was shown by Volhard's criteria, filtered fraction data and renal biopsy findings. From lymph-node biopsies from patients, from the pharyngeal exudate of patients and carriers, and from three samples of cow's milk, 16 strains of beta-haemolytic streptococci of Lancefield group C, type *zooepidemicus* subtype lactose-positive, were isolated.

The aetiological role of the streptococcus isolated in this outbreak was confirmed by autoserodiagnosis (precipitation tests with Lancefield's antigens and patients' sera) which in this case proved more valuable than antistreptolysin-O titration as evidence of infection.

Two of the 85 patients, 7 months after the acute phase of illness, show nephrotic oedema and hypertension as chronic sequelae.

We are pleased to express our gratitude to Dr M. T. Parker and Dr W. R. Maxted for much advice, and confirmation of our serological and biochemical findings.

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Trachoma vaccine field trials in The Gambia

By SHIONA SOWA, J. SOWA, L. H. COLLIER
and W. A. BLYTH

M.R.C. Trachoma Unit, Lister Institute of Preventive Medicine, London, S.W.1, and Medical Research Council Laboratories, Fajara, The Gambia

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There have been many reports of attempts to induce immunity against ophthalmic infection by the trachoma/inclusion conjunctivitis (TRIC) agents. Vaccines prepared from these members of the genus *Chlamydia* have been tested for their power to prevent infections artificially induced in simian species and in human volunteers, and for their therapeutic and prophylactic efficacy against naturally acquired trachoma in man. Collier (1966) reviewed the problems of producing and testing trachoma vaccines and the results of field trials in various countries. Comparison of these reports, some of which were conflicting, was difficult because of the diversity of vaccines used and of the test conditions; but the general conclusions were that although a measure of protection could be secured, it was usually of short duration, and that no fully effective vaccine then existed. This opinion was reinforced by subsequent articles published in 'Conference on Trachoma and Allied Diseases' (1967) and by our experiments on baboons (Collier & Blyth, 1966*a*, *b*; Collier, Blyth, Larin & Treharne, 1967). Lack of space forbids a detailed review of these papers; but although opinions still differ about the respective merits of live and inactivated vaccines, dosage and the use of various adjuvants, the general experience is that immunity is comparatively short-lived; and that under certain circumstances vaccination may increase both the attack rate in naturally acquired trachoma and the severity of response to artificial challenge. The main exception to these generalizations is the contention of Guerra, Buogo, Marubini & Ghione (1967) that in Ethiopia protective and therapeutic effects were still demonstrable $2\frac{1}{2}$ years after vaccination. This trial was, however, characterized by a high proportion of cures of the infected controls; and like those of other workers, the vaccine used was no more than partially effective.

Our first field trial in The Gambia (Collier, Sowa, Sowa & Blyth, 1963) indicated that a live vaccine given to trachomatous children diminished the severity of the disease in about one-third of those vaccinated, but this effect was of short duration. The present paper describes Trials II and III.

Trial II was a test of the prophylactic efficacy of mineral-oil adjuvant vaccine prepared from a locally isolated strain of trachoma (MRC-187); in Trial III, we tested an aqueous suspension of two strains of trachoma (ASGH and SA-2) isolated respectively in the United States and in Saudi Arabia. Although these vaccines did not induce good immunity, some of the clinical findings and their

modification by vaccination are of interest; for this reason, and because of the expense and difficulty of mounting field trials in developing countries, we report this experimental work in the hope that our methods will be of value to others. The trials were undertaken before the Fourth W.H.O. Scientific Group on Trachoma Research (1966) had recommended standardized methods of clinical examination and scoring for use in field studies of trachoma; our scoring system was, however, similar in principle to that recommended, and our use of the slit-lamp permitted more precise observations than those made by the naked eye or the binocular loupe suggested by the W.H.O. Group for large-scale studies.

MATERIALS AND METHODS

Vaccine for Trial II

TRIC agent

The vaccine was prepared from strain TRIC/WAG/MRC-187/OT (abbreviation: MRC-187) isolated from a Gambian child suffering from early trachoma (Tr I) with micropannus.

Preparation

For the first dose, TRIC agent was purified from yolk sacs infected with the 6th chick embryo passage; sacs were shaken with phosphate-buffered saline (Dulbecco & Vogt, 1954) and strained through gauze to remove the membranes. The crude filtrate was treated with 0·5 % (w/v) trypsin (Difco 1:250) for 30 min. at 37° C., and then subjected to differential centrifugation (Collier, 1961); the elementary bodies were finally deposited at 8000*g* for 20 min., and resuspended in sucrose–potassium glutamate (Bovarnick, Miller & Snyder, 1950) containing streptomycin sulphate 200 μg./ml. The second dose, made some months later, was

Table 1. *Trial II: characteristics of MRC-187 vaccines*

	First dose	Second dose
Total elementary bodies ($\log_{10}$/ml.)	9·8	9·4
50 % egg lethal dose ($\log_{10}$/ml.)	5·4	4·2
Titre of group complement-fixing antigen*	1280	Not tested
Total nitrogen (mg./100 ml.)	216·0	31·5

* Reciprocal of dilution giving 50 % fixation with an optimal dilution of antiserum and 2 M.H.D. complement.

prepared from 11th chick embryo passage material. The method was similar except that purification was much improved by slowly adding to the crude yolk sac suspension an equal volume of 2 M-KCl at 0° C. with constant stirring. After centrifugation at 8000 *g* for 20 min. to remove yolk material, the deposit containing the elementary bodies was resuspended as described above. Dummy vaccines were prepared from normal yolk sacs by similar methods. These and the vaccine proper were stored at − 70° C. until the day of use. The characteristics of the vaccines before freezing are given in Table 1. Elementary bodies were counted by the dark-ground method of Reeve & Taverne (1962). The *Chlamydia* group antigen content of the first dose vaccine was determined by chess-board titration

of a boiled sample against serum from a sheep infected with enzootic abortion; the use of antibody from this source obviated cross-reactions with antibody to yolk sac. The nitrogen content was determined by the micro-Kjeldahl method.

Safety tests

Vaccines and dummy preparations were tested for the presence of aerobic and anaerobic bacteria (Collier *et al.* 1963) and mycoplasma. They were also tested for extraneous viruses by inoculation into HeLa cell cultures and by intraperitoneal injection into 12 adult mice; and for toxicity by intramuscular injection into three guinea-pigs. No cytopathic effects were observed in the HeLa cells; all the animals remained well for 21 days after inoculation, and no lesions were detected at autopsy.

Oil adjuvant

On the day of use, the vaccines and control preparations were emulsified in an equal volume of a mixture of 9 parts light mineral oil (Drakeol 6 VR, Pennsylvania Refinery Co.) and 1 part of mannide mono-oleate (Arlacel A, Atlas Powder Co.). Arlacel A was proved to be non-toxic by a mouse test (Berlin, 1962). The water-in-oil emulsion of vaccine was prepared and tested as described by Collier & Blyth (1966*b*).

Vaccine for Trial III

TRIC agents

The vaccine was made from a mixture of the 'fast-killing' variants (Reeve & Taverne, 1963) of trachoma agents TRIC/2/SAU/HAR-2/OT ('SA-2'; Murray *et al.* 1960) and TRIC/ /USA-Cal/Cal-2/OT ('ASGH'; Hanna, Jawetz, Thygeson & Dawson, 1960).

Table 2. *Trial III: characteristics of the ASGH and SA-2 components of the vaccine*

	ASGH	SA-2
Total elementary bodies (log_{10}/ml.)	9·0	9·3
50 % egg lethal dose (log_{10}/ml.)	6·0*	7·4
Infective units in HeLa Cells (log_{10}/ml.)	7·3	7·3
Total nitrogen (mg./100 ml.)	12·5	11·0

* After storage at −70° C. for 4 months.

Preparation

The purified suspensions of ASGH and SA-2 were prepared respectively by Evans Medical Ltd. and Pfizer (Great Britain) Ltd. The methods were identical, and based on those described for Trial II, except that treatment with trypsin was omitted and an extra cycle of centrifugation in molar KCl was introduced. The final volumes were adjusted so that the infective titres in cell cultures (Furness, Graham & Reeve, 1960) were the same. Table 2 gives the characteristics of each component. These tests were made before freezing, except for the egg titration of ASGH; the original egg titration gave an irregular result, and the recorded titre was obtained by a later test on a frozen sample. The dummy preparation was made

in the Evans Medical laboratory by a similar method. Both vaccine proper and control material were frozen at $-70°$ C. after manufacture. They were transported to The Gambia and stored until the day of use in liquid nitrogen.

Safety tests were similar to those for the Trial II vaccine.

Location and size of the trials

From the trachoma prevalence rates observed in the village of Marakissa during 1959 and subsequently (Sowa, Sowa, Collier & Blyth, 1965), Dr I. A. Sutherland (M.R.C. Statistical Unit) calculated the numbers of non-trachomatous children needed to demonstrate a significant degree of protection by vaccines with differing potencies. For example, in a trial limited to 40 vaccinated children and 40 controls, a vaccine would have to be 100 % effective in preventing trachoma for the result to be statistically significant with a 1-year follow-up, and 75 % effective with a 2-year follow-up. With 100 children in each group, the corresponding figures are

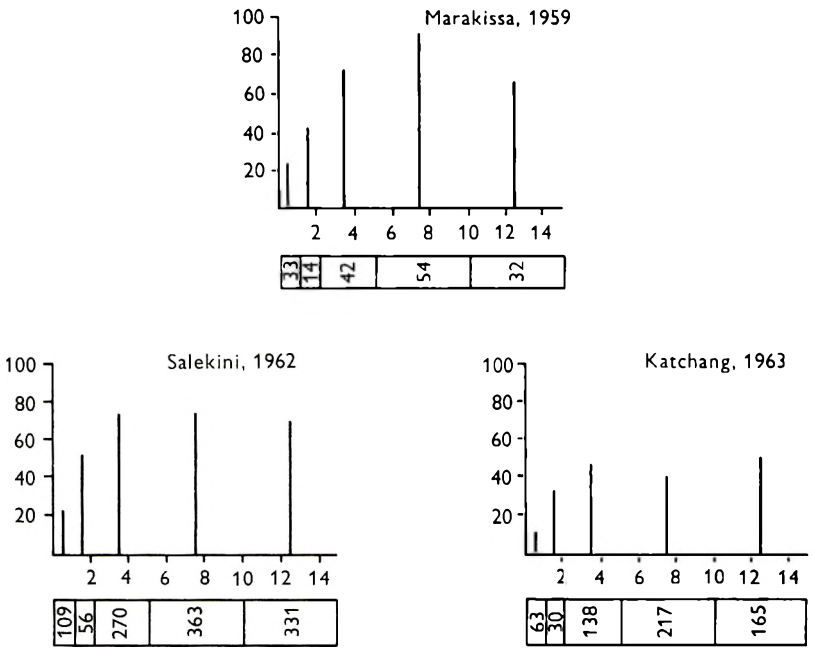

Fig. 1. Prevalence of clinical trachoma (including Tr D) in three Gambian villages. Ordinates: percentage of trachoma. Abscissae: age in years, below which are shown the age groups examined and the total numbers of children in each category; the bars indicating prevalence are placed at the midpoint of each age group.

65 % if observed for 1 year, and 50 % if observed for 2 years. These estimates allow for the high infant mortality rate in The Gambia, which may be as high as 40 % within the first 2 years of life. We hoped to recruit about 300 subjects for Trial II; the Marakissa surveys suggested that this number of children aged from 6 months to 9 years who had not yet acquired trachoma would be provided by a total population of approximately 3000. In case the prevalence of trachoma and losses during the trial proved higher than expected, a somewhat larger population was

desirable, and the villages of Salekini (population 3500) and Katchang (population 1100) were chosen. They are situated on the north bank of the River Gambia about 50 miles (80 km.) from its mouth, and are 15 miles (24 km.) apart. Preliminary surveys showed that the prevalence of trachoma in Salekini was very similar to that in Marakissa, but was substantially lower in Katchang (Fig. 1).

Diagnostic criteria

Trachoma and its various stages were diagnosed in accordance with the main recommendations of the W.H.O. Expert Committee on Trachoma (1962). In brief, *Trachoma dubium* (Tr D) implies clinical signs suggestive of early infection, but follicles and corneal changes are not yet visible or not typical of trachoma, and TRIC agent is not demonstrated in the conjunctiva. Trachoma Stage I (Tr I) means that immature follicles are present on the upper tarsal conjunctiva, including the central area; early corneal changes are usually present, but if not, the diagnosis of Tr I is still permissible if TRIC agent can be isolated. Stage II (Tr II) indicates the presence of well-developed mature soft follicles, papillary hyperplasia and pannus and infiltrates extending from the upper limbus. Stage III (Tr III) is marked by the onset of conjunctival scarring; some or all of the signs of Stage II may be present. A diagnosis of Stage IV (Tr IV) indicates that the follicles and infiltrates have been entirely replaced by scar tissue, and that the disease is healed and no longer infectious (although further changes in cicatrization may follow).

Children without clinical evidence of trachoma are divided into two categories. Those with completely normal eyes are referred to as normal (N); those with minor signs, such as papillae, with or without scanty follicles restricted to the outer angles are given the non-committal diagnosis of 'Abnormal' (Ab).

Selection of children

In Trial II children aged from 6 months to 2 years were examined with an illuminated × 10 loupe; all older children were examined with a slit-lamp. Those with signs suggestive of trachoma were dismissed forthwith, and those with completely normal eyes were admitted; conjunctival scrapings from children diagnosed as Ab were examined for inclusions and were inoculated into chick embryos. When TRIC agent was demonstrated by either method, the child was regarded as trachomatous and dismissed from the trial.

In Trial III the children were selected by slit-lamp examination and, when appropriate, by tests for TRIC agent, from those born in the 2 years since the start of Trial II, and from some older children who were not available at the time of the previous trial. The proportion of children under 2 years of age was thus greater than in Trial II.

Scoring system

The clinical findings in Trial II are analysed only in terms of diagnostic categories; the physical signs were scored according to severity, but the results are not given here because the slit-lamp was not used for all examinations and the

scores are thus not strictly comparable. For Trial III, in which the slit-lamp was used throughout, the system of Collier *et al.* (1963) was used to assess the severity of trachoma acquired after the start of the trial (Table 3). When the physical signs varied in the two eyes, the score for the eye with the most advanced lesions was used in the analysis of results.

Table 3. *Scoring system for physical signs of trachoma*

Physical sign	Degree of involvement	Score
Conjunctival follicles	Scattered follicles over whole of upper tarsal conjunctiva or a few confined to circumscribed area	1
	Follicles over whole of upper tarsal conjunctiva, but not confluent	2
	Confluent follicles over whole of upper tarsal conjunctiva.	3
Papillary hyperplasia	Slight, without cellular infiltration	1
	*Moderate; normal vessels appear hazy	2
	*Pronounced; conjunctiva thickened and opaque, normal vessels obscured	3
Limbal follicles	*One to three typical follicles	1
	*More than three but entire upper lunula not involved	2
	Follicles involving one half or more of the corneal circumference	3
Superficial punctate keratitis	Small localized subepithelial punctate lesions	1
	Punctate lesions affecting approximately half the corneal area	2
	Numerous punctate lesions scattered over entire cornea	3
Pannus	Extension of vessels from upper limbus 0·5 to < 2·0 mm	1
	2·0 to < 4·0 mm	2
	≥ 4·0 mm	3
Herbert's pits	As limbal follicles, but scores given negative values	−1 to −3
Conjunctival scars	Fine scarring of upper tarsal conjunctiva	−1
	*Moderate readily recognizable scarring with no shortening or distortion of the upper tarsus	−2
	Dense scarring of upper tarsus with or without distortion	−3

N.B. Signs recorded as very slight but definite (i.e. less than 1) are scored + or −0·5.
* Criteria and scoring identical with those of W.H.O. Scientific Group on Trachoma Research (1966).

Tests for TRIC agent

The methods for detecting the agent in conjunctival scrapings by staining with iodine and by inoculation into chick embryos were those of Sowa *et al.* (1965). These tests were made on all children with signs suggestive of active trachoma at the follow-up examinations indicated in Table 4.

Complement-fixation tests

In Trial II only, serum samples from children aged 2 years and over were held at 4° C. until tested for antibody to group antigen; titrations were done on Perspex plates similar to those described by Fulton & Dumbell (1949). The heated antigen was prepared from infected yolk sacs; the end-point was taken as the dilution of serum giving 50% fixation with an optimum concentration of antigen and 2 M.H.D. of complement (Collier & Blyth, 1966*a*).

Table 4. *Trials II and III: summary of vaccination and follow-up procedures*

	Trial II		Trial III	
Type of vaccine	Oil adjuvant		Aqueous suspension	
Strain of TRIC agent	MRC-187		SA-2*f* ASGH*f*	
First dose	0·5 ml. intramuscular in buttock, May 1963		0·5 ml. deep subcutaneous in arm, January 1965	
Second dose	0·5 ml. intramuscular in opposite buttock 6 months later		0·5 ml. deep subcutaneous in opposite arm 3 weeks later	
Follow-up examinations (months after 1st dose)	6	12	12	24
Clinical examination	× 10 loupe	× 10 loupe or slit-lamp	Slit-lamp	Slit-lamp
Tests for inclusions	No	Yes	Yes	Yes
TRIC agent isolation attempted	No	Yes	No	No

Randomization and 'double-blind' procedures

A punch card was prepared for each child in the trial. The cards were sorted into age groups and those in each group were thoroughly shuffled and allocated alternately to vaccine and control sets. The vaccine and control preparations were identified only by colour code, and the cards were marked correspondingly. For follow-up examinations fresh unmarked cards were prepared showing only the child's name and number; the examiner was therefore unaware of the preparation given and the previous clinical findings.

Methods of vaccination

In Trial II two intramuscular doses each of 0·5 ml. were given at an interval of 6 months, the first into the right buttock and the second into the left.

In Trial III two deep subcutaneous doses each of 0·5 ml. were given at an interval of 3 weeks, the first into the deltoid area of the right arm and the second into the left.

Reaction to vaccination

In Trial II the vaccinated and control children in the first batch were inspected at 24 and 48 hr. In Trial III about 20 children in each group were examined at 24, 36 and 48 hr.

The vaccination and follow-up procedures used in both trials are summarized in Table 4.

RESULTS

Trial II

Composition of control and vaccinated groups. Table 5 gives the age distribution of the children selected for this trial, and the clinical diagnoses before vaccination.

Reactions to vaccination. No local or general reactions were observed in the samples of children examined. We received only one report of an untoward reaction; some time after the second dose, the mother of a vaccinated child said that an abcess had developed at the injection site. It had healed without treatment by the time we were notified.

Table 5. *Trial II: age distribution of control and vaccinated children*

Clinical diagnosis at start*	Nos. of children in age groups				
	0–11 months	12–23 months	2–4 years	5–9 years	Totals
Controls					
N	33	14	36	11	94
Ab	19	6	13	1	39
Totals	52	20	49	12	133
% of group total	39·1	15·0	36·8	9·0	—
Vaccinated					
N	36	11	36	11	94
Ab	16	9	12	2	39
Totals	52	20	48	13	133
% of group total	39·1	15·0	36·0	9·8	—

* For explanation of the abbreviations used in this and subsequent tables see 'Diagnostic criteria', page 703.

First follow-up examination at 6 months. No microbiological tests were made on this occasion. In interpreting the results of this and all other follow-up examinations, *Trachoma dubium* (Tr D) is counted as trachoma. On this basis, 17 (14·4 %) of the control group had acquired trachoma compared with 7 (5·9 %) of the vaccinated group (Table 6). Fisher's exact test for 2×2 tables (1958) shows that this difference borders on significance at the 5 % level of probability ($P = 0{\cdot}053$).

Second follow-up examination at 12 months. Table 7 shows that 25·9 % of the controls had acquired trachoma, compared with 34·3 % in the vaccinated group; the difference is not significant. This result is reflected in the tests for TRIC agent (Table 8). In Salekini, one-third of those examined were positive in both vaccinated

and control groups. By contrast, in Katchang only one conjunctival scraping (from a control subject) yielded TRIC agent; this point will be discussed later.

Irrespective of whether they belonged to the vaccinated or control group, children originally diagnosed as 'Abnormal' acquired trachoma more frequently than those with completely normal eyes at the outset (Tables 6 and 7); by Fisher's exact test this difference was significant at the 6-month follow-up ($P = 0{\cdot}028$) and even more so at 1 year ($P = 0{\cdot}0003$). However, an analysis of variance in which

Table 6. *Trial II: conversions to clinical trachoma at first follow-up examination*

		Clinical diagnosis at Six months after first dose				Percentage conversions to trachoma (including Tr D)
Start		N	Ab	Tr D	Tr I	
Controls						
N	86	44	33	9	—	10·5
Ab	32	4	20	7	1	25·0
Totals	118	48	53	16	1	14·4
Vaccinated						
N	83	32	48	2	1	3·6
Ab	34	7	23	4	—	11·8
Totals	117	39	71	6	1	5·9

Table 7. *Trial II: conversions to clinical trachoma at second follow-up examination*

		Clinical diagnosis at Twelve months after first dose						Percentage conversions to trachoma (including Tr D)
Start		N	Ab	Tr D	I	II	III	
Controls								
N	78	43	22	6	4	1	2	16·7
Ab	30	4	11	10	4	1	—	50·0
Totals	108	47	33	16	8	2	2	25·9
Vaccinated								
N	74	32	21	13	2	3	3	28·4
Ab	31	5	11	10	3	1	1	48·4
Totals	105	37	32	23	5	4	4	34·3

all children who acquired trachoma (including Tr D) were scored 1, and those who did not (including subjects converting to or remaining Ab) were scored 0, showed that the prophylactic effect of vaccination on children with an initial diagnosis of Ab did not differ from that in children with normal eyes.

Antibody response to vaccination. If serum titres of less than 1/20 are disregarded, a positive antibody response to vaccination was observed in only two of 24 subjects tested 6 months after the first dose, and in four of 19 tested 6 months after the second dose of vaccine (Table 9).

Table 8. *Trial II: tests for TRIC agent in conjunctiva at second follow-up examination*

Clinical diagnosis	Controls		Vaccinated	
	No. tested	TRIC positive*	No. tested	TRIC positive*
		Salekini village		
Ab	14	3	8	1
Tr D	6	2	10	3
Tr I	7	3	4	2
Tr II	2	1	3	3
Tr III	1	1	3	—
Totals	30	10	28	9
		Katchang village		
Ab	12	—	10	—
Tr D	11	1	13	—
Tr I	2	—	2	—
Tr II	—	—	3	—
Tr III	—	—	1	—
Totals	25	1	28	—

* Inclusions demonstrated in conjunctiva, and TRIC agent isolated in chick embryos, except for three subjects in whom only one of these tests was positive.

Table 9. *Trial II: results of complement-fixation tests on serum samples*

Months after vaccinations ...	Nos. of children with titre*				Not tested
	< 10	10	20	40	
Controls					
0	29	0	0	0	0
6	25	0	0	0	4
12	21	1	0	0	7
Vaccinated					
0	29	0	0	0	0
6	24	2	2	0	1
12	19	4	3	1	2

* Reciprocal of serum dilution giving 50 % fixation with 2 MHD complement and an optimal concentration of *Chlamydia* group antigen.

Trial III

Composition of control and vaccinated groups. The age distribution (Table 10) may be compared with that in Trial II (Table 5). In Trial III, a somewhat higher proportion of children was aged 2 years and under.

Reactions to vaccination. There were no reactions in the 19 control subjects examined, but in 12 of 21 children receiving vaccine proper, mild or moderate local swelling and inflammation appeared within 24 hr. and subsided within the next day or so, without lymphadenitis.

First follow-up examination at 1 year. The overall rates of conversion to clinical trachoma (Table 11) were virtually identical for both vaccinated and control

subjects. The attack rate of over 60% was about twice that in Trial II (Fig. 2). Isolation of TRIC agent was not attempted in this trial; but the incidence of conjunctival inclusions in Salekini was not affected by vaccination. In Katchang, only two subjects, both in the control group, were inclusion-positive (Table 12).

Table 10. *Trial III: age distribution of control and vaccinated children*

Clinical diagnosis at start	Nos. of children in age groups 0–11 months	12–23 months	2–4 years	Totals
Controls				
N	40	12	10	62
Ab	16	11	5	32
Totals	56	23	15	94
% of group total	59·6	24·5	15·9	—
Vaccinated				
N	36	25	9	70
Ab	17	9	3	29
Totals	53	34	12	99
% of group total	53·5	34·3	12·1	—

Table 11. *Trial III: conversions to clinical trachoma at first follow-up examination*

Start		Clinical diagnosis at Twelve months after first dose N	Ab	Tr D	I	II	III	IV	Percentage conversions to trachoma (including Tr D)
Controls									
N	45	10	7	3	3	16	3	3	62·2
Ab	26	4	5	1	1	11	4	—	65·4
Totals	71	14	12	4	4	27	7	3	63·4
Vaccinated									
N	52	12	11	5	1	16	5	2	55·8
Ab	27	1	4	1	6	14	1	—	81·5
Totals	79	13	15	6	7	30	6	2	64·6

Second follow-up examination at 2 years. Again, the trachoma conversion rate in the vaccinated group was similar to that in the controls (Table 13). Although in Salekini there was a higher proportion of inclusion-positive subjects among vaccinated children, the difference between them and the controls was not statistically significant. As before, there were fewer inclusion-positive subjects in Katchang (Table 14).

At 2 years, but not at 1 year, the conversion rate was significantly higher in children initially diagnosed as Ab than in those with normal eyes ($P = 0{\cdot}020$ by Fisher's exact test); but, as in Trial II, analysis of variance revealed no significant difference in the prophylactic effect of vaccination on these two categories.

Table 12. *Trial III: tests for conjunctival inclusions at first follow-up examination*

Clinical diagnosis	Controls		Vaccinated	
	No. tested	Inclusion-positive	No. tested	Inclusion-positive
Salekini village				
Ab	1	—	—	—
Tr D	1	—	2	—
Tr I	3	—	4	2
Tr II	16	8	23	6
Tr III	5	—	4	—
Totals	26	8	33	8
Katchang village				
Ab	4	—	6	—
Tr D	3	1	4	—
Tr I	1	—	3	—
Tr II	11	1	7	—
Tr III	—	—	—	—
Totals	19	2	20	—

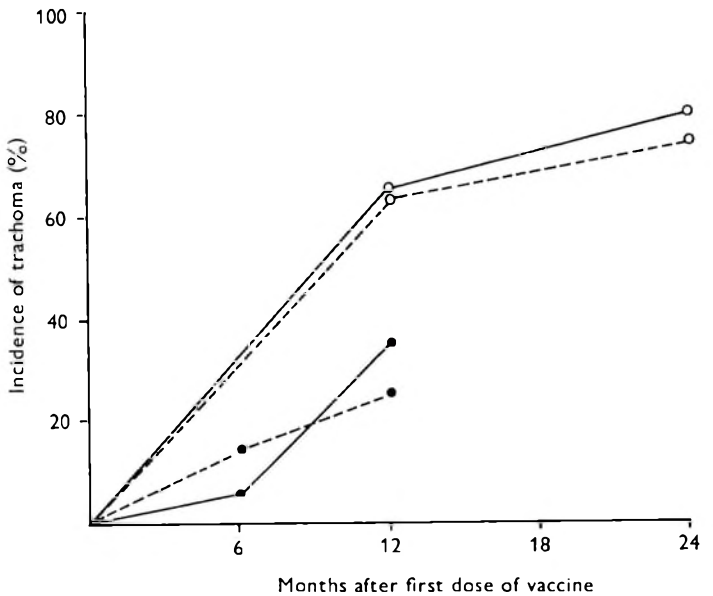

Fig. 2. Trials II and III: trachoma attack rates in vaccinated and control children. Closed circles = trial II; open circles = trial III; continuous lines = vaccinated children; broken lines = control children.

Influence of vaccination on course and severity of trachoma. Table 11 shows that at 1 year there was little difference between the numbers of vaccinated and control children progressing to cicatricial trachoma (Tr III and Tr IV). Two years after vaccination the proportion of subjects with scarring was higher in the controls; of 47 with trachoma, 18 were Tr III or Tr IV, compared with nine of 55 vaccinated children who acquired trachoma (Table 13). By Fisher's exact test the difference is significant ($P = 0{\cdot}034$).

The mean clinical score of severity did not differ in the vaccinated and control groups at 1 year. At 2 years after vaccination it was significantly higher in the vaccinated group (Table 15).

Table 13. *Trial III: conversions to clinical trachoma at second follow-up examination*

		Clinical diagnosis at Twenty-four months after first dose						Percentage conversions to trachoma
Start	N	Ab	Tr D	I	II	III	IV	(including Tr D)
Controls								
N	38	6	6	—	—	17	5	4	68·5
Ab	25	4	—	—	—	12	8	1	84·0
Totals	63	10	6	—	—	29	13	5	74·6
Vaccinated								
N	47	6	7	1	1	23	6	3	72·4
Ab	22	—	1	—	—	21	—	—	95·5
Totals	69	6	8	1	1	44	6	3	79·7

Table 14. *Trial III: tests for conjunctival inclusions at second follow-up examination*

	Controls		Vaccinated	
Clinical diagnosis	No. tested	Inclusion-positive	No. tested	Inclusion-positive
Salekini village				
Ab	1	—	—	—
Tr D	—	—	—	—
Tr I	—	—	—	—
Tr II	15	8	21	17
Tr III	3	1	3	—
Totals	19	9	24	17
Katchang village				
Ab	3	—	—	—
Tr D	—	—	—	—
Tr I	—	—	1	—
Tr II	6	—	13	1
Tr III	4	1	—	—
Totals	13	1	14	1

Table 15. *Trial III: comparison of clinical scores in control and vaccinated children who acquired trachoma*

Months after vaccination	Group	No. of children	Mean score	Student's *t*	Degrees of freedom	*P*
12	Control	45	4·100	−0·8749	94	> 0·100
	Vaccinated	51	4·569			
24	Control	47	3·085	−2·3529	100	< 0·025
	Vaccinated	55	4·255			> 0·020

Relation between clinical score and presence of conjunctival inclusions. It is generally accepted that inclusions are more likely to be found in severe than in mild trachoma (see, for example, Nichols, Bobb, Haddad & McComb, 1967; Tarizzo, Nabli & Labonne, 1968), and our findings support this contention. Table 16 relates the clinical scores at the two follow-up examinations to presence or absence of inclusions; no distinction is made here between vaccinated and control subjects. At both examinations the mean score in inclusion-positive subjects was about twice that in inclusion-negative children—a highly significant difference.

Table 16. *Trial III: relation of clinical score to presence or absence of conjunctival inclusions*

Months after vaccination	Inclusion-positive (+) or negative (−)	No. of children	Mean score	Student's *t*	Degrees of freedom	*P*
12	+	18	7·000	5·7287	95	< 0·001
	−	79	3·696			
24	+	28	6·910	9·5190	68	< 0·001
	−	42	3·083			

Table 17. *Trial III: differences between the Salekini and Katchang populations in terms of clinical scores for children who acquired trachoma*

Months after vaccination	Population		No. of children	Mean score	Student's *t*	Degrees of freedom	*P*
12	Salekini	control	26	4·461	1·2247	43	> 0·100
	Katchang		19	3·605			
	Salekini	vaccinated	34	5·147	2·1206	49	< 0·050 and > 0·025
	Katchang		17	3·411			
24	Salekini	control	29	3·690	2·8302	45	< 0·010 and > 0·005
	Katchang		18	2·111			
	Salekini	vaccinated	33	5·258	3·4960	53	< 0·001
	Katchang		22	2·750			

Table 18. *Trials II and III: numbers of children dead or absent at follow-up examination*

		Months after first dose of vaccine					
		Trial II			Trial III		
		0	6	12	0	12	24
Controls	Dead	—	12	2	—	16	6
	Absent	—	3	8	—	7	2
	Remaining	133	118	108	94	71	63
Vaccinated	Dead	—	13	2	—	15	7
	Absent	—	3	10	—	5	3
	Remaining	133	117	105	99	79	69

Difference in severity of trachoma in Salekini and Katchang. The average clinical severity of trachoma acquired during the trial period was greater in Salekini than in Katchang (Table 17). This difference was not pronounced in the control children examined at the first follow-up examination, but was statistically significant in the vaccinated children; and 2 years after vaccination, the average scores in both control and vaccinated children in Salekini were nearly twice those in the corresponding children in Katchang. These findings probably account for the difficulty of detecting TRIC agent in the Katchang population. To confirm that our failure to demonstrate TRIC agent was not the result of sampling under adverse field conditions, 14 children with active trachoma were brought from Katchang to the laboratory and carefully examined for TRIC agent; inclusions were found in one child only.

Efficiency of follow-up. Table 18 shows the losses of children due to death and absenteeism in both trials. The villagers attributed most of the deaths to measles and malaria.

DISCUSSION

Live vaccines were used in these trials because in our laboratory their immunogenicity in baboons was consistently better than that of inactivated preparations (Collier, 1961; Collier & Blyth, 1966*a*, *b*; Collier *et al.* 1967). This may in part be accounted for by our observation that 'fast-killing' TRIC agents multiplied in a primate host after parenteral injection, with a presumed increase in antigenic stimulation (Collier & Smith, 1967). Nevertheless, good short-term immunity was also induced in baboons by live vaccines made from 'slow-killing' strains which may multiply to a much lesser extent *in vivo* (Collier & Mogg, 1969).

A mineral oil vaccine prepared from strain MRC-187 did not perform well in baboons (Collier & Blyth, 1966*b*), but was nevertheless used in Trial II in the hope that the immunity induced would be sufficient to overcome the small infectious dose received in naturally acquired infection, although it was inadequate against the more severe challenge given under laboratory conditions. A barely significant measure of protection was demonstrable 6 months after the first dose, but not after 12 months, despite a second dose given 6 months after the first. Our findings thus agree with those in a field trial in Saudi Arabia reported by Snyder *et al.* (1964), but contrast with those of Wang, Grayston & Alexander (1967), the efficacy of whose mineral oil vaccine they attributed to its content of more than 10^8 elementary bodies per ml.—a condition that was adequately met by our vaccine (Table 1). The serological findings in Trial II (Table 9) confirm our observation in baboons that mineral oil adjuvant vaccine does not induce a prolonged rise in complement-fixing antibody.

In Trial III, the mineral oil adjuvant was abandoned and a live vaccine of greater purity and higher infective titre was used. The ASGH and SA-2 strains of trachoma were selected as representatives of the two main serotypes defined by the mouse toxicity protection test (Bell & Theobald, 1962). The 'fast-killing' (*f*) variants of each strain (Reeve & Taverne, 1967) were used, since yolk sacs can be

harvested comparatively soon after inoculation and the elementary bodies can be purified much more easily than from chick embryos dying late after inoculation with 'slow-killing' (*s*) strains. In one respect, this decision was unfortunate because Graham (1967) later showed that the $s \rightarrow f$ transformation tends to be associated with loss of antigenic specificity, so that the Trial II vaccine cannot be considered as truly bivalent. However, Graham also pointed out that in mice *f* mutants are more immunogenic than the parent strains, perhaps because they are more invasive; and we ourselves obtained good evidence of the immunogenicity of one such strain in our baboon experiments.

We should have preferred an interval longer than 3 weeks between the first and second doses in Trial III, but difficulties of organization made this impossible. The mild local reactions to subcutaneous vaccine were probably due to multiplication of TRIC agent; similar lesions were observed in baboons (Collier & Smith, 1967). At the first follow-up 1 year after vaccination, there was no difference between vaccinated and control children in the incidence or severity of trachoma; the attack rate was double that in Trial II (Fig. 2), probably because the average age of the Trial III population was lower (cf. Tables 5 and 10). Two years after vaccination, the trachoma conversion rates were still similar in both vaccinated and control groups; but in vaccinated children the proportion progressing to cicatricial trachoma was smaller, and the clinical severity greater. Deleterious effects of trachoma vaccination have now been reported by a number of workers. In a field trial in Taiwan, Woolridge *et al.* (1967) observed over a 2-year period a significantly higher attack rate in children receiving a monovalent oil adjuvant vaccine; but a more commonly reported phenomenon is an increased severity of infection induced by conjunctival challenge of monkeys or baboons previously given vaccines of low potency, vaccines made from TRIC agents differing antigenically from the challenge inoculum, or vaccines with mineral oil adjuvant (for example, Wang *et al.* 1967; Mordhorst, 1967; Collier & Blyth, 1966*b*). These more severe responses are usually ascribed to delayed hypersensitivity induced by vaccination; Wang and his colleagues provide evidence that the allergen is the TRIC agent itself rather than egg material contained in both vaccine and challenge inoculum. Although delayed hypersensitivity is the most immediately obvious explanation of our findings, it is hard to understand why it was not apparent 1 year after vaccination; perhaps the physical signs in vaccinated children who acquire trachoma are the resultant of two forces: a resistance to infection that decreases comparatively quickly and a delayed-type hypersensitivity that may be maintained for long periods. In Trial III, the increased severity on the one hand and failure to heal by cicatrization on the other are probably related, since trachomatous scarring is normally preceded by a diminution of inflammation and follicular hyperplasia.

Another interesting feature of these trials is the increased likelihood of acquiring trachoma in children with an initial diagnosis of 'Abnormal' (Ab), compared with those having completely normal eyes at the outset. Some Ab children may have been suffering from early trachoma, and TRIC agent was in fact isolated from 3 of 14 in Trial II; nevertheless, the physical signs were not at all consistent with

this diagnosis, and it may be that the minor abnormalities observed predispose to TRIC infection.

The pronounced difference between the populations of Salekini and Katchang in terms of severity of trachoma and presence of TRIC agent in the conjunctiva might be explained by environmental factors. In both villages the tribal pattern, general living standard and way of life are similar; but Salekini, with 3500 inhabitants, occupies about the same area as Katchang, whose population is only a third of this. The differences observed may thus be due to overcrowding. In their masterly report on the epidemiology of trachoma in Saudi Arabia, Nichols and his colleagues (1967) draw attention to the paramount importance of poor living conditions as a factor in prolonged infection, which in turn is closely related to severity of disease, persistence of TRIC agent in the conjunctiva, and disabling sequelae.

In reiterating the hope that the methods used in these trials will be useful to others we draw attention particularly to three points. First, the establishment of an efficient follow-up organization permitted considerable economy in the size of the trials; second, examination by slit-lamp, which, given adequate immobilization, is possible with very young babies and greatly improves the accuracy of observation; and third, the use of a scoring system to facilitate the application of statistical tests to the analysis of results. Although the value of scoring systems is generally agreed, the method of application is still open to argument. For example, an important difference between our method and that proposed by the W.H.O. Scientific Group on Trachoma Research (1966) is that we assign negative values to scores for regressed limbal follicles ('Herbert's pits') and cicatrization, regarding them as signs of healing, and in a different category from signs such as follicles and pannus. We think that this procedure is justifiable when assessing the influence of a vaccine or drug on the course of trachoma; in such instances the onset of scarring might be held to represent a beneficial effect of the treatment. By contrast, an epidemiological study of the 'relative gravity' of trachoma in a given population (W.H.O. Expert Committee on Trachoma, 1962) entails evaluation of the prevalence of disabling sequelae; in this case, addition of scores for cicatrization to the total would give a truer picture of the situation. In fact, cicatricial lesions were not a prominent feature of the disease in the very young children in our trials; and our conclusions are unaffected even when scores for Herbert's pits and scarring are added to those for other lesions.

In conclusion, our findings support the view that the prophylactic efficacy of trachoma vaccines prepared by conventional methods falls well short of what is desirable; for much of the argument in favour of vaccination depends on the assumption that a high degree of protection can be maintained for long periods with a minimum of reinforcing doses. Production methods will have to be reappraised, perhaps along the lines of making much more highly concentrated vaccines at an economic cost. It is also clear that the complex mechanism of specific immunity to trachoma needs further study; and the possibility that vaccination decreases rather than enhances resistance to infection must be eliminated.

SUMMARY

The ability of two live trachoma vaccines to protect against naturally acquired infection was tested in young Gambian children. With a mineral oil adjuvant vaccine prepared from a Gambian strain of trachoma (MRC-187) a barely significant measure of protection was demonstrable 6 months after the first dose, but not at 1 year, despite a reinforcing dose given 6 months after the first. In a later trial an aqueous vaccine prepared from the 'fast-killing' variants of strains 'SA-2' and 'ASGH' failed to induce immunity. Two years after vaccination, the proportion of vaccinated children progressing to cicatricial trachoma was less than in the controls, and the average severity of the disease in terms of clinical score was greater; vaccine-induced hypersensitivity may have contributed to this result.

Irrespective of whether they had received trachoma vaccine, children with completely normal eyes at the outset were less likely to acquire trachoma than those with slight conjunctival folliculosis or papillary hyperplasia. In children acquiring trachoma, there was a highly significant positive correlation between severity of the disease and the presence of conjunctival inclusions. The pattern of trachoma differed significantly in the two villages used in both trials; the prevalence, severity and proportion of inclusion-positive subjects were all higher in the village with the greater population density.

An efficient follow-up organization, use of a slit-lamp for clinical observations, and a scoring system for recording physical signs are all desirable for trachoma vaccine field trials.

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Investigations into the contamination of Ceylon desiccated coconut

By KAMINI MEEDENIYA*
Ceylon Coconut Board, 11 *Duke Street, Colombo* 1, *Ceylon*

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INTRODUCTION

The contamination of Ceylon desiccated coconut with *Salmonella* and the steps taken to eradicate the contamination were discussed in a previous paper (Velaudapillai, Nitiananda & Meedeniya, 1963). A list of salmonella serotypes isolated from desiccated coconut in the first 3 months of the setting up of the Ceylon Coconut Board Laboratory was also given. Although the hygiene of the product improved tremendously in the following years, samples of desiccated coconut received in the laboratory from a few mills (factories) showed continuous contamination with different salmonella serotypes, and consequently these mills sustained a heavy financial loss. The most persistent serotypes were two strains of *Salmonella senftenberg* (one positive and the other negative for H_2S production), *S. typhimurium*, *S. tennessee* and *S. cubana*. Other contaminants frequently found were *S. paratyphi B*, *S. bareilly* and *S. waycross*.

Investigations were carried out into the reasons for the frequent and persistent contamination as well as to try to trace the source of contamination and to find out at what stage in the manufacturing process the contamination was taking place.

The processing of desiccated coconut can be divided into three main stages: (1) the picking of the fruit and the removal of the outer husk. (2) The preparation of the coconut kernel prior to the disintegration of the coconut meat. (3) The disintegration, drying and packing of the product. The regulations which now govern the manufacture of desiccated coconut (Coconut Products Ordinance, 1961) make it compulsory that stages (2) and (3) be carried out in completely separate parts of the mill, and that there be no access from one part to the other. At stage (2) the outer shell is removed by means of a small hatchet and the brown testa is pared away with a special type of knife to leave only the white kernel. The kernel is then cut up roughly and put into metal tanks containing chlorinated water where the kernel is washed (the section in which this takes place is known as the 'wet' section). The washed kernel is then placed in baskets which are hooked on to a screw conveyor, or placed in a slatted conveyor which takes it through a tank of very hot water (95–100° C.) in 90 sec., one end of the tank is in the wet section, and the other in the dry section (stage 3). The operations at stages (1) and (2) are

* Present address: Food Hygiene Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9.

carried out manually and the tank of near boiling water is used to sterilize the coconut.

At stage (3) the coconut kernel which comes off the conveyor is sent down a chute into a hopper which feeds the disintegrator or cutter where it is milled into minute pieces (disintegrated). The disintegrated coconut is collected into aluminium bins placed under the cutter and taken to the Driers. The driers are of two types; the old-fashioned, tray-type drier as described by Galbraith, Hobbs, Smith & Tomlinson (1960) where the drying process can take up to 45 min. for each load, and the newer, semi-automatic type where the coconut is fed into the top of a machine in which very hot air circulates. After about 10–15 min. the coconut emerges from the bottom of the machine, dried. The coconut is dried to about 2% moisture content and then placed on cooling tables. The cooled desiccated coconut is then graded into medium and fine cuts by mechanical sifters from which they are collected into Kraft paper bags ready for export. Grading and packing is done in a separate room which by regulation has to be strictly vermin proof. All manual handling of coconut is forbidden. The entrances to the section where stage (3) is carried out (the 'dry' or 'sterile' section as it is sometimes called), have foot baths containing disinfectant and any person entering this section is expected to use these and also to wash his hands with soap and water which is provided at the entrance. Workers from the 'wet' section are prohibited from entering. One would therefore expect that the coconut at stage (3) would be entirely free of pathogens, if not sterile. However, our investigations revealed that this was not so.

METHODS

Samples of fibre and soil were collected from the places where the husk was removed before transport to the mill. However, as many of the mills were supplied by contractors who gather husked nuts from the estates in the area, this was not always possible.

Samples of material which might harbour salmonellas were collected from inside and around the mill premises. Particular attention was paid to animal faeces, and soil from the yard where the nuts were stored before processing. Samples of water from the wells supplying the mill were collected aseptically and examined for salmonellas and faecal coliform bacilli.

Samples of coconut fibre, parings and kernel were collected at the different stages of processing, and the hands of individual workers were swabbed. As no single worker was ever shown to be the only person in the mill with contaminated hands, all workers are referred to collectively and not individually in the results given in Table 1.

Samples collected at the mills were brought back the same day and examined for salmonellas in the Ceylon Coconut Board Laboratory. These were incubated in tetrathionate and selenite F enrichment broths at 37° C. for 18–24 hr. and plated on bismuth sulphite agar (Difco) and S.S. agar (Oxoid). After 24 hr. incubation at 37° C. suspicious colonies were picked into combined urea-soft agar-Kligler medium (Velaudapillai, 1962) and incubated overnight. Bismuth sulphite plates were

Table 1. *Isolations of salmonellas at the desiccated coconut mills*

Mill	Dung	Soil	Water from drains	Stage of coconut processing: 2	2–3	3*a*	3*b*	3*c*	3*d*	3*e*	Hands
A	2/5 *senft.*	0/6	NE	20/25 *senft.*	0/10	7/10 *senft.*	7/10 *senft.*	10/20 *senft.*	10/20 *senft.*	0/2	NE
B	None	1/9 unident.	NE	11/25 *new.* 3 *para. B* 1 *senft.* 7	0/3	2/5 *senft.*	2/4 *senft.*	NE	1/1 *senft.*	NE	NE
C	0/3	0/3	0/3	0/12	0/2	1/1 *senft.*	3/3 *senft.*	3/4 *senft.*	2/3 *senft.*	NE	3/3 *senft.*
D	2/9 *senft.* 1 *bar.* 1	0/4	0/4	0/10	0/3	0/3	0/5	NE	0/4	0/1	NE
E	0/2	0/2	0/2	4/5 *unident.*	0/1	0/1	2/4 *senft.*	NE	3/5 *senft.*	NE	1/4 *senft.*
F	1/4 *ten.*	4/10 *ten.*	2/3 *ten.*	7/15 *ten.*	0/2	6/8 *ten.*	4/5 *ten.*	3/6 *ten.*	2/2 *ten.*	1/3 *ten.*	NE
G	None	0/1	0/2	3/12 *senft.*	0/1	0/2	0/6	0/1	2/2 *senft.*	NE	NE
H	None	0/2	0/2	0/6	0/1	0/1	0/2	NE	1/1 *ty.mur.*	NE	NE
I	None	NE	NE	0/7	NE	2/2 *para. B*	2/2 *para. B*	1/1 *para. B*	3/5 *para. B* 2 *bar.* 1	NE	2/3 *para.* B
J	None	NE	0/1	0/4	0/1	1/1 *cubana*	3/3 *cubana*	0/1	0/3	1/1 *cubana*	NE

Material examined at different stages of process:

Stage 2. Fibre from shell-removing section, parings, water and coconut from wash tanks.

Stage 2–3. Coconut pieces after immersion in boiling water for 90 sec.

Stage 3*a*. Wet disintegrated coconut meat from cutter.

Stage 3*b*. Wet coconut meat and swab from collecting bin and implements in use.

Stage 3*c*. Partially dried coconut meat from desiccator/drier.

Stage 3*d*. Dry coconut from cooling tables, sifter (grading) and packed bags.

Stage 3*e*. Swabs from hands of workers at cutter.

Hands. Swabs from hands of workers at desiccators or automatic driers.

senft. = *S. senftenberg* (H_2S+ in A, B, C and G; H_2S- in D, E and F); *new.* = *S. newport*; *para. B* = *S. paratyphi B*; *bar.* = *S. bareilly*; *ten.* = *S. tennessee*; *ty.mur.* = *S. typhimurium*; NE = not examined; unident. = unidentified.

incubated for a further 24 hr. Organisms showing salmonella-like reactions on Kligler medium were identified serologically.

Over 20 mills which sent salmonella-contaminated samples of desiccated coconut were examined in this way, with several sets of samples collected on different days. Tables 1 and 2 give the results of investigations carried out at 10 mills. These mills are considered to be the most representative of all the results obtained. There were some mills where salmonellas were not isolated at any stage of the processing although some of the samples of desiccated coconut, the final product sent to the laboratory, were found to be contaminated.

Table 2. *Number of isolations of salmonella serotypes from routine samples of desiccated coconut from the ten mills shown in Table* 1, *July* 1962 *to August* 1967

Salmonella serotype	Mills									
	A	B	C	D	E	F	G	H	I	J
S. senftenberg H_2S+	40	29	37	.	.	.	7	.	.	.
S. senftenberg H_2S-	.	.	.	49	29	4	.	.	.	.
S. newport	.	2	.	.	.	.	.	.	.	.
S. paratyphi B	.	1	1	.	.	.	.	.	11	3
S. poona	.	.	2	.	.	.	.	.	.	.
S. bareilly	.	.	.	1	.	.	.	.	4	.
S. tennessee	.	.	.	.	.	25	.	.	.	.
S. typhimurium	.	.	.	.	.	.	.	14	.	.
S. litchfield	.	.	.	.	.	.	.	5	.	.
S. cubana	.	.	.	.	.	.	.	.	.	9

Table 3. *Salmonella serotypes isolated from desiccated coconut*

**S. paratyphi B*	*S. butantan*	*S. newport*
**S. typhimurium*	*S. chester*	*S. poona*
**S. senftenberg* H_2S+	*S. cubana*	*S. oslo*
**S. senftenberg* H_2S-	*S. ferlac*	*S. perth*
**S. waycross*	*S. frintrop*	*S. rubislaw*
**S. bareilly*	*S. hvittingfoss*	*S. simsbury*
S. angoda	*S. lanka*	*S. tennessee*
S. adelaide	*S. litchfield*	*S. welikada*

* Most frequent contaminants.

It was not possible to collect stool samples from mill workers for examination for carriers owing to the non-cooperation of the workers who were suspicious that they would lose their jobs. On one occasion the management of Mill D sent some samples collected into Stewart transport medium. No salmonellas were isolated.

A list of salmonella serotypes isolated from desiccated coconut in the Ceylon Coconut Board Laboratory is given in Table 3.

The frequency of isolation of five of the more commonly isolated serotypes is shown in Table 4. The average number of routine samples collected each week from a mill was 3–4 if the mill was not contaminated; if the mill was contaminated, daily samples were collected (5–6/week). Most mills work an average of about 9 months steadily. The off-peak period is December to March when many mills work only a few days in each month.

Heat resistance studies were carried out on strains of *S. senftenberg* producing H_2S (1) and not producing H_2S (2), and also on *S. typhimurium* and *S. bareilly* isolated from desiccated coconut. Cultures suspended in neutral phosphate buffer were sealed in ampoules in 0·2 ml. amounts and heated in a water bath at different temperatures. Survivors were counted on blood agar plates and the time taken for a tenfold reduction (90 %) of numbers was noted. This is the D value (decimal reduction time) for a particular temperature and serotype. Table 5 gives the D values at 57 and 60° C. for the three serotypes.

Table 4. *Salmonella serotypes most frequently isolated from desiccated coconut*

	Number of times isolated (from no. of mills)				
	1962 July–Dec.	1963 Jan.–Dec.	1964 Jan.–Dec.	1965 Jan.–Dec.	1966 Jan.–Dec.
S. senftenberg H_2S+	32 (3)	100 (9)	23 (6)	8 (3)	12 (3)
S. senftenberg H_2S-	5 (1)	1 (1)	34 (3)	4 (2)	21 (4)
S. typhimurium	21 (3)	3 (3)	3 (1)	2 (2)	0
S. bareilly	3 (1)	10 (5)	1 (1)	0	7 (4)
S. paratyphi B	17 (8)	18 (9)	3 (3)	1 (1)	24 (7)
S. waycross	22 (16)	11 (6)	3 (3)	3 (2)	11 (8)

Table 5. *Decimal reduction time (D value) at* 57 *and* 60° C.

	Time taken in seconds to reduce the viable count by 90 % at	
	57° C.	60° C.
S. senftenberg H_2S+	105	15
S. senftenberg H_2S-	60	12
S. typhimurium	120	30
S. bareilly	100	24

Pigs are common in the areas of major production of desiccated coconut and could have access to some mill premises, therefore pig faeces collected from the animal slaughter house at Colombo were examined for salmonellas. Faeces from a portion of the rectum were squeezed into a sterile jar after slaughter. In the laboratory the specimen was divided into two portions and incubated in selenite and tetrationate liquid enrichment broths for 18 hr. before plating on S.S. and bismuth sulphite agar. Specimens from 93 animals were examined and salmonellas were isolated from 28 (30 %).

RESULTS

The results of ten mills selected as typical of the rest are summarized in Tables 1 and 2.

Mill A

Samples of desiccated coconut were found to be contaminated with *S. senftenberg* in October 1962 and this heavy contamination continued for about 2 months. It was the first mill to show persistent contamination with *S. senftenberg* (40 isolations

in 11 months); more than 20 visits were paid to the mill and as many sets of samples were collected.

After the isolation of *S. senftenberg* from the droppings of cattle and other unidentified animals—probably polecats—the yard where the nuts were stacked before use was cleaned and tarred in early 1963. The heavy contamination then ceased but occasional samples were still found to be contaminated up to August, 1963. Since then no contaminated coconut has been found at this mill (August 1967).

Mill B

The first contaminant detected was *S. newport* (2 samples) in June 1963, subsequently *S. paratyphi B* (1 sample) and later *S. senftenberg* (H_2S+), which proved to be the most persistent contaminant, were found. This mill was not hygienic and has stopped production.

Mill C

The salmonella serotype first detected here was *S. poona* (2 samples) followed by *S. paratyphi B* (1 sample) and subsequently *S. senftenberg* which was persistently isolated (37 times). Salmonellas were isolated from wet coconut meat coming direct from the cutter, and it was recommended that the cutter be dismantled and thoroughly cleaned preferably by immersion in the sterilizing tank. This was done and appeared to eliminate the contamination. It was also recommended that the cutter should be mounted on metal stands and all wood round the cutter should be removed (before this was done salmonellas were isolated from all parts of the dismantled cutter). No contamination was detected after the recommendations were carried out until August 1967.

Mill D

The condition of the surroundings was poor, as the fibre pit, the site where the coconuts were husked and the cattle shed were all very near to the desiccated coconut mill. Salmonellas were not isolated from the husking site or from stage (2) of the manufacture, but were isolated from cattle dung (*S. senftenberg*) and from a pet monkey on the premises (*S. bareilly*). *S. bareilly* was the first contaminant isolated from this mill, in August 1963 from desiccated coconut; *S. senftenberg* (H_2S-) was isolated later in 1964 and this contamination persisted until August 1967.

Mill E

The desiccated coconut was contaminated in January 1964 with *S. senftenberg* (H_2S-). The mill is situated about 80 miles from Colombo and consequently it was not investigated as thoroughly as one would have wished. No evidence of a source of *S. senftenberg* contamination was found outside the mill but several unidentified salmonella-like organisms (dulcite and citrate positive) were isolated from fibre, parings and wash tanks (Stage 2).

Mill F

In May 1965 *S. tennessee* was isolated 12 times in 4 weeks, but after the dismantling and boiling of the cutter rings and tightening up of hygienic practices

the contamination was eliminated. In the following year an H_2S- strain of *S. senftenberg* appeared and the mill stopped production indefinitely. Table 1 shows the results of the investigation into *S. tennessee* contamination. *S. tennessee* was isolated from cattle dung which may have been the source of contamination.

Mill G

S. senftenberg (H_2S+) was occasionally isolated from desiccated coconut samples from this mill. Contamination was neither persistent nor heavy. *S. senftenberg* was isolated from fibre and parings in the 'wet' section and from desiccated coconut. There is no other clue as to a possible source.

Mill H

This mill is also about 50 miles from Colombo and investigation was inconvenient. The first contaminant found in 1962 was *S. typhimurium* which was very persistent. The organism was isolated from desiccated coconut only and not from earlier stages in manufacture. The mill was closed for some years. In 1966 when it was reopened the desiccated coconut was found to be contaminated with *S. litchfield*. While the second infection was being investigated *S. weltevreden* was isolated from well-water but as the serotype was different, the water was not considered to be the source of contamination, although the probability of there being mixed salmonella serotypes in the water could not be ruled out.

Mill I

Samples gave a mixed contamination with *S. bareilly* and *S. paratyphi B* on about 11 consecutive days in October 1966; the contamination disappeared after the cutter was dismantled and boiled.

Mill J

S. cubana was persistently isolated from routine samples of desiccated coconut from this mill. Table 1 gives the results of only one visit to the mill. It was found that the chute leading to the cutter, the coconut pieces going down the chute, the hands and feet of the worker attending the cutter and the floor surrounding it were contaminated with *S. cubana*, but not the coconut directly from the sterilizing tank. It is possible that the worker or workers brought this contamination into the dry section where it flourished in the favourable environment of the cutter and surroundings.

DISCUSSION

The results of these investigations showed that in the great majority of instances contamination of the coconut took place before drying and not after. The contamination could often be traced back to the 'wet' section of the mill where the coconuts were prepared for processing, and in some instances to animal droppings found in the mill yard.

The results also showed that the main site of contamination inside the mill was the cutter or disintegrator. With the exception of one or two mills, there is only one cutter at each mill, and all the coconut processed at the mill must pass through it before going on to be dried.

The cutter, which disintegrates the pared and sterilized coconut into very small pieces, is made up of two cutting (also called grinding) rings with numerous teeth. These work together at high speed to shred the coconut. During this process some of the milk from the coconut is extracted and remains in the small interspaces. This milk contains fat, sugars, protein and minerals and provides a good source of nutrition for the bacteria. Once in the cutter, the salmonellas are difficult to dislodge. Neither boiling water nor disinfectants can reach the interspaces, and fresh coconut going through the cutter provides increasing nutriment for the salmonellas. It was found that to rid the cutter of salmonellas, it had to be dismantled and the separate parts scrubbed and boiled. As this piece of equipment weighs several hundred pounds, it could only be boiled in the sterilizing tank. This was a tedious process resulting sometimes in the loss of a working day, and was generally carried out only when contamination was detected at the mill. The usual daily practice was to flush it out with the water from the sterilizing tank. Disinfectants were not encouraged as they tended to flavour the coconut. Dismantling and boiling the cutter was successful in the case of Mill C where 37 consecutive samples were found to be contaminated before the cutter was cleaned. After cleaning the product remained free from contamination during the period of investigation up to August 1967. At other mills, however, e.g. Mills B, D and E, the measure was only temporarily successful; the plants were thought to have become recontaminated.

Table 1 shows that when wet, disintegrated coconut meat was contaminated, the bins used for collecting the wet meat, the implements used for handling it, the hands of the workers at the cutter (Mills F, J) and even at the desiccators (Mills C, E) were contamined. Recontamination of fresh batches of coconut, from the hands of these workers, would also take place. The workers could become infected and even act as symptomless excretors. As previously stated this aspect could not be investigated.

The disintegrated coconut meat is sometimes dried in old tray-type desiccators at 85–95° C. for 30–45 min. Operation of these desiccators requires some handling. Coconut is also dried in new semi-automatic driers which require less handling, at even higher temperatures (95–100° C.) but for a shorter time. The moisture content is reduced to about 2 % in this process. The numbers of bacteria are considerably reduced during drying, but not all the vegetative forms are killed. Desiccated coconut after grading and packing had total bacterial counts up to about 10,000/g. and some coliform counts of over 1000/g. Table 1 shows that salmonellas did survive the drying process. A possible explanation is that, although the temperatures in the desiccator are in the range 85–95° C., some of the coconut may reach this temperature only momentarily, if at all. Heat penetration is not uniform, for as the coconut dries it tends to clump together, and salmonellas can survive in these clumps. Laboratory experiments in which about 50 organisms/gram were introduced into wet coconut gave a recovery of over 11 organisms/gram after drying at oven temperatures of 75–90° C. for 30 min. No systematic counts of salmonellas in naturally contaminated samples of desiccated coconut were made but there are indications that in some instances they could be as low as 1/100 g.

This shows that if salmonella organisms are present in the coconut before desiccation in sufficiently high numbers, some will be present in the dried product.

In five of the ten mills shown in Table 1, the same salmonella serotypes were found on the mill premises, or in the stages before sterilizing, as were isolated from the cut coconut meat before drying, and from the desiccated product. It is concluded that contamination is somehow finding its way into the cutter from the outside. Theoretically this should not happen as all the pared coconut must pass through a tank of near-boiling water after which there should be no more handling before it passes along a chute into the cutter. Precautions are also taken to prevent contamination coming in to the dry section in other ways. The problem is how does the cutter become contaminated?

One possibility is that contaminated coconut reaches the cutter despite the sterilization tank. Our investigations showed that salmonellas were absent from the coconut coming out of the sterilizing tank when it was properly operated. However, there is a possibility that certain heat-resistant strains of *Salmonella* were surviving this. This seemed most probable with the two strains of *S. senftenberg* which are the most common contaminants of desiccated coconut. But heat-resistance studies carried out on four strains of *Salmonella* in the laboratory showed that this was not likely. The D values given in Table 5 for these strains when heated in phosphate buffer at 57 and 60° C. with initial cultures of approximately 10^8 organisms/ml. show that the organisms would be destroyed in a matter of minutes at 60° C.

However, Jensen (1945) and Yesair, Boher & Cameron (1946) have shown that when micrococci, for example, are heated in fat their resistance to heat is greater than when heated in water or nutrient broth solution. The fat on the surface of the coconut could have a protective effect on the bacteria. This would be true if the coconut pieces, which are sometimes in the shape of half cups, are cupped together when immersed in the sterilization tank, and so prevent a proper circulation of the boiling water between the surfaces.

T. Velaudapillai (personal communication) suggests that the organisms are only 'shocked' in the sterilizing tank, and recover in the favourable medium of the coconut in the cutter.

In the Phillipines, which is the other large production centre for desiccated coconut, the coconut is sterilized by passing through water at 80° C. for 8–10 min. (Schaffner, Mosbach, Bibit & Watson 1967). This is considered to be satisfactory.

Another likely explanation is that owing to careless supervision the temperature of the water in the sterilizing tank is not strictly maintained at over 95° C. This is likely to happen when long hours are worked at peak periods of production, and has been known to occur.

A further possibility is that as salmonellas are in the mill environment, workers even inside the mill and looking after the cutter and desiccators pick up the organism on their hands, and bring it into the dry section where it thrives in the favourable surroundings of the cutter. It has been shown in Table 1 that hands of workers in this area were contaminated, as well as implements, bins and tables.

Sources of contamination

The investigations led to the conclusion that contamination of the coconuts took place on the mill premises and was due chiefly to infection from animal excreta, and that the organisms multiplied and were encouraged during the stages of manufacture in the 'wet' section. This was passed through to the dry section chiefly to the cutter, and there was another build-up of bacteria in the cutter.

The desiccated coconut industry in Ceylon is situated in the main coconut growing districts of the island, to the north and north-east of Colombo, in rural areas. Many domestic animals are reared in these parts in a rather haphazard fashion. Poultry, cattle and pigs are the most common, and these animals can and do forage in the coconut estates. Cattle are often tethered very near to desiccated coconut mills. Most transportation is by bullock-drawn carts, and cattle droppings are thus scattered around the area. Dogs and semi-wild animals such as polecats and other small creatures could also have access to mill premises at night.

Table 6. *Salmonella serotypes isolated from pig faeces*

S. javiana	*S. give*
S. bareilly	*S. chester*
S. senftenberg H_2S-	*S. stanley*
S. typhimurium	

Table 6 gives a list of salmonella serotypes isolated from pigs, and Schmid & Velaudapillai (1963) isolated *S. paratyphi B*, *S. typhimurium*, *S. virchow*, *S. dublin* and *S. gallinarum* from domestic animals in Ceylon. *S. bareilly*, *S. senftenberg* (H_2S-) and *S. typhimurium* were the serotypes found in both pigs and desiccated coconut; while *S. paratyphi B* and *S. typhimurium* found in other domestic animals have also been isolated from desiccated coconut. In the course of our investigations *S. senftenberg* strains, H_2S+ and H_2S-, were isolated from cattle droppings, and *S. senftenberg* (H_2S+) was isolated from the droppings of an unidentified animal. *S. tennessee* was isolated from cattle droppings and *S. bareilly* from the dung of a pet monkey found in the mill premises. While it may be argued that the animals were contaminated from the coconut, they could still become a reservoir of infection.

It was not possible to investigate whether humans were the chief source of contamination, which they may have been, particularly in the case of *S. paratyphi B* and *S. typhimurium*. Only once was a salmonella isolated from well water—*S. weltevreden* from Mill I.

Whether the source of contamination was animal or human, the contamination of the cutter is the most important factor to be avoided from the point of view of the mill. Not only does the coconut passing through become contaminated, but the contamination is transferred to implements and workers and so to the entire mill, and results in a continuous circle of contamination. This is probably the reason why some mills found it so difficult to eliminate salmonellas despite cleaning and boiling of the cutter. It must be emphasized that to obtain a salmonella-free product with any degree of certainty, the cutter must be regularly and efficiently cleaned, and some method of carrying this out must be evolved.

A special mention must be made of *S. senftenberg* which was the most frequent and persistent serotype found in desiccated coconut; this serotype was also the predominant organism mentioned in connexion with the contamination of Phillipine coconut, where 75% of the salmonellas isolated were found to be *S. senftenberg* (Schaffner *et al.* 1967).

SUMMARY

Investigations were carried out at the desiccated coconut manufacturing 'Mills' in Ceylon to attempt to trace the source and sequence of salmonella contamination of desiccated coconut. It was found that one of the sources of contamination was animal excreta found on the mill premises—the yard where the nuts were stacked. Contamination was passed through the successive stages of preparation for manufacture and into the cutter which became the focal point of contamination within the dry section of the mill. The coconut appeared, in the majority of cases, to have been contaminated before drying. Some of the organisms survived the drying process. Two strains of *S. senftenberg* were found to be the most frequent contaminants of desiccated coconut.

These investigations were carried out with the assistance of the laboratory and field staff of the Ceylon Coconut Board, for whose co-operation many thanks are due; thanks are also due to Dr T. Velaudapillai formerly of the Medical Research Institute, Colombo for many helpful suggestions; to Dr Joan Taylor, Salmonella Reference Laboratory, Colindale, for identification of the salmonella serotypes; and to Dr Betty C. Hobbs, Food Hygiene Laboratory, Colindale, for reading the manuscript.

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The serological response and long-lasting resistance against infection with louping-ill virus in sheep immunized with a highly attenuated tick-borne encephalitis virus

By V. MAYER, D. BLAŠKOVIČ, E. ERNEK and H. LIBÍKOVÁ
The Institute of Virology, Slovak Academy of Sciences, Bratislava

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Infections with louping-ill (Li) virus, belonging to the tick-borne encephalitis (TE) complex of the B group of arboviruses, represent an important veterinary and human public health problem, mainly in Scottish natural foci of Li, in northern England and in Ireland. The Li virus, transmitted by the tick *Ixodes ricinus*, causes not only economic losses in flocks of sheep, but is known also as an aetiological agent in human infections.

To protect sheep against Li, intensive studies of the immunizing effect of a formolized virus vaccine were undertaken (Edward, 1947; Williams & Thorburn, 1961). The use of the live TP-21 strain (Smith, 1956) of the Malayan Langat virus, showing greatly lowered virulence for sheep, is not recommended by O'Reilly *et al.* (1965) because of a weak antibody response.

In the course of the investigation of immunogenic and antigenic properties of the monkey- and mouse-attenuated clone, designated Hy-HK 28 '2', of the TE (Western subtype) virus, a distinct protective effect in immunized animals against challenge with various virulent strains of different members of the TE complex was observed. Even a single dose of the attenuated TE virus, administered subcutaneously, elicited in large domestic milk-giving animals seroconversions into positivity accompanied by a high and long-lasting resistance (Mayer *et al.* 1967; Blaškovič *et al.* 1969). This marked immune response was observed mainly in goats, animals important in the epidemiology of milk-transmitted, human alimentary infections with the TE virus.

The aim of the present study was to investigate the degree of the resistance which develops in sheep, immunized with a single dose of the attenuated TE virus. These experiments were also intended to study the specific immune response, including the persistence of resistance to challenge with virulent Li virus in a long-term study. The investigations of the influence of the time factor on the degree of the resistance developed were expected to show to what extent the Hy-HK 28 '2' virus, differing in the character of at least 8 genetic markers (Mayer, 1966; Mayer & Rajčáni, 1968) from the usually encountered highly virulent TE virus strains, is capable of eliciting a long-persisting immune state in domestic animals. In this study it concerns sheep, which under natural conditions participate in the ecological cycle of TE and similarly also of Li virus.

The work presented was performed as a part of an extensive field trial of experi-

mental immunization of domestic milk-giving animals (goats, cattle and sheep) using the attenuated TE virus, with the final aim to collect information regarding the possibility of preventing alimentary human TE infections. Results of this field trial are described in detail elsewhere (Blaškovič *et al.* 1969).

MATERIALS AND METHODS

Viruses

For immunization of sheep, the virus clone, designated Hy-KH 28 '2', derived from the prototype Czechoslovak strain 'Hypr' of the TE virus (Western subtype) (Pospíšil, Jandásek & Pešek, 1954) was used. This particular clone, described in detail elsewhere (Mayer, Slavik & Libíková, 1967*a*) is avirulent for 6–8 g. white mice (Děčín breed) after subcutaneous inoculation and for young *M. mulatta* and *M. radiata* monkeys after intrathalamic or intranasal administration (Mayer & Rajčáni, 1967; 1968). The virus was used in the form of 10 % mouse brain suspension with the titre of $10^{8\cdot0}$LD 50/ml. after intracerebral and $10^{1\cdot0}$LD 50/ml. after subcutaneous inoculation of young mice.

For challenge of immunized sheep and infection of control, non-immune animals the 'Li 1959' strain of the Li virus (from the collection of viruses, Institute of Virology, Bratislava, strain originally supplied by Dr McCallum, London) was used. This virus strain was originally isolated from the brain of a sick lamb and underwent 7 intracerebral passages. The virus titre in 10 % mouse brain suspension was $10^{8\cdot2}$/ml.

Animals

Ninety-seven sheep, 1–2 years old, were used in this study. These animals, from which 74 were used for the immunization experiment and 23 served as controls (placebo), belonged to one herd, grazing on the southern slopes of Tribeč Mountains (south-west Slovakia), a locality known as a natural focus of TE.

Before the experiment 11 sheep from the larger group of animals (14·8 %) and 6 from the 23 control animals (26 %) showed the presence of virus-neutralizing antibodies (VNA) against TE virus in their serum (Tables 1, 2). As control for the challenge experiments, 4 animals of the same age as the immunized sheep, but without detectable VNA, were chosen.

Viraemia

During the first days after the challenge, the blood from all animals was assayed for the presence of the virus. The isolation experiments were performed by intracerebral inoculation of mice. Each blood sample was administered immediately after collection into one litter of 8–10 newborn mice. Virus in ill mice of the primary passage was demonstrated definitely by means of a second passage. The isolated virus was identified by the virus-neutralization test using specific immune serum. Blind passages were performed on the 10th day after infection by inoculating a 10 % mouse brain suspension from primary passage into further litters of suckling mice. The intracerebral route was invariably used.

Virus-neutralization tests

Titres of specific VNA in sheep sera were determined in cell cultures of Detroit-6 cells and Salk cynomolgus heart cells using a cytopathic variant of TE virus (Libíková, 1963). The initial dilution of the inactivated serum was 1/4. Blood samples were collected at intervals from the jugular vein. Separated sera were stored frozen until they were examined.

Table 1. *Virus-neutralizing antibody in sheep before immunization, and 6 weeks, 9 months and 12 months after immunization with one dose, containing 10^7 mouse ICLD 50, of attenuated tick-borne encephalitis virus*

	Before immunization		After immunization					
			6 weeks		9 months		12 months	
Antibody titre	No. of sheep	(%)	No. of sheep	(%)	No. of sheep	(%)	No. of sheep	(%)
< 4	63	85	9	13	3	5	3	6
4	10	14	21	30	15	24	14	27
8	0	—	22	31	21	34	16	31
16	1	1	16	23	12	20	10	19
32	0	—	1	1	7	11	7	13
64	0	—	1	1	1	2	0	—
⩾ 128	0	—	0	—	2	3	2	4
Total no. of animals	74	—	70	—	61	—	52	—
Antibody present in	11	15 %	61	87 %	58	95 %	49	94 %

Table 2. *Virus-neutralizing antibody titres in the control group of sheep before and 6 weeks and twelve months after administration of placebo*

	Before placebo		6 weeks after placebo		12 months after placebo	
Titre of virus-neutralizing antibodies	No. of sheep	(%)	No. of sheep	(%)	No. of sheep	(%)
< 4	17	74	13	76	11	78
4	3	13	3	18	2	14
8	2	9	1	6	1	7
16	1	4	0	—	0	—
32	0	—	0	—	0	—
Total number of animals	23	—	17	—	14	—

RESULTS

Experimental immunization of sheep

Seventy-four 1- to 2-year-old sheep were subcutaneously injected with one 1 ml. dose of the attenuated Hy-HK 28 '2' virus, in the form of 1 % infected mouse brain suspension, containing 10^7 mouse ICLD 50. The remaining 23 sheep were

given, also subcutaneously, one dose of 1 % normal mouse brain suspension, which served as placebo.

The evaluation of the whole experiment showed a striking immune response to TE virus in animals given the Hy-HK 28 '2' clone. The application of this clone stimulated the production of specific VNA to virulent TE virus to such an extent that specific VNA were observed 6 weeks after the injection in 87 % of sheep, including those where VNA were detected before the immunization (15 %). Nine or 12 months after the administration of the virus, the incidence of VNA reached 94 %.

VNA at titres of 1/4–1/16 were present 6 weeks after immunization in 84 % of sheep, but they were not detected in 13 % of animals. Nine months after immunization, VNA were absent in only 5 % of 61 sheep examined, and in 90 % of the animals they reached titres of 1/4–1/32. In two sheep VNA were found at a titre as high as or higher than 1/128. Similar serological results were also found with sera collected during the 12th month after immunization, when VNA titres of 1/4–1/32 were found in 90 % of the animals examined and the same two sheep as before showed the high titres ($\geq$ 1/128) (Table 1).

Table 3. *Immune response in sheep on the 6th week after administration of attenuated tick-borne encephalitis virus*

Effect of immunization on virus-neutralizing antibody	Number of sheep*	(%)
Seroconversion from negative to positive	50/59	85
Seronegative after immunization	9/59	15
Pre-existing antibody titres increased	9/11	82
Pre-existing antibody titres not increased	2/11	18

* Numerator = no. of animals showing effect; denominator = no. of animals examined.

When considering the type of immune response (Table 3), it is interesting to note that the serological conversion from negative to positive and the increase of the pre-existing VNA titres of the VNA was observed in approximately the same percentage, i.e. in 85 and 82 %.

From the 23 placebo-injected sheep only 14 remained to the end of the 12th month. From them only 3 animals showed the presence of VNA (Table 2). In this number is included also 1 sheep, in which a clear seroconversion to positive was observed.

Challenge of the one-shot immunized sheep

Four immunized sheep were selected on the basis of their VNA titres. Care was taken to choose animals which had rather low VNA titres, i.e. titres most frequently observed after immunization with the live virus. The selected sheep and three further control animals (without specific serum antibodies) were challenged with 10^5 ICLD 50 of virulent Li virus administered subcutaneously in the form of diluted 10 % mouse brain suspension.

Table 4. *Viraemia and virus-neutralizing antibody in sheep immunized with one dose of* 10^7 *ICLD*50 *of attenuated tick-borne encephalitis virus clone HY-HK* 28 '2', 324 *days before challenge with* 10^5 *ICLD*50 *of virulent louping-ill virus, and in control unimmunized sheep similarly challenged*

Sheep no.		Before challenge	Days after challenge												
			2	3	4	5	6	7	8	9	10	11	12	14	16	21
657	Imm.*	4	–	–	–	–	–	–	–	–	–	–	–	.	.	.
			8	–	128	.	128	.	.	256	.	.	256	256	256	256
604	Imm.	4–8	–	–	–	–	–	–	–	–	–	–	–	.	.	.
			4–8	.	4–8	.	32	.	.	64	.	.	128	128	256	256
607	Imm.	4–8	–	–	–	–	–	–	–	–	–	–	–	.	.	.
			4–8	.	4–8	.	32	.	.	64	.	.	128	128	128	128
623	Imm.	8	–	–	–	–	–	–	–	–	–	–	–	.	.	.
			16	.	32	.	128	.	.	128	.	.	128	256	256	256
701	Cont.*	< 4	–	–	+	+	+	–	–	–	–	–	–	.	.	.
			< 4	.	< 4	.	4	.	.	16	.	.	16	16	16	16
711	Cont.	< 4	–	+	+	+	+	–	–	–	–	–	–	.	.	.
			< 4	.	< 4	.	< 4	.	.	8	.	.	8	8	4	4
732	Cont.	< 4	< 4	.	< 4	.	8	.	.	64	.	.	64	64	64	64

* Imm. = immunized. Cont. = control unimmunized animal.
The upper line for each animal shows viraemia. – = virus not detected in blind passage on unweaned mice. + = virus detected.
The lower line shows reciprocals of the neutralizing antibody titres.

Edward (1947) reported that the administration of 10^4–10^6 mouse LD 50 of the Li virus caused death in 66 % of sheep inoculated. The challenge was performed in January, as advised by the same author since the winter is claimed to be the most favourable season for the establishment of experimental infection.

During the first days after the challenge, the blood from all animals was assayed for the presence of virus. In the sheep immunized with the Hy-HK 28 '2' virus, no viraemia was observed between the 2nd and 12th day after challenge with louping-ill virus, irrespective of the actual level of VNA. In control previously non-immunized sheep, viraemia started on the 3rd–4th day after challenge and lasted for as long as 4 days. These results indicate the multiplication of the virus in the organism of non-immune animals (Table 4).

Furthermore, a very marked difference between the challenged immunized and control sheep was observed in their VNA titres. In immunized animals the titres of VNA increased considerably during 2–3 days after the injection of the challenging virus and, starting from the 6th day, they reached relatively high titres which were further maintained. High titres of specific VNA were observed in all immunized animals (Table 4). The immune response in control animals was considerably less pronounced. They developed VNA only later and in lower titres.

The temperature response and clinical observation revealed no significant differences between the immunized and control animals.

DISCUSSION

The results of experiments described above indicate that even a single dose of the monkey-attenuated virus was able to elicit in sheep an immune response lasting for at least 11 months. This immune response, although caused by the TE virus (Western subtype), was specific also against the serologically related louping-ill virus. The investigations of VNA showed that animals immunized with the attenuated virus acquired a considerable resistance against infection with a virulent virus, even when humoral antibodies were present at rather low titres. The relatively low VNA titres were observed also in goats, which similarly exhibited a high resistance against the challenging virus (Mayer *et al.* 1967*b*).

The steep increase of VNA titres observed during the first days after challenge of immunized animals, and the fact that this good anamnestic response (together with a satisfactory degree of resistance) was observed even when the challenging virus was administered on the 324th day after the one-shot immunization, seems to indicate that a highly monkey- and mouse-attenuated virus, as exemplified by the Hy-HK 28 '2' clone, could also serve as a good antigenic stimulus for sheep.

These experiments, which have rather an informative character, will need to be completed on a larger number of challenged animals.

The increase of the percentage of serologically positive sheep (from 87 to 94 %) and of the animals with the higher VNA titres, in the period between 6 weeks and 9 months after immunization, may be due either to contact of the sheep with virus circulating in nature, or to a slower immune response of some sheep to the administration of the attenuated virus. The hypothesis concerning the supposed contact

of animals during the spring and summer period with the virus circulating under natural conditions seems to be, in the majority of cases, the more probable, because the grazing locality is known as a natural focus of TE. This assumption is supported also by the relatively high number of sheep showing the presence of specific VNA before immunization. Thus it seems that the immunity conferred by immunization could be maintained or even increased by small booster doses of virus transmitted by ticks, parazitic on sheep.

The results obtained indicate that the immunization of sheep, raised and moving freely in geographic areas known as natural foci of TE or Li, could exert its effect at various levels. By preventing the detectable multiplication of the tick-transmitted virus in the organism of vaccinated animals (as shown by the absence of viraemia) the immunization (*a*) protects the sheep against clinically apparent forms of Li and (*b*) hinders the infection of other, still non-virophoric ticks, simultaneously infesting the same host animal, on which the infectious, virus-transmitting ticks are parasitic.

Thus the vaccination of sheep could be considered also as a limiting factor in the spread of the virus in nature, which has epizootiological and epidemiological implications.

SUMMARY

The vaccination of sheep with one dose of the monkey- and mouse-attenuated tick-borne encephalitis virus (the Hy-HK 28 '2' clone) causes seroconversion from negative into positive in 85 % of animals. In sheep with pre-existing virus-neutralizing antibodies an increase of their titres was observed in 81 %. The antibodies persisted for at least 12 months after the vaccination and during the summer period of grazing the number of serologically positive animals even increased.

The vaccinated animals, in contrast to the non-immune control sheep, developed no viraemia after challenge with the virulent louping-ill virus, performed 11 months after immunization.

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Toxoplasma antibodies in sera from Hong Kong

By G. B. LUDLAM
Public Health Laboratory, Leeds, England
AND SIMON K. K. WONG AND C. ELAINE FIELD
Paediatric Department, Queen Mary Hospital, Hong Kong

(*Received* 4 *June* 1969)

Before carrying out the Sabin–Feldman dye-test on the sera of children in Hong Kong suspected of toxoplasmosis, sera from a number of the population were tested to determine the incidence of toxoplasma antibodies and thus to help in estimating the significance of positive results obtained in suspected cases. Surveys on the incidence of toxoplasma antibodies have been reported from many parts of the world but we think our results have proved sufficiently unusual and unexpected to justify publishing them here.

METHODS

As it was obviously impracticable to test a true random sample of the Hong Kong population, sera from the following groups were tested for toxoplasma antibodies: 32 adult females (30 antenatal and two others), 35 male patients suffering from various complaints and aged 20–48 years, 46 men working as butchers or slaughterers, and nine children aged 1 week to 17 years mainly suspected of toxoplasmosis. Sera from 31 pigs, imported from China and slaughtered in Hong Kong, were also tested.

The sera were despatched in the frozen state and received still frozen at the Leeds Public Health Laboratory where they were maintained at $-20°$ C. until tested. They were examined by the Sabin–Feldman dye-test by the technique routinely employed in the laboratory.

RESULTS

Table 1 shows the results obtained. Out of a total of 113 adult human sera tested only seven were positive (6·2 %). The titres obtained with the seven positive human sera were all low, five positive at 1/8 and two at 1/32. The 22 positive pig sera included six with titres of 1/512 to 1/8192. Sera from nine children of all ages mainly suspected of toxoplasmosis were all negative.

DISCUSSION

At the onset of this investigation 30 antenatal sera from Hong Kong were tested and all gave negative dye-tests. In Britain the incidence of toxoplasma antibodies is such that one would have expected about 10 positive results in 30 antenatal

sera. Further sera were therefore tested and although a few positive results were found the incidence still remained very low, even among meat workers, who are normally found in other parts of the world to have a higher incidence than the rest of the population. The results strongly suggest there may be an exceptionally low incidence of toxoplasma infection in the population of Hong Kong. Clinical cases are rare.

Table 1. *Toxoplasma dye-test antibodies in man and pig in Hong Kong*

Group	Total	No. positive	% positive
Adult females	32 } 67	0 } 2	0 } 3
Adult males	35 }	2 }	5·7 }
Male meat workers	46	5	10·9
Children (1 week–17 years)	9	0	0
Pigs	31	22	71

An incidence as low as this has never previously been reported in a large tropical city and only rarely in other environments. Feldman & Miller (1956) reported an incidence of 6 % in Navajo Indians and 11 % in Icelanders, and toxoplasmin skin tests were all negative in 183 persons living round two Egyptian oases (Rifaat *et al.* 1965).

Findings in Europe and the Americas generally range from 17 % in Portland, Oregon (Feldman & Miller, 1956) to 84 % in France (Desmonts, Couvreur & Ben Rachid, 1965). The only exception we have found to this is an incidence of 0·8 % in 478 blood donors in Cracow, Poland (Starzyk, 1959). The incidence in Africa is also high, but although not much work has been reported from the Far East such figures as are available suggest that the incidence there may be rather lower. Murakami (1964) found an incidence of 12·6 % in Nagasaki City, Sery *et al.* (1959) reported an incidence of only 2·9 % positive skin tests in Vietnam, and V. Zaman (personal communication) found 17 % of blood donors in Singapore positive by the haemagglutination test.

A low incidence is usually associated with a hot dry climate or with cold conditions, but the climate of Hong Kong is hot and wet in the summer. The incidence tends to be lower in towns than in the country but the incidence in Hong Kong is apparently much lower than that normally reported from Western urban areas. When one considers the size of Hong Kong and its closely packed population it would appear that spread by person-to-person contact cannot be a common mode of transmission. The high incidence of antibodies in the pigs examined (71 %) indicates an important potential source of infection and pork is a common food in Hong Kong. French workers consider that the high incidence in their country may be due to the eating of undercooked meat. Perhaps the Chinese population in Hong Kong escape infection from pork by their custom of eating pork in small lumps and only when well cooked. It is their belief that eating undercooked pork may cause 'madness' in the consumer. Undercooked fish and beef, on the contrary, are considered relatively harmless to the consumer. This could also account for their low incidence of *Taenia solium* infection in spite of heavy infestation of the local pigs. Further study of the cooking methods and social customs of the people

combined with more detailed serological studies of special groups is needed in attempting to explain the low incidence of infection. Whatever the explanation, it may be there is a common cause affecting the Far East as a whole, for so far there have been no reports of a high incidence comparable with the high rates found in Europe, tropical Africa and Central and South America.

SUMMARY

Toxoplasma antibodies were absent from the sera of 32 adult Hong Kong women and were present in only two of 35 adult males and five of 46 meat workers, an overall incidence of 6·2%. This is the lowest incidence recorded in a large tropical city and lower than the incidence reported for most areas of the world, whether rural or urban. The reasons for this are unknown. Of 31 sera from swine imported from China and slaughtered in Hong Kong, 22 (71%) were positive for toxoplasma antibodies.

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Selection for resistance to myxomatosis in domestic rabbits (*Oryctolagus cuniculus*)

By W. R. SOBEY

Commonwealth Scientific and Industrial Research Organization, Division of Animal Genetics, P.O. Box 90, *Epping N.S.W.* 2121

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The course followed by a disease resulting from the infection of an animal by a pathogen invariably displays some variation, and selection in a population for resistance or susceptibility generally results in an advance in the direction of selection (Gowen, 1951; Hutt, 1958). A build up of innate resistance to myxomatosis in the wild rabbit population after the release and spread of myxoma virus in Australia in 1950 was both predicted and found (Fenner, 1953; Marshall & Fenner, 1958; Marshall & Douglas, 1961). A programme of selection for resistance to myxoma virus in domestic rabbits was begun in this laboratory in 1954. It was planned to obtain estimates of the genetic factors underlying resistance, and in time, to build up a strain of genetically resistant rabbits for evaluating the usefulness of new virus strains, collected from the field, or produced as a result of laboratory manipulation. The present paper describes the progress made by selection.

MATERIALS AND METHODS

Rabbits

Domestic rabbits donated by, or purchased from, other scientific institutions made up the initial population. Complete breeding records were kept, each animal being identified by a number tattoed in the ear. Animals were housed in wire cages in a brick building, and fed on a dry pellet diet and water *ad lib.*, supplemented by fresh green feed. Heating was provided to give a minimum temperature of 70° F in winter, but no cooling was available and during hot spells in summer the temperature often reached 95° F. during the day. In July 1963 a temperature controlled room for housing animals under test became available. The temperature régime adopted was a constant $72 \pm 4°$ F. raised to $85 \pm 3°$ F. for 24 hr. 3–4 days after infection (Sobey, Menzies, Connolly & Adams, 1968). Rabbits were weaned at 8 weeks of age and tattoed. Rabbits were infected with the appropriate virus after the age of 16 weeks; this was considered a sufficient interval from birth to obviate the effects of maternal antibodies (Fenner & Marshall, 1954).

Virus strains

Four strains of virus were used, the highly virulent Standard laboratory strain (SS) Aust/Corowa/12-52, Uriarra (U) (Aust/Corowa/2-53-1, Fenner & Marshall, 1957) and two strains of KM 13 which will be referred to as KM 13/1 (Aust/

Corowa/12-52/2, Fenner & Marshall, 1957) and KM13/2 (Aust/Corowa/12-52A, Marshall, 1959). Although a standard inoculation dose of 500 lesion-forming units, (L.F.U., Sobey *et al.* 1966) was aimed at, this was found on titration to vary between 100 and 1000 L.F.U. in different tests.

Grade

The time interval between infection and when an animal could be used for breeding varied widely in animals which recovered from infection. Bucks were often sterile, or became fertile only after extended periods of time (Sobey & Turnbull, 1956). For this reason it was found inconvenient to record data on a generation basis and a system of grading was introduced. Unselected laboratory rabbits were graded 0. If a rabbit of grade 0 (or any other grade) recovered from infection it was allotted a grade of 1 (or its previous grade plus 1). The offspring of a 0×1 mating were graded 0·5, and 0·5 grade rabbits which recovered from infection became grade 1·5, and so on. A grade was thus a generation equivalent. It was possible for a rabbit to have two gradings one before and one after it had been exposed to infection. The pre-inoculation grade will be given in the text.

Selection was based in the first instance on an animal's ability to survive virus infection. While the percentage mortality was high it was necessary to breed, where possible, from all surviving rabbits and from unchallenged selected does. When the percentage mortality was low, as during 1960–1, criteria other than just survival were resorted to. The range of symptoms increased with decreased mortality, from rabbits which showed only a clearly demarcated lesion at the site of injection to rabbits which were very severely diseased. On the basis of symptoms the following classes were devised and used as a basis for selection:

(*a*) Reaction at the site of injection only.
(*b*) Mild eye reaction usually in the form of small and isolated inflamed areas.
(*c*) Eyelids red and swollen but without exudate.
(*d*) Eyes swollen and closed with exudate.

The survival time (S.T.) of all rabbits which died was recorded. For the purposes of statistical analysis, each rabbit was given a score equal to the time it lived in days after injection with virus: animals which survived infection with SS were given a score of 30 and those which survived any of the more attenuated virus strains a score of 60.

EXPERIMENTAL RESULTS

Response to selection

The response to selection, using each of the four strains of virus is illustrated in Fig. 1 in which recovery is plotted against grade. The number of animals tested with each strain at each grade is given in Table 1. SS/1 represents Standard strain before temperature control for testing and SS/2 Standard strain with temperature controlled and an elevated temperature 3–4 days after infection. Environmental variation was evident even during the period 1963–8 when temperature control was available for testing animals. This is illustrated in Fig. 2 where the percentage recovery from infection with the four strains of virus is plotted

against time and the average annual mean grade. These data also show the times during which the different strains were used. During 1956 and 1957 a severe outbreak of *Pasteurella multocida* infections depleted rabbit numbers and also adversely affected the ability of rabbits to recover from myxomatosis. The data for 1956 and 1957 have, for this reason, been omitted from Fig. 1. No explanation can be offered for the trough in 1965; unselected animals also showed a depressed S.T. during this period.

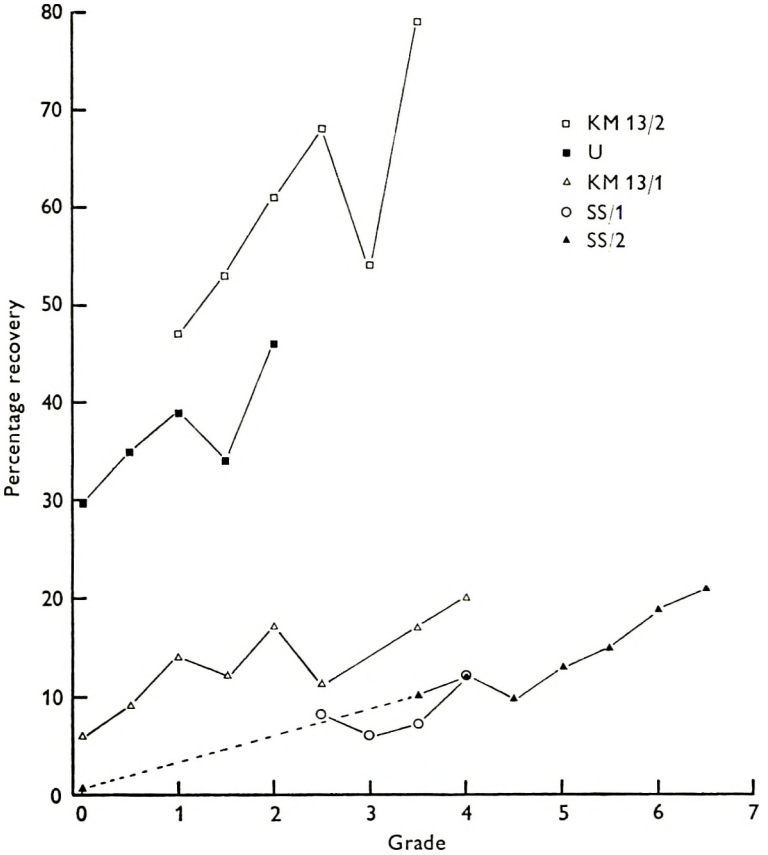

Fig. 1. The percentage recovery plotted against grade for the four virus strains used during selection. SS/1 denotes animals tested before and SS/2 those tested after temperature control for testing became available.

Table 1. *The number of animals tested with each virus in the different grades*

(SS/1 denotes the animals tested before and SS/2 those after temperature control for testing to Standard laboratory strain of virus.)

Virus strain	No. of animals tested in each grade													
	0	0·5	1·0	1·5	2·0	2·5	3·0	3·5	4·0	5·4	5·0	5·5	6·0	6·5
U	297	207	261	250	41	—	—	—	—	—	—	—	—	—
KM13/2	—	—	28	107	313	213	78	24	—	—	—	—	—	—
KM13/1	178	264	578	509	201	46	—	54	76	—	—	—	—	—
SS/1	—	—	—	—	—	48	118	293	164					
SS/2	138	—	—	—	—	—	—	67	297	316	331	256	153	34

Because of overlapping generations any one grade was tested over about a 3-year period and, as seen in Fig. 1, most of the environmental variation has been ironed out, and response to selection appears to be linear for all four virus strains. Clearly selection in the earlier stages with attenuated viruses raised the level of resistance of the animals to the fully virulent SS virus.

Maternal antibodies

The consequences of passive immunity were examined by Fenner & Marshall (1954) up to the age of 47 days. The offspring from does with maternal antibodies were found to have a higher percentage recovery, an extended survival time and

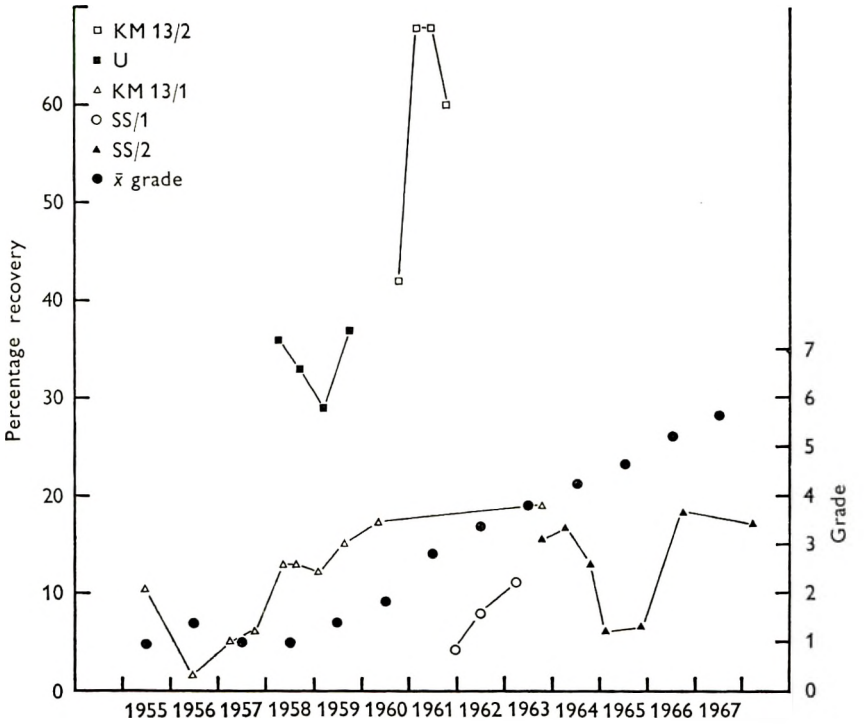

Fig. 2. Percentage survival of the animals and their mean annual grade, illustrating the viruses used for selection in time, and the large variation due to environmental factors. Each point represents the mean of 100–300 animals tested in two to four experiments. Between 1958 and 1967 there was an average annual increment of 0·5 of a grade.

Table 2. *The percentage recovery and mean survival times of offspring from does which had recovered from KM* 13/1 *with a post-inoculation grade of* 1 *and offspring from does not exposed to virus with a pre-inoculation grade of* 1.

(Data collected between 1957 and 1960).

Offspring from	No. of animals	% recovery	$\bar{x}$ S.T.
Recovered does	268	13·1	26·3
Uninfected does	204	13·7	25·7

a lowered rate of infection by mosquito bite when compared with the progeny of animals without maternal antibodies. To ensure that passive immunity was not influencing the recovery rate in selected animals challenged between 16 and 23 weeks of age the offspring from does which had recovered from KM 13/1 with a post-inoculation grade 1 were compared with the offspring from unchallenged does with a pre-inoculation grade 1, with respect to percentage survival and mean survival time. The results, shown in Table 2, demonstrate that by the age of 16–23 weeks, maternal antibodies no longer influenced the course of the disease.

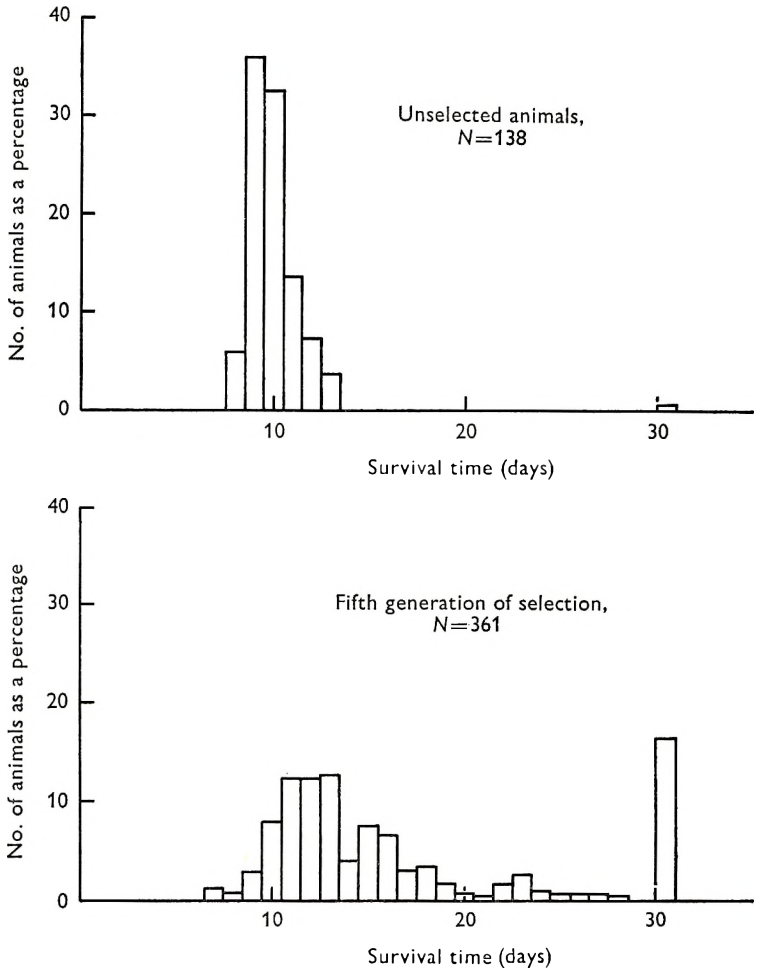

Fig. 3. Changes in the distribution of survival time with selection, illustrating the extended survival times of animals with increased genetic resistance. All animals were infected with the standard laboratory strain of myxoma virus.

Heritability analysis

Intra-sire correlation

The basis of selection was quantal (death or recovery) and heritability (h^2) could not be estimated by direct parent-offspring comparison. Survival time (S.T.) within virus strains is probably a valid index of genetic resistance; as shown in Fig. 3

the mean S.T. increases with increased selection. It is probably valid to compute the h^2 of resistance using S.T. as an index where the percentage recovery is low, and recoveries are allotted an arbitrary S.T. However, the bimodality of the S.T. distribution is exaggerated as the percentage recovery increases, and at some point the assumption of a normal distribution must become untenable. On the assumpsion that a percentage recovery of 10% was not excessive, h^2 was estimated by intra-sire correlation (Lerner, 1950) on data collected between 1957 and 1960, where KM 13/1 was used. Each buck was mated to five does selected at random and the S.T.'s of four of her offspring selected at random recorded for h^2 analysis. The data were grouped into two lots, each consisting of the results from 320 offspring of 16 bucks and 80 does; the first lot had a 5% and the second a 10% recovery rate. The average grade rose from 0·3 for females and 1·0 for males in the first lot to 1·2 and 1·7 in lot 2. The results are presented in Table 3.

Table 3. *Estimates of heritability (h^2) of resistance to myxomatosis. Intra-sire correlations based on log_{10} survival times as an index of resistance after infection with KM* 13/1.

Lot no.	Estimated h^2	Estimated 95% limits	D.F.	% recovery within lot
1	Sire 0·41	0–1	15	5
	Dam 0·37	0·13–0·53	64	—
2	Sire 0·22	0–1	15	10
	Dam 0·83	0·75–0·88	64	—
Total 1 and 2	Sire 0·33	0–0·62	31	7·5
	Dam 0·64	0·40–0·70	128	—

In the first lot both sire and dam components of h^2 are about 40%, whereas in lot 2 there is an elevated dam component 83% and a lowered sire component 22%. The sire components of h^2 are based on relatively few animals and the difference is well within the limits of error. The elevated dam h^2 in lot 2, however, appears to be outside the limits of experimental error, suggesting an increase in the dam component with time, i.e. with increased selection. This apparent maternal effect cannot be accounted for by maternal antibodies (see Table 2). A possible explanation could be that the apparent maternal effect is a litter effect due to seasonal variation, more pronounced in lot 2 than in lot 1 because of the time of year at which litters were tested. Individuals within litters were invariably infected at the same time so that any variation affecting survival, such as seasonal effect, would increase between-litter variation or spuriously decrease within-litter variation. This would, in estimates of intra-sire correlations, tend to elevate the dam component of h^2. The frequency distribution of the time at which litters were tested is shown in Fig. 4. It will be seen that in lot 1 the majority of litters were tested during the warmer months of the year, 42% during the summer months of December, January and February and only 14% during the winter months June, July and August; whereas in lot 2 23% were tested in the summer and 30% in the winter. The differential effect of the times of testing litters is reflected in the range of mean survival times over the year; 18·2–25·6 days for lot 1 and 16·0–34·0 days for lot 2.

Achieved heritability measured by probit analysis

Resistance is an all-or-none character and in the absence of some quantitative index of resistance does not lend itself to parent/offspring comparison. A method of determining the heritability of all-or-none characters has been given by Robertson & Lerner (1949) using the heterogeneity chi-squared in determining the genetic

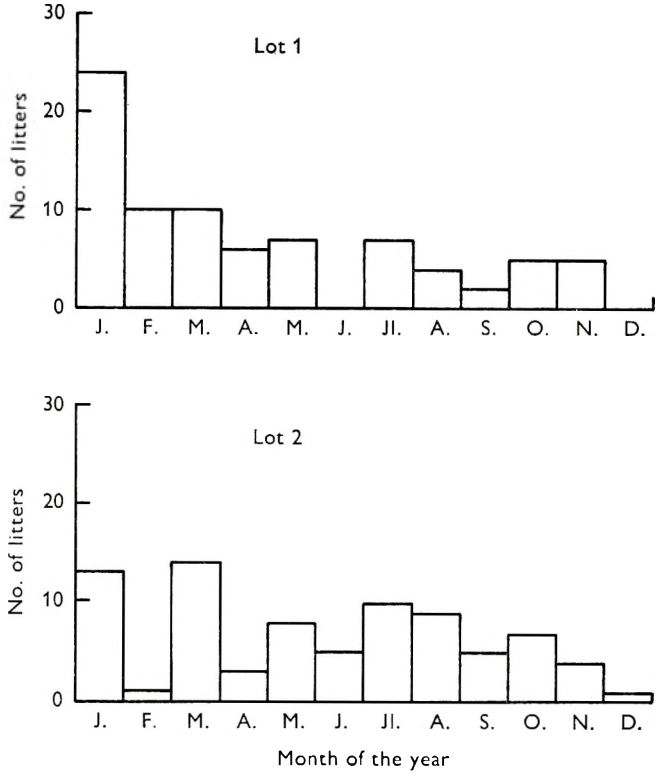

Fig. 4. The number of litters tested at different months of the year for two lots each of 80 litters. 42 % of litters were tested during the summer months (D.J.F.) and 14 % during the winter months (J.J.A.) in lot 1 and 23 and 30 % respectively in lot 2.

variance. The subclass numbers in the present data were too small for the above method to be used. Using the same basic proposition

$$\text{heritability} = \frac{\text{genetic improvement}}{\text{phenotypic selection differential}}$$

and the probit transformation, the following method was used to calculate heritability.

$$h^2 = \frac{\Delta P_0}{\frac{1}{2}(Is + Id)},$$

where Is and Id are the selection differentials of sire and dam respectively, and ΔP_0 is the difference in mean phenotype between the parental and filial generations. Is and Id are estimated from the percentage of survivors in the population of

which the parent was a member when it was infected with virus to test its resistance. These tests were done in batches of 30–150 animals and the selection differential of an individual is measured from the percentage survival of the batch in which it was tested. The percentage is expressed as the amount in standard deviations by which the mean of that fraction of a normal distribution would exceed the mean of the whole distribution, on the assumption that whatever is responsible for survival is a normally distributed quality with a sharp cut-off

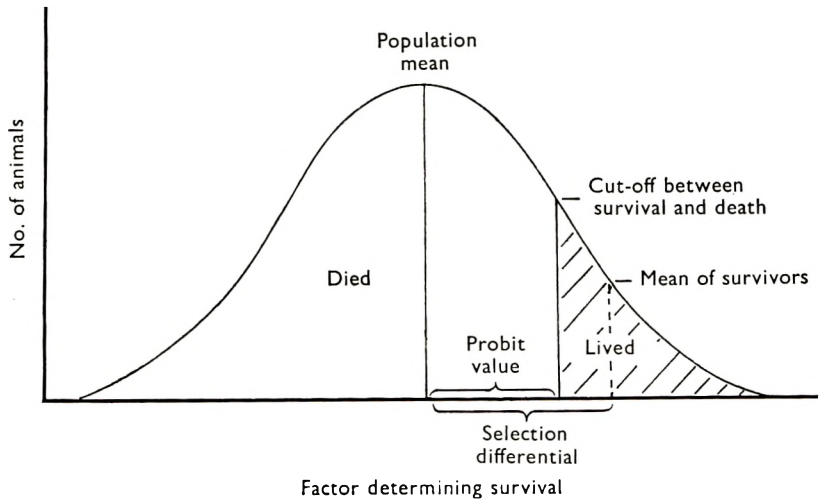

Fig. 5. An assumed normally distributed factor determining survival indicating the basis of the probit value and selection differential.

separating values adequate for survival from those leading to death (see Fig. 5). ΔP_0 is estimated by subtracting the mean survival of the parental population from the mean survival value of the filial populations. The survival value of each parent, estimated by converting the percentage of survivors in the population in which it was tested into probits, is weighted for the number of offspring each parent had for the purpose of estimating mean survival value. The filial survival value is the percentage surviving in a test batch converted into probits. The probit values indicate where the mean of the two populations falls relative to the cut-off between life and death and so the difference between the two in probits.

All offspring were tested with SS virus but as not all parents were tested with the same virus it was necessary for calculating ΔP_0 to equate the different viruses used, as shown in Fig. 6. Table 4 shows the results of 30 tests. Columns 5 and 6 are the selection differentials, columns $(8-4)$ is the estimate of ΔP_0. Columns 2 and 3 are not comparable to 5 and 6 because they have been adjusted for the virus used to test the different sires and dams. Fig. 7 is a histogram of the values of h^2 estimated from each of the 30 tests, involving 1228 animals, with a mean h^2 of 0·36.

It is of interest to note that most of the negative values of heritability in Table 4 occurred in tests made in 1964/5 when the whole level of recovery was lowered by some unknown environmental effect.

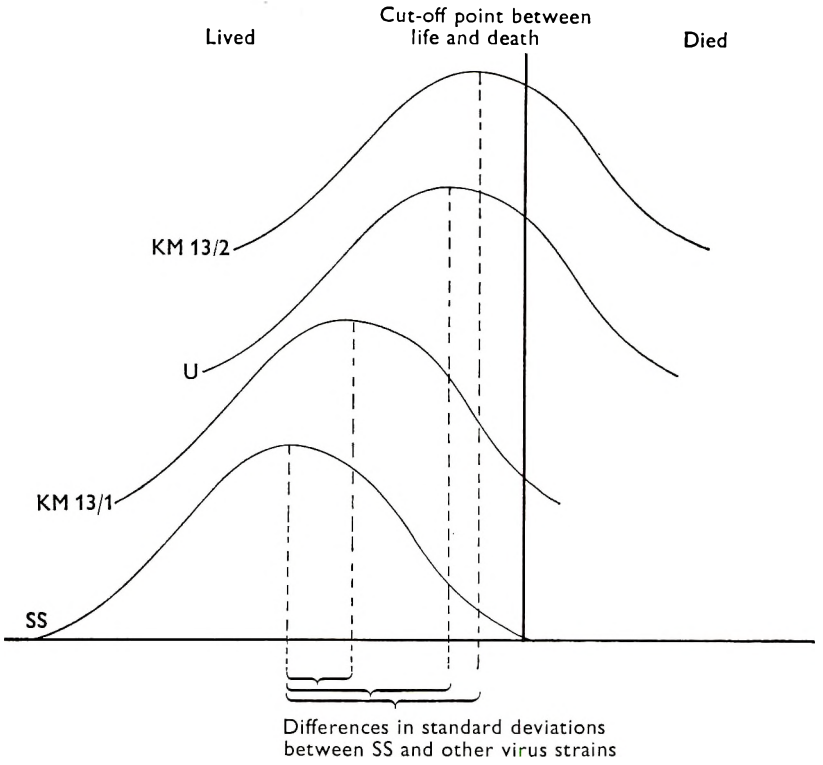

Fig. 6. The distributions of the assumed factor determining survival for the different strains of virus relative to the cut-off point between death and survival, determining the relation of KM 13/1, KM 13/2 and U to SS.

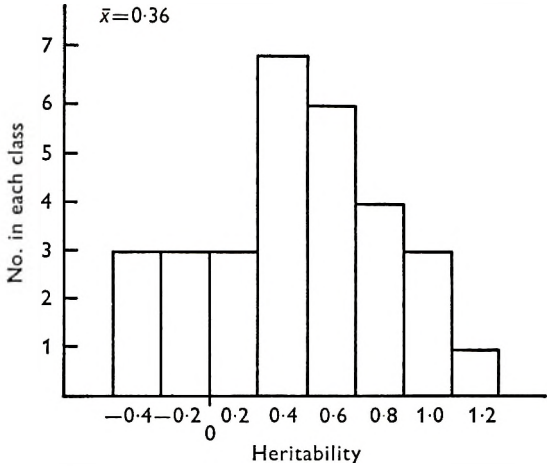

Fig. 7. The distribution of the heritabilities calculated from 30 different tests by probit analysis with a mean of h^2 of 0·36.

Table 4. *Estimate of heritability (h^2) using an all-or-none probit analysis*

Date of expt.	Sire probit	Dam probit	$\bar{x}$	Sire S.D.	Dam S.D.	$\bar{x}$	Offspring probit	h^2
17. i. 61	3·20	3·54	3·37	1·25	1·57	1·41	3·92	0·39
16. ii. 61	3·48	3·91	3·70	1·48	0·87	1·18	4·33	0·53
22. iii. 61	3·38	3·55	3·47	1·40	0·95	1·17	4·42	0·81
13. ix 61	3·38	3·34	3·28	0·71	0·96	0·84	4·07	0·94
1. xi. 61	3·30	3·48	3·39	0·75	0·61	0·68	3·84	0·66
22. xi. 61	3·15	3·55	3·35	0·72	0·69	0·71	4·03	0·96
3. i. 62	3·25	3·58	3·42	0·67	0·74	0·71	3·92	0·71
21. ii. 62	3·31	3·44	3·38	0·58	0·58	0·58	3·80	0·72
14. iii. 62	3·29	3·30	3·30	0·58	0·67	0·63	3·59	0·46
14. xi. 62	3·44	3·59	3·52	0·95	0·98	0·97	3·96	0·45
30. i. 63	3·63	3·58	3·61	0·91	0·95	0·93	3·87	0·28
6. iii. 63	3·51	3·47	3·49	0·96	1·11	1·04	4·13	0·62
31. vii. 63	3·54	3·68	3·61	1·03	1·07	1·05	4·16	0·52
1. xi. 63	3·74	3·64	3·69	1·27	1·24	1·25	3·51	−0·14
9. xii. 63	3·55	3·35	3·45	1·01	1·16	1·09	4·05	0·55
10. xii. 63	3·79	3·46	3·58	1·08	1·71	1·40	5·18	1·14
11. xii. 63	3·53	3·62	3·58	1·16	1·29	1·18	4·28	0·59
13. i. 64	3·66	3·42	3·54	1·26	1·23	1·24	3·72	0·15
13. iii. 64	3·60	3·56	3·58	1·26	1·37	1·32	3·98	0·30
10. iv. 64	3·58	3·72	3·65	1·20	1·50	1·35	4·15	0·37
15. vi 64	3·60	3·47	3·60	1·33	1·24	1·28	3·87	0·21
17. vii. 64	3·55	3·86	3·71	1·33	1·64	1·49	3·69	−0·01
14. viii. 64	3·65	3·79	3·72	1·70	1·20	1·48	3·85	0·08
16. x. 64	3·51	3·85	3·68	1·12	1·25	1·18	3·24	−0·37
20. xi. 64	3·59	3·98	3·79	1·24	1·54	1·39	3·31	−0·35
11. xii. 64	3·60	3·76	3·68	1·30	1·70	1·50	4·07	0·26
5. ii. 65	3·79	3·86	3·83	1·26	1·51	1·38	3·63	−0·14
2. iv. 65	3·96	4·13	4·04	1·52	1·39	1·45	3·59	−0·31
18. vi. 65	4·08	3·83	3·96	1·36	1·45	1·41	4·38	0·02
								$\bar{x}$ 0·36

DISCUSSION

Selection for resistance resulted in a steady increase in the percentage of animals able to survive myxomatosis. The inoculation dose of 500 L.F.U. was high, and it is probable that at a lower dose rate, of say 5 L.F.U., the resistance achieved would be reflected by a much higher percentage recovery. The generation increment of 0·5 of a grade per year was low for an animal such as the rabbit, capable of achieving a generation turnover in 6–12 months. The reduced turnover was caused by the necessity to wait until an animal reached 4 months of age before it could be tested, the further interval while it recovered sufficiently from the disease to be capable of breeding, and the necessity of including in the breeding stock unchallenged selected does.

Determining the heritability of resistance with any degree of accuracy proved impossible. The use of survival time as an index of resistance and its utilization in intra-sire correlation is severely limited by the bimodality of the distribution even at low recovery rates. The method does however reveal an elevated dam component of heritability. This has been interpreted as a 'litter effect' because members of a litter were invariably tested at the same time, and within-litter

variation would be decreased relative to between-litter variation by environmental fluctuations. The limited data available, with overlapping generations, did not allow existing techniques for dealing with an all-or-none character to be used. The estimation of realized heritability by converting percentage survivors into probits used in the present work, while not precise, at least gives an acceptably valid estimate of heritability. The method is affected by the vicissitudes of the environment in that the selection differential is calculated on the basis of animals surviving in one year and the genetic improvement, in part-at-least, on animals surviving in following years. As has been shown (Fig. 2) year-to-year variation in survival is often marked. The accuracy of the above estimate of heritability will increase with the number of tests on which the mean is based and the number of years over which the tests extend.

The degree of genetic resistance to myxomatosis attained by wild rabbits in Australia will vary considerably depending mainly on the number of epizootics experienced. In measuring the genetic resistance the many factors affecting the course of the disease such as strain of virus, dose, route of inoculation, temperature conditions etc. (see Fenner & Ratcliffe (1965) for a detailed review) must be taken into account, particularly if results from different laboratories are to be compared. A recent finding that the age at which rabbits with some genetic resistance are infected is of considerable importance to their chance of recovery (Sobey, unpublished data) adds to the above list of variables. Further, the effects of the variables listed appear to be exaggerated in rabbits with increased genetic resistance. Current investigations are aimed at developing a suitable procedure for comparative studies in assessing the genetic resistance of wild rabbit populations.

SUMMARY

1. Response to selection was achieved with all strains of Myxoma virus used.

2. Heritability of resistance to myxomatosis was determined by intra-sire correlation using survival time as an index and by an all-or-none probit analysis. Both resulted in an estimate of heritability of about 35–40 %.

3. The ability of animals to survive myxomatosis varied widely with environmental variation in time.

I am indebted to W. Menzies for his very able assistance in all aspects of the work; to Dorothy Conolly for her critical assistance with data analysis, and to Dr J. M. Rendel for suggesting the probit analysis for determining heritability.

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