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Immunogenicity of experimental trachoma vaccines in baboons

I. Experimental methods, and preliminary tests with vaccines prepared in chick embryos and in HeLa cells

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INTRODUCTION

Trachoma is a form of chronic follicular conjunctivitis, usually associated with corneal lesions, and with a tendency to cicatrization in the later stages. Untreated, it is liable to impair vision, and is responsible for a vast amount of ophthalmic disability, mainly in tropical and sub-tropical countries. The causal agent is a member of the psittacosis–lymphogranuloma group of obligate intracellular micro-organisms; it was first isolated with certainty by T'ang, Chang, Huang & Wang (1957), who inoculated conjunctival scrapings into the chick embryo yolk sac. It is closely similar to—if not identical with—the agent causing inclusion conjunctivitis and inclusion blennorrhoea, first isolated by Jones, Collier & Smith (1959). For convenience, the trachoma/inclusion conjunctivitis micro-organisms are jointly referred to as TRIC agents (Gear, Gordon, Jones & Bell, 1963) and the strain designations used in this paper follow the system suggested by these authors and by Collier (1963).

Although the earlier stages of trachoma can be treated effectively with sulphonamides and some antibiotics, notably the tetracyclines, the difficulty of applying mass therapy to primitive communities for long periods prompted research into the possibility of developing a preventive vaccine. Collier (1961) showed that baboons are susceptible to conjunctival infection by the MRC–4 strain of inclusion conjunctivitis (formerly known as LB4); and that two spaced subcutaneous doses followed by an intravenous injection of a live vaccine prepared from this strain conferred virtually complete protection against challenge by the conjunctival route. Replacement of the final intravenous dose by a third subcutaneous injection conferred partial immunity.

The researches described here are an extension of this work, and are part of a collaborative investigation of trachoma vaccine by the Medical Research Council's Trachoma Research Unit, Pfizer Ltd., Evans Medical Ltd., and the Lister Institute of Preventive Medicine, under the auspices of the National Research Development Corporation.

The first two papers in this series deal with experimental methods, and with studies by the M.R.C. Trachoma Research Unit on live vaccines prepared in

chick embryos or in HeLa cells; the third describes collaborative experiments by the Unit and by Pfizer Ltd. with inactivated vaccines. The methods of challenge, clinical and microbiological examinations and scoring of results were devised by the Unit, and were identical in both laboratories.

MATERIALS AND METHODS

General plan of experiments

Groups of young baboons were inoculated with various TRIC vaccines, and challenged, usually 10 days after the final dose, by rubbing live TRIC agent into the upper and lower conjunctival sacs of one eye (Collier, 1961). The course of the subsequent infection was compared with that in control animals challenged similarly.

TRIC agents

The trachoma and inclusion conjunctivitis strains used are shown in Table 1. In the text, 'fast-killing' variants that kill chick embryos comparatively quickly and grow readily in cell cultures (Reeve & Taverne, 1963) are distinguished from their 'slow-killing' parent strains by the suffix *f* (Taverne, Blyth & Reeve, 1964*a*).

Table 1. *Designations of trachoma and inclusion conjunctivitis (TRIC) agents*

Full designation	Abbreviation	Reference
TRIC/ /GB/MRC–1/G (formerly LB 1)	MRC–1/G	Jones, Collier & Smith, 1959
TRIC/2/SAU/HAR–2/OT (formerly SA 2)	SAU/HAR–2	Murray *et al.* 1960
TRIC/ /WAG/MRC–221/OT	MRC–221	Collier, Sowa, Sowa & Blyth, 1963
TRIC/ /GB/MRC–4/ON (formerly LB 4)	MRC–4	Jones, 1961; Jones & Collier, 1962

It should be noted that strain MRC–4 used for the vaccine experiments was isolated from the eye of an English baby with neonatal inclusion conjunctivitis; inoculated into the eye of an adult volunteer it induced a syndrome indistinguishable from trachoma (Jones & Collier, 1962). The course of infection in the baboon conjunctiva has been described in detail (Collier, 1961, 1962).

Yolk sac vaccines

Yolk sac vaccines described in this and the subsequent two papers were suspensions of heavily infected membranes collected soon after death of the embryos. They were partially purified by centrifugation, using methods similar to those of Collier (1961), and were finally suspended in phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954) or in sucrose potassium glutamate (SPG) (Bovarnick, Miller & Snyder, 1950); 1–5 ml. of antigen were obtained from each gram (wet weight) of yolk sac. The vaccines were stored in 1 ml. amounts in ampoules at $-60°$ C. or $-70°$ C. until required.

HeLa cell vaccines

The seed material was a suspension of MRC–4 *f* that had been passaged 18 times in chick embryos, and then 3–8 times in HeLa cells.

HeLa cells were grown in Roux bottles with 50 ml. of medium (Hanks' saline containing 10% calf serum, 5% human serum, 0·5% lactalbumen hydrolysate and streptomycin 100 μg./ml.). Monolayers were seeded with a dilution of MRC–4 *f* that formed inclusions in 80–100% of cells; 42–55 hr. later the cells were suspended in their medium by shaking with glass beads, deposited by centrifugation at 8000 *g* for 20 min., and resuspended in one-eighth of the original volume with SPG containing 10% human serum. They were then treated for 5 min. with ultrasonic vibrations from a Dawe disintegrator with a power output of 60 W. at a frequency of 20 kc./sec. (Furness & Fraser, 1962). After centrifugation at 8000 *g* for 20 min., the deposits were finally suspended in SPG containing streptomycin at 500 μg/ml. All manipulations, including centrifugation and ultrasonic treatment, were done at 0–4° C. One Roux bottle yielded 0·5–1·0 ml. of vaccine; each batch was prepared on the day of use.

Challenge inocula

Inocula were crude or partially purified yolk sac suspensions in PBS or SPG, and were held at −60° C. until used.

Titrations of infectivity and lethality in chick embryos

Serial tenfold dilutions of TRIC suspensions were made in PBS or SPG, and each inoculated in 0·3 ml. amounts into the yolk sacs of at least five 7-day chick embryos. Specificity of death was confirmed by examining Giemsa stained yolk sac smears for elementary bodies. Titres were calculated by Thompson's (1947) method in terms of infectivity (EID50/ml.) or lethality (ELD50/ml.).

Infectivity titrations in HeLa cells

The method of Furness, Graham & Reeve (1960) was used; titres are expressed as inclusion forming units (IFU) per ml.

Complement fixation tests

Serum antibodies fixing complement with psittacosis–lymphogranuloma–trachoma (PLT) group antigen were titrated on Perspex plates similar to those described by Fulton & Dumbell (1949). Sera from baboons immunized with yolk-sac antigens were tested with HeLa cell antigens and vice versa.

Blood samples were taken from the femoral vein; sera were stored at 4° C. with 0·25% sodium azide as preservative. To remove anticomplementary activity, sera were diluted 1/5 on the day of test with 1/10 Richardson's preserved complement and incubated at 37° C. for 60 min.; they were then inactivated at 56° C. for 30 min.

Yolk sac group antigen. A 20% suspension of yolk sacs heavily infected with the MRC–1/G strain of TRIC agent was centrifuged at low speed to remove coarse

particles; it was then treated with an equal volume of 2M-KCl and centrifuged at 8000 *g* for 30 min. to remove adventitious protein, which was discarded with the supernatant. The deposited elementary bodies were suspended in 1 ml. calcium-magnesium saline (Reeve & Taverne, 1962) for each yolk sac used, and heated at 100° C. for 20 min. After adding sodium azide to a final concentration of 0·25%, the antigen was stored at 4° C. Control antigen was prepared in the same way from normal yolk sacs.

HeLa cell group antigen was made from cells heavily infected with the SAU/HAR–2*f* strain by a method similar to that used for HeLa cell vaccine, except that the diluent was PBS containing 5% sodium glutamate, and, after heating at 100° C. for 20 min., the final suspension was freeze-dried for storage.

Both yolk sac and HeLa cell antigens were used at optimally effective concentrations determined by titrating them against ascending dilutions of known positive sera.

Complement. Richardson's preserved complement was diluted to contain two minimal haemolytic doses (MHD) per unit volume used in the test.

Diluents. Sera were diluted in calcium–magnesium saline, and antigen and complement in calcium-magnesium saline containing 2% inactivated human serum.

Haemolytic system. Sheep red cells were thrice washed and diluted to 0·25% with 0·85% saline. They were sensitized for 5 min. at room temperature with an equal volume of haemolysin diluted to contain 10 MHD per unit volume used in the test.

Antibody titrations. The unit volume was 0·02 ml. dispensed by dropping pipettes. One vol. each of serum, complement and antigen were added in this order to the appropriate squares on the Perspex plates, which were then left at 4° C. overnight in a moisture saturated atmosphere. On the following day 2 vols. of sensitized sheep cells were added to each square, and the plates were put in the humidity box at 37° C. for 60 min. The serum titre was taken (by interpolation when necessary) as the highest dilution giving 50% lysis. Appropriate controls for all reagents were included in each test.

Baboons

Young baboons (*Papio cynocephalus*) of either sex weighing 3–4 kg. were used. All manipulations were done under general anaesthesia induced by giving 15–20 mg. phencyclidine hydrochloride ('Sernylan', Parke Davis and Co. Ltd.) intramuscularly.

Conjunctival inoculations, and examinations for inclusion bodies

The methods of Collier (1961) were used, except that checking of negative or doubtful conjunctival scrapings by a second examiner was omitted; it was found that the very small number of false negatives revealed by this check did not justify the considerable extra work involved.

Scoring system for assessing the severity of response to conjunctival challenge

The use of a scoring system for assessing the severity of conjunctival infection was suggested by Dawson, Jawetz, Thygeson & Hanna (1961). In our experiments arbitrary numerical scores, which are amenable to statistical analysis, were allotted to the various physical signs induced by the challenge. Scores for signs of inflammation and for follicular hyperplasia were recorded separately, since these lesions appear to be expressions of different pathological processes.

Signs of inflammation comprise (*a*) external oedema of the lids, (*b*) purulent discharge, (*c*) conjunctival hyperaemia and (*d*) conjunctival infiltration (recognized by loss of transparency, oedema and thickening). Each of these signs was allotted a score of 1, 2 or 3 according to severity, 0 if absent. Conjunctival hyperaemia and infiltration were scored separately for the upper and lower lids.

Follicular hyperplasia was scored separately for upper and lower lids, using the same scale as that for inflammation. Superficial translucent follicles characteristic of 'non-specific folliculosis' were sometimes present in normal animals and were ignored (Collier, 1961.)

Inclusion bodies. The presence of inclusions in whatever numbers was allotted the maximum score of 3, since their finding constitutes an important sign of the specificity of infection.

Table 2 shows how individual scores were derived, and is an example of a severe infection.

Table 2. *Example of use of scoring system for baboons infected with TRIC agents by the conjunctival route*

(This example refers to one examination of one animal.)

Physical sign		Score	
Slight external oedema	Upper lid	1	Total score for signs of inflammation = 10
Moderate discharge		2	
Severe hyperaemia		3	
Moderate infiltration		2	
Moderate hyperaemia	Lower lid	2	
No infiltration		0	
Moderate follicular hyperplasia, upper lid		2	Total score for follicular hyperplasia = 3
Slight follicular hyperplasia, lower lid		1	
Inclusion bodies present (upper and/or lower lid)			= 3
			Total score = 16

Analysis of scores

In most experiments, the animals were examined 1, 2, 3, 4 and 6 weeks after challenge. The cumulative score for each animal for all examinations up to and including the 4th week after challenge is the basis for interpreting the results, and, except when otherwise stated, comprises four successive examinations. By the 6th week spontaneous regression of physical signs diminished the differences between control and vaccinated animals, and inclusion of the scores at this final examination yielded no additional information.

For each group the square roots of the cumulative scores for each animal up to

and including the 4th week were summed and divided by the number of animals in the group to give the mean score in terms of square roots. Using the square roots of the scores, significance of differences between immunized and control animals in their response to challenge was tested by the analysis of variance. In the tables of results, the least significant difference at the level $P = 0{\cdot}05$ is given for comparison with the actual differences.

Examination of the range of the transformed scores indicated a slightly higher overall variability in the immunized groups than in the control groups, and suggested looking for individual differences in response within groups of immunized animals. This was done by making an estimate of error from all experiments and calculating the least significant difference at the $P = 0{\cdot}05$ level between the transformed score for single animals and the mean of the transformed scores for control animals in the same experiment. These differences were added and subtracted from the corresponding mean score for the control group, and the resulting limits transformed back to the original score, to give 95% confidence limits to the scores of individual animals. Thus individual scores smaller than the lower limit suggested protection by the vaccine; and those greater than the upper limit suggested an enhanced response to challenge.

DOSE OF TRIC AGENT USED FOR CHALLENGE

A strain of trachoma agent (MRC–221) and one of inclusion conjunctivitis (MRC–4) were each titrated in parallel in the baboon conjunctiva and in the chick embryo yolk sac. Both strains had been propagated exclusively in the yolk sac in which they had the growth characteristics of 'slow-killing' strains. MRC–221 was used at the 5th passage; the MRC–4 suspension was a pool of 2nd and 3rd passage membranes. Serial tenfold dilutions were each inoculated into groups of 5 or 6 chick embryos, and into 3–5 baboons. For the baboon inoculations, cotton-wool throat swabs were soaked in the diluted suspensions and rubbed across the conjunctival surfaces of the upper and lower lids of one eye, using twelve strokes for each lid. It was estimated that each animal received approximately 0·1 ml.; the infective titre of each of the undiluted suspensions was $10^{5{\cdot}0}$ EID50/ml., so that animals inoculated with a 1/10 dilution received approximately 1000 EID50.

With the higher dilutions, some animals had slight physical signs unaccompanied by formation of inclusion bodies; in others, scanty inclusions were found in the complete absence of physical signs. With MRC–4, the less dilute suspensions induced more severe infections and inclusions were more frequently found; this effect was less obvious with MRC–221. For the purpose of these experiments, induction of specific infection was assumed only when inclusions were demonstrable at some stage of infection. The numbers of animals infected with various dilutions of each strain are given in Table 3. The estimates of 50% end-points are open to the objection that the highest dilutions of both strains induced infection in one or more animals, and that the lowest dilution of MRC–221 failed to infect all the animals in the group. Assuming however that the next dilutions higher than those actually used would have failed to infect any animals, and that

undiluted MRC–221 suspension would have infected the whole group, estimates of 50% end-points (Reed & Muench, 1938) gave values of $10^{3\cdot8}$/ml. and $10^{4\cdot0}$/ml. respectively for MRC–221 and MRC–4. Thus for both strains, one baboon infectious dose (BID 50) is roughly equivalent to 10–20 EID 50. These results may be compared with those of Dawson *et al.* (1961); about 3 ELD 50 (egg lethal doses) of trachoma strain BOUR were equivalent to a minimal 'inclusion producing dose' in cynomolgus monkeys, whereas the corresponding figure for trachoma strain ASGH was more than 1000 ELD 50. Bell, Murray, Carroll & Snyder (1963) found that for the SAU/HAR–2 strain of trachoma the infectivity end-point for chick embryos approximated closely to that for the human eye.

Table 3. *Parallel titrations of TRIC agents in baboon conjunctivae and in chick embryo yolk sacs*

Strain	Dilution	No. infected / Total	Titre* BID 50/ml.	Titre EID 50/ml.	BID 50 / EID 50
MRC–221	10^{-1}	3/4	$10^{3\cdot8}$	$10^{5\cdot0}$	16
	10^{-2}	3/5			
	10^{-3}	3/5			
	10^{-4}	1/3			
MRC–4	10^{-1}	5/5	$10^{4\cdot0}$	$10^{5\cdot0}$	10
	10^{-2}	4/5			
	10^{-3}	3/5			

* Assuming 0·1 ml used to infect each animal.
BID 50 = 50% baboon infective dose.
EID 50 = 50% egg infective dose.

The findings with our strains suggest that to induce satisfactory infection with reasonable certainty in all animals of a given control group it is necessary to use an inoculum of at least 1000 EID 50, and this was done in the vaccine experiments to be described. Challenge of this order is undoubtedly more severe than anything likely to be encountered with naturally occurring infections, but the use of more dilute inocula would entail an impractically large number of baboons in order to demonstrate significant differences in behaviour between vaccinated and control groups.

IMMUNIZATION WITH LIVE AQUEOUS VACCINES

Antigen prepared in yolk sac

Experiment 2: immunization and challenge with strain MRC–4

The first experiment in this series was described by Collier (1961). Experiment 2 was also reported in part, but since the scoring system was not then in use, and additional information about the duration of immunity has since been gained, it is now given in greater detail.

The vaccine was made from yolk sacs infected with the 5th egg passage of MRC–4; its infective titre was $10^{5\cdot2}$ EID 50/ml. The vaccination schedule is given in Table 4; for subcutaneous inoculations, 0·5 ml. was injected into the thoracic aspect of each axilla, with the object of stimulating more than one group of lymph nodes.

The challenge inoculum was a partially purified pool of 2nd and 4th passage MRC–4 with an infective titre of $10^{4\cdot9}$ EID50/ml., and was inoculated into the right eyes of all animals by the conjunctival route 10 days after the final dose of vaccine.

Table 4. *Experiment 2: vaccination with aqueous suspension of live MRC–4 grown in yolk-sac: challenge with MRC–4*

No. of baboons	Day vaccinated	Dose of vaccine	Mean score ($\sqrt{}$) at 24 days after challenge	Difference from mean score ($\sqrt{}$) of control group	L.S.D.† ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
6	0, 7, 14	1·0 ml. s.c., 1·0 ml. i.v.	1·41	−4·04	1·65	6/6
6	0, 7, 14	1·0 ml. s.c.	3·18	−2·27	1·65	3/6
6	—	No vaccine	5·45	—	—	—

95 % confidence limits on scores for individual vaccinated animals: upper = 62, lower = 9.
* That is, with individual scores of 9 or less.
† L.S.D. = least significant difference.

Response to conjunctival challenge

By way of example, individual scores for animals in one of the vaccinated groups and for the controls at each examination are given in Table 5 (but for reasons of space are not shown for subsequent experiments). Table 4 gives the transformed mean scores for vaccinated and control groups, and the least significant differences between them; it also shows the number of animals in the vaccinated groups deemed to have been protected against conjunctival challenge.

Two successive subcutaneous doses of live MRC–4 followed by an intravenous dose almost completely protected all six baboons against challenge with the homologous strain; physical signs were slight or absent, and scanty inclusions were found in only one animal 46 days after challenge (not shown in Table 5). By contrast, the unvaccinated animals all had severe or moderately severe conjunctival infection, and all were inclusion-positive at two or more examinations. Of the six baboons receiving three successive subcutaneous doses, three were protected to a significant degree.

Rechallenge after 15 months

Fifteen months after vaccination, the surviving animals were challenged in their left eyes with a pool of 3rd and 4th passage MRC–4 containing $10^{4\cdot0}$ EID50/ml.

Table 6 shows that the immunity in both vaccinated groups had by now waned considerably. Only one baboon of those receiving an intravenous dose still resisted challenge to a significant extent, even though the challenge dose was 10 times less on this occasion. One of the three animals originally protected by three subcutaneous injections died in the interim period; the remaining two failed to resist the second challenge.

Table 5. *Experiment 2: individual scores of animals immunized with two subcutaneous and one intravenous dose of MRC–4 (group A) and unvaccinated controls (group B)*

Baboon No.	Lesion	Days after challenge 0	4	9	17	24	Cumulative totals
		Group A					
29	I*	0	0	0	0	0	0
	F	0	0	0	0	0	0
	IB	0	0	0	0	0	0
	Totals	0	0	0	0	0	0
30	I	0	0	0	0	0	0
	F	0	0	0	0	0	0
	IB	0	0	0	0	0	0
	Totals	0	0	0	0	0	0
31	I	0	3	0	0	0	3
	F	0	0	0	0	1	1
	IB	0	0	0	0	0	0
	Totals	0	3	0	0	1	4
32	I	0	4	1	0	0	5
	F	0	0	2	1	0	3
	IB	0	0	0	0	0	0
	Totals	0	4	3	1	0	8
33	I	0	3	0	2	0	5
	F	0	0	2	0	0	2
	IB	0	0	0	0	0	0
	Totals	0	3	2	2	0	7
34	I	0	0	0	1	0	1
	F	0	0	0	0	0	0
	IB	0	0	0	0	0	0
	Totals	0	0	0	1	0	1
		Group B					
41	I	0	13	9	6	1	29
	F	0	2	1	2	4	9
	IB	0	3	3	3	0	9
	Totals	0	18	13	11	5	47
42	I	0	0	0	2	2	4
	F	0	0	0	0	1	1
	IB	0	3	3	0	0	6
	Totals	0	3	3	2	3	11
43	I	0	4	4	4	3	15
	F	0	0	0	2	1	3
	IB	0	3	3	0	3	9
	Totals	0	7	7	6	7	27
44	I	0	3	2	2	0	7
	F	0	0	2	2	2	6
	IB	0	0	3	3	3	9
	Totals	0	3	7	7	5	22
45	I	0	7	7	5	2	21
	F	0	0	2	2	3	7
	IB	0	3	3	3	0	9
	Totals	0	10	12	10	5	37
46	I	0	6	5	8	7	26
	F	0	0	0	2	3	5
	IB	0	3	3	3	3	12
	Totals	0	9	8	13	13	43

* I = Signs of inflammation; F = follicular hyperplasia; IB = inclusion bodies.

Table 6. *Experiment 2: rechallenge with MRC–4, 15 months after vaccination*

No. of baboons	Original vaccination	Mean score ($\sqrt{}$) at 28 days after challenge	Difference from mean score ($\sqrt{}$) of control group	L.S.D. † ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
6	2 × 1·0 ml. s.c. 1 × 1·0 ml. i.v.	3·00	−1·29	1·14	1/6
5	3 × 1·0 ml. s.c.	3·88	−0·41	1·18	0/5
4	None	4·29	—	—	—

95 % confidence limits on scores for individual vaccinated animals: upper = 46, lower = 3.
* That is, with individual scores less than 3.
† L.S.D. = least signicant difference.

Table 7. *Experiment 2: comparison of complement-fixing antibody titres with conjunctival responses to challenge*

Baboon nos.	Route of vaccination		Reciprocal CF titres: days after 1st dose of vaccine 24*	48	473	Cumulative score at 28 days after challenge
29	2 s.c. + 1 i.v. dose		320	160	20	0
30			640	80	< 10	0
31			1280	640	10	4
32			250	320	20	8
33			1280	160	< 10	7
34			1280	640	< 10	1
		Means†	690	254	4	3·3
35	3 s.c. doses		1280	2560	20	2
36			1280	640	< 10	4
37			80	< 10	ND	7
38			450	640	< 10	21
39			120	640	< 10	11
40			640	320	< 10	26
		Means	407	245	4	11·8
41	No vaccine		< 5	40	ND	47
42			< 5	< 10	ND	11
43			< 5	20	< 10	27
44			< 5	160	30	22
45			< 5	20	< 10	37
46			< 5	ND	ND	43
		Means	< 5	19	3	31·2

* That is, on day of challenge.
† Means of antibody titres are geometric. ND = Not done.

Serological response to vaccination and challenge

Before vaccination, sera from all the animals used in this experiment failed at a dilution of 1/5 to fix complement with group antigen. Ten days after the final dose, i.e. on the day of first challenge, the antibody titres in the group receiving two subcutaneous and one intravenous dose ranged from 1/250 to 1/1280, and were on the average somewhat higher than in baboons given three subcutaneous doses (Table 7). By the sixth week after vaccination, serum titres in most of the vaccin-

ated animals had fallen 2- to 8-fold, but in some the titres had increased slightly. The finding at this time of small amounts of antibody in the control animals suggests that the increases were due to the stimulus of challenge; similar responses were observed in human volunteers inoculated with trachoma by the conjunctival route (Collier, Duke-Elder & Jones, 1958; 1960). By the time the second challenge was given, 15 months later, little or no complement fixing antibody was found in either vaccinated or control animals.

Table 7 also shows that there was no obvious inverse correlation between the formation of complement fixing antibody and the conjunctival response to challenge as measured by individual cumulative scores.

Antigen prepared in HeLa cells

Experiment 3*: immunization with strain MRC–4* f*: challenge with MRC–4*

Although antigens grown in HeLa cells could not be used in man, the use of vaccines prepared from other cell cultures is a possibility; at present, only 'fast-killing' variants of TRIC agents can be readily propagated in cell cultures and, accordingly, this experiment was done to determine whether MRC–4 *f* grown in HeLa cells retained the immunogenicity of its parent strain. The minimum dose necessary to confer immunity was also investigated.

The vaccines were freshly prepared on the day of use as described under 'Materials and Methods'. On day 0, one group of five baboons received 0·5 ml. of undiluted vaccine subcutaneously into each axilla; this was repeated on day 7. On day 14, 1 ml. was given intravenously. The same schedule was followed for two more groups, which received respectively vaccine diluted 1/10 and 1/100 on each occasion. The undiluted vaccines contained respectively $10^{8\cdot0}$, $10^{7\cdot9}$ and $10^{8\cdot2}$ IFU/ml. and 167, 208 and 300 mg. total nitrogen per 100 ml.

Reactions to vaccination. A week after the first subcutaneous dose, all the baboons receiving undiluted vaccine had nodules approximately 2·5 cm. in diameter at the sites of injection; two of those receiving 1/10 vaccine had smaller nodules, but there were no local reactions in baboons inoculated with 1/100 vaccine. The nodules all regressed without necrosis during the following month.

The challenge inoculum prepared from the fourth yolk sac passage of MRC–4 had an infective titre of $10^{5\cdot9}$ EID 50/ml., and was inoculated into the right eyes of both vaccinated and control animals 11 days after the final intravenous dose of vaccine.

Response to conjunctival challenge

The total score for the group receiving undiluted vaccine was significantly less than that for the controls (Table 8), and two of out five animals showed convincing evidence of protection in terms of their individual scores. By contrast, the scores of animals receiving 1/10 vaccine did not differ significantly from those of the controls; nor was there evidence of protection in animals receiving 1/100 vaccine, one of whom had a score significantly higher than the upper 95% confidence limit for this experiment.

Serological response to vaccination

Before vaccination, sera from all the animals used in this experiment failed at a dilution of 1/5 to fix complement in the presence of group antigen. Table 9 shows that the mean antibody titres in animals receiving respectively 1/1 and 1/10 vaccine did not differ significantly; the mean serum titre of those given 1/100 vaccine was somewhat lower. As in the previous experiments, there was no relation between the titre of circulating antibody at the time of challenge and the severity of infection induced by conjunctival inoculation.

DISCUSSION

Methods for testing trachoma vaccines were recently reviewed by Collier (1966), who pointed out that none of the characteristics that can be assayed in the laboratory has yet been correlated with the results of field trials; and that since only primates appear to be susceptible to conjunctival inoculation with TRIC agents, experiments of the sort described here are, short of field trials in man, the most direct method available of ascertaining immunogenicity in relation to conjunctival infection. Nevertheless, the size and scope of such experiments are limited by expense and by the difficulty of handling large numbers of primates. To derive valid conclusions from comparatively small numbers of monkeys or baboons, in which there may be substantial individual variation in response, the clinical and microbiological examinations must be conducted in a uniform manner, and the results interpreted by a method that gives some assurance of objectivity.

Statistical analysis of the results of Expt. 2, previously reported in part by Collier (1961), confirmed that live MRC–4 grown in the yolk sac confers immunity to conjunctival challenge with a heavy dose of the homologous strain; and that in terms of the proportion of animals protected, 2 subcutaneous and 1 intravenous dose were more effective than 3 subcutaneous doses. However, a second challenge 15 months later showed that the immunity induced shortly after vaccination had largely disappeared even though the infective titre of the challenge inoculum was 10 times less than that on the first occasion. In this connexion, it is noteworthy that in trachomatous children the diminution in the severity of disease induced by vaccination is also short-lived (Collier *et al.* 1963).

Yolk-sac vaccines tested in simians by other workers usually consisted of inactivated TRIC agent, and will be discussed in the third paper of this series. There are, however, a few reports of the use of live vaccines in monkeys. For example, Grayston *et al.* (1960) contend that 4 successive doses of vaccine made from strain TW–29 modified the response to challenge with TW–10 given 2 weeks after the final dose; the route of immunization is not stated. Dawson *et al.* (1961) immunized small groups of rhesus monkeys with crude yolk-sac suspensions of various TRIC agents; they gave three intramuscular injections at weekly intervals, each containing approximately $10^{6\cdot0}$ to $10^{7\cdot7}$ ELD 50/ml. In assessing the results, scores were assigned to the degree of follicular hyperplasia induced by challenge. By this criterion, and from examinations for inclusion bodies, they concluded that

Table 8. *Experiment* 3*: vaccination with aqueous suspensions containing graded doses of live MRC–4* f *grown in HeLa cells: challenge with MRC–4*

No. of baboons	Dilution of vaccine	Mean score ($\sqrt{}$) at 28 days after challenge	Difference from mean score ($\sqrt{}$) of control group	L.S.D.† ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
5	1/1	3·28	−1·62	1·59	2/5
5	1/10	4·10	−0·80	1·59	0/5
6	1/100	5·27	+0·37	1·51	0/6††
6	No vaccine	4·90	—	—	—

95% confidence limits on scores for individual vaccinated animals: upper = 53, lower = 6.
* That is, with individual scores of 6 or less.
† L.S.D. = least significant difference.
†† One animal had a significantly high score (67).

Table 9. *Experiment* 3*: comparison of complement-fixing antibody titres with conjunctival responses to challenge*

Baboon nos.	Dilution of vaccine	Reciprocal CF titre 25 days after 1st dose of vaccine*	Cumulative score at 28 days after challenge
228	1/1	640	10
229		1280	24
230		640	15
231		1280	4
232		1280	6
		Means† 971	11·8
234	1/10	640	7
235		1280	35
236		640	14
237		1280	9
238		1280	27
		Means 971	18·4
240	1/100	1280	67
241		1280	19
242		640	21
243		1280	22
244		320	27
245		80	21
		Means 570	29·5
246	No vaccine	< 10	17
247		< 10	28
248		< 10	22
249		< 10	13
250		< 10	32
251		< 10	36
		Means < 10	24·7

* That is, on day of challenge
† Means of antibody titres are geometric.

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strains BOUR, ASGH, Apache–1 and IC–Cal–1 modified the response to challenge with a small dose of BOUR given 8 days after the final dose of vaccine, but did not induce solid immunity.

Until now, there have been no reports of the use of TRIC vaccines prepared in cell cultures. Recent findings (Graham, 1965) suggest that the 'fast-killing' variant strains differ from their parent 'slow-killing' strains in the degree of cross-protection they give against pulmonary infection of mice; nevertheless, the 'fast-killing' strain MRC–4*f* used for immunization in Expt. 3 induced immunity against its parent strain in two of five monkeys receiving undiluted vaccine. This vaccine was not titrated in chick embryos, but had an average titre of about 10^8 inclusion-forming units/ml. Taverne, Blyth & Reeve (1964*b*) showed that for *f* strains the infectivity titre in HeLa cells approximates to the 50% lethal dose in chick embryos (which for these strains is similar to the 50% infective dose) and that only about ten elementary bodies of *f* strains constitute one ELD50 for chick embryos; but with 'slow-killing' (*s*) strains, like that used for preparing the yolk sac vaccine, the ratio of total particles to infectivity is likely to be approximately 5000:1. Thus although the undiluted HeLa cell vaccine contained about 1000 times more infective TRIC agent than the yolk sac material and, by inference, a similar or greater number of elementary bodies, it was less effective in terms of immunogenicity against MRC–4. In an experiment to be described later, a HeLa cell vaccine with an infective titre approximately 10 times higher than that of the vaccines used in the tests reported here protected all of six baboons against conjunctival challenge with the parent *s* strain.

The serological results suggest that the serum titre of PLT group complement fixing (CF) antibody is not directly related, if it is related at all, to the conjunctival response to infection. Similar findings have been reported by others; thus Bietti, Guerra, Felici & Vozza (1962) and Khaw *et al.* (1963) found no correspondence between the titres of CF antibodies in vaccinated human volunteers and the course of conjunctival infection after challenge with TRIC agent. Collier *et al.* (1963) observed that the beneficial effect of vaccination on a proportion of children with active trachoma was unrelated to CF antibody titre. It is noteworthy that Blyth, Reeve, Graham & Taverne (1962) concluded from studies on rabbits immunized with TRIC agent that 'production of antibody fixing complement with group antigen does not parallel that of neutralizing antibody'. Nevertheless, they found that sera with low titres of CF antibody were unlikely to contain much neutralizing antibody. The animals in our experiments were not tested for neutralizing antibody; but in these and other experiments to be reported subsequently, it is interesting that a number of baboons that were protected against conjunctival challenge had comparatively low titres of CF antibody.

Our observation that CF antibodies induced by vaccination do not persist for long confirms the findings of Grayston *et al.* (1963) and Collier *et al.* (1963) in children given experimental trachoma vaccines.

SUMMARY

Parallel titrations of a strain of trachoma (MRC–221) and one of inclusion conjunctivitis (MRC–4) in the baboon conjunctiva and in chick embryos suggest that ten to twenty 50% egg infective doses are equivalent to one 50% baboon infective dose; but that at least 1000 egg infective doses are needed to induce moderate or severe infections in all of a given number of baboons.

For vaccine experiments in baboons, a system of scoring physical signs and presence of inclusion bodies was devised; the significance of differences in vaccinated and control animals in their response to conjunctival challenge was determined by analysis of variance. An aqueous suspension of live MRC–4 grown in the yolk sac was given as two subcutaneous doses and one intravenous dose at weekly intervals, and protected all of six baboons challenged with the homologous strain; three similarly spaced subcutaneous doses were less effective. The immunity induced by this vaccine waned considerably during the ensuing 15 months. Vaccine prepared from a live 'fast-killing' variant of MRC–4 grown in HeLa cells was less effective than MRC–4 itself in protecting baboons against infection with the parent strain.

Although both yolk sac and HeLa cell vaccines induced the formation of antibody fixing complement with trachoma group antigen, the serum titres in individual animals at the time of challenge were unrelated to the degree of protection; during a 15 month observation period there were pronounced falls in the titres of antibody induced either by vaccination or by challenge with egg-grown TRIC agent.

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