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Immunogenicity of experimental trachoma vaccines in baboons.

II. Experiments with adjuvants, and tests of cross-protection

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INTRODUCTION

In the preceding paper (Collier & Blyth, 1966) we showed that although aqueous suspensions of strain MRC–4 grown in the chick embryo yolk sac protected baboons against conjunctival challenge shortly after vaccination, little or no immunity was demonstrable a year or so later; the investigation of adjuvants reported here was undertaken in an attempt to improve the immunogenicity of the vaccine.

As well as duration of immunity, an important consideration in devising a trachoma vaccine is the extent of cross-protection between different strains. In baboons, several trachoma agents proved insufficiently pathogenic for use as conjunctival challenge (Collier, 1961, 1962); but a few strains, notably MRC–187. were later found to induce moderately severe infections, and a full cross-protection test was made with this strain and the MRC–4 strain of inclusion conjunctivitis. MRC–187 was also used to test the immunity induced by an oil adjuvant trachoma vaccine against challenge with the homologous strain.

The trachoma/inclusion conjunctivitis (TRIC) agents used in these experiments are shown in Table 1. Except where otherwise stated, the experimental and statistical methods are those described previously (Collier & Blyth, 1966).

Table 1. *Designation of trachoma agents used in Experiments* 4–9

Full designation	Abbreviation	Reference
TRIC/ /WAG/MRC–17/OT (formerly G 17)	MRC–17	Sowa & Collier, 1960
TRIC/ /WAG/MRC–1/OT (formerly G 1)	MRC–1/OT	Collier & Sowa, 1958
TRIC/ /WAG/MRC–187/OT	MRC–187	—
TRIC/ /WAG/MRC–062/OT	MRC–062	—

EXPERIMENTS WITH MINERAL OIL ADJUVANTS

Experiments 4–7 were parallel tests of the immunogenicity of 'aqueous' vaccines and mineral oil adjuvant vaccines prepared from them.

Aqueous vaccines were partially purified suspensions of live TRIC agent in phosphate buffered saline (PBS) (Dulbecco & Vogt, 1954) or in sucrose potassium glutamate (SPG) (Bovarnick, Miller & Snyder, 1950). They were stored in 1·0 ml.

amounts at −60° C. until required. Their characteristics are summarized in Tables 2 and 4.

Adjuvant vaccines. Nine volumes of light mineral oil (Drakeol 6 VR, Pennsylvania Refinery Company) were mixed with 1 volume of an emulsifying agent, mannide monooleate (Arlacel A, Atlas Powder Company); the mixture was sterilized by filtration at 7 lb./sq.in. positive pressure through a Gradocol membrane (A.P.D. 0·6 μ). On the day of use, 1 vol. aqueous vaccine, thawed rapidly from −60° C., was added to 1 vol. Drakeol/Arlacel A mixture; except in Expt. 7 (see below) the vaccine was emulsified in an M.S.E. blender at 2000 rev./min. for 3 min.

Table 2. *Aqueous yolk-sac vaccines used for Experiments* 4–6

	Experiment no.		
	4	5	6
Strain	MRC–187	MRC–187	MRC–062
Chick embryo passage	11	6	4
Infectivity titre ($\log_{10}$ EID50/ml.)	4·4	4·8	5·2
Total particle count ($\log_{10}$/ml.)	9·4	9·8	11·9
Reciprocal titre of complement-fixing group antigen	ND	1280	512
Total nitrogen (mg./100 ml.)	31·5	216·0	ND

EID50 = 50% egg infective dose.
ND = not done.

at 4° C. To ensure that a true water-in-oil emulsion had formed, vaccines were subjected to the 'drop test', whereby a drop of this type of emulsion retains its integrity when expelled on to the surface of cold water. They were also tested for stability on horizontal centrifugation at 380 ***g*** for 10 min.

Challenge inocula were aqueous suspensions of 'slow-killing' TRIC agents (Reeve & Taverne, 1963) grown in the yolk sac.

Experiment 4*: vaccination with live MRC*–187 *grown in yolk sac; challenge with MRC*–187

Aqueous vaccine. On days 0 and 7, five baboons were given 0·5 ml. subcutaneously into the thoracic surface of both axillae; an intravenous dose of 1·0 ml. was given on day 14.

Adjuvant vaccine. On day 0, five baboons were each given 0·25 ml. intramuscularly into each buttock. The total dose of 0·50 ml. was equivalent to 0·25 ml. of aqueous vaccine.

Control vaccines. Three animals received dummy aqueous vaccine, and three more were given dummy adjuvant vaccine, prepared from normal yolk sac and given in the dosages used for the vaccines proper.

Challenge. On day 24, all baboons were challenged by conjunctival inoculation of MRC–187 in its 11th yolk sac passage, with an infectivity titre of $10^{6·7}$ EID50/ml.

Results

The scores for both control groups were similar, and are considered together in Table 3, which shows that neither aqueous nor adjuvant vaccine exerted a protective effect; indeed, two animals receiving adjuvant vaccine had high scores suggesting an enhanced response to challenge.

Table 3. *Experiment* 4*: vaccination with aqueous and mineral oil suspensions of live MRC*–187 *grown in yolk sac: challenge with MRC*–187

Nos. of baboons	Vaccine	Mean score (√) at 29 days after challenge	Difference from mean score (√) of control group	L.S.D.‡ ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
5	Aqueous	3·71	−0·60	0·98	0/5
5	Adjuvant	5·89	+1·58	0·98	0/5†
6	Normal yolk sac	4·31	—	—	—

95% confidence limits on scores for individual vaccinated animals: upper, 45; lower, 3.
* That is, with individual scores of 3 or less.
† Two animals had significantly high scores (45 and 48).
‡ L.S.D. = least significant difference.

Experiment 5*: vaccination with live MRC*–187 *grown in yolk sac: challenge with MRC*–187

The vaccines and challenge inoculum were similar to those used in Expt. 4, except that the antigen was made from the 6th chick embryo passage (Table 2).

Aqueous vaccine was given to six baboons as $2 \times 0{\cdot}5$ ml. doses subcutaneously on days 0, 8 and 14, followed by 0·5 ml. intravenously on day 38.

Adjuvant vaccines were given to six baboons in a single intramuscular dose of 0·5 ml. (equivalent to 0·25 ml. aqueous vaccine) on day 0.

Controls. As in the previous experiment, two groups of three baboons received respectively dummy aqueous and adjuvant vaccines prepared from normal yolk sac.

Challenge. In Expt. 4, the failure of the adjuvant vaccine to protect might have been due to premature challenge before immunity had developed; in Expt. 5, challenge was delayed until day 70. The suspension of MRC–187 used was 11th yolk sac passage material with a titre of $10^{6{\cdot}3}$ EID50/ml.

Results

There was again no evidence of protection either by aqueous or by adjuvant vaccine; on this occasion no vaccinated animals had scores significantly higher than those of the controls.

Experiment 6*: vaccination with live MRC*–062 *grown in yolk sac; challenge with MRC*–4

Aqueous vaccine was given intramuscularly to five baboons on days 0, 14 and 28; each dose of 1·0 ml. was given as 0·5 ml. into each buttock.

Adjuvant vaccine was given to two groups each of five baboons. One group was given $2 \times 0{\cdot}5$ ml. intramuscularly on day 0, as for aqueous vaccine; the other received a second similar dose on day 28. This vaccine was serially diluted in SPG

and titrated in chick embryos; allowing for its two-fold dilution, the titre of $10^{4.7}$ EID50/ml. was close to that of the aqueous vaccine (Table 2), and showed that there was no inactivation of TRIC agent during the emulsifying process.

Control animals received no vaccine.

Challenge. All animals were inoculated on day 71 with pooled 2nd and 5th passage MRC–4. The titration in chick embryos of this material was unsatisfactory, but it induced infection of adequate severity in the baboon conjunctiva.

Results

For all the vaccinated animals, the group scores were higher than for the controls, but not significantly so. One animal receiving two doses of adjuvant vaccine had an individual score significantly higher than the upper 95% confidence limit for this experiment.

Rechallenge after 11 months

The baboons that had received two doses of adjuvant vaccine and the control group were again challenged 11 months after the first dose of vaccine to determine whether late immunity had developed. The inoculum was MRC–4 in its 4th yolk sac passage with an infectivity titre of $10^{5.9}$ EID50/ml. There were again no significant differences between the scores of the vaccinated and control animals.

Experiment 7: vaccination with live MRC–4 f grown in HeLa cells; challenge with MRC–4

The object of this experiment was to determine whether the rather disappointing result with aqueous HeLa cell vaccine (Collier & Blyth, 1966) could be improved by using a higher concentration of MRC–4 *f* or by incorporating a mineral oil adjuvant. It was done before Expts. 4–6, and made use of a different method for preparing adjuvant vaccine.

Aqueous vaccines were prepared from heavily infected HeLa cell monolayers (Collier & Blyth, 1966). On days 0 and 8, six baboons each received $2 \times 0{\cdot}5$ ml. subcutaneously, given as in Expt. 4, and 1·0 ml. intravenously on day 15. The characteristics of the vaccines, which were made freshly on each occasion, are given in Table 4.

Adjuvant vaccines were prepared by repeatedly expelling the mixed oil and aqueous phases from a 10 ml. syringe fitted with a no. 1 needle. The first use of this method resulted in an unstable oil-in-water (O/W) emulsion, which was nevertheless given intramuscularly to a group of six baboons; on day 0 each received 0·5 ml. of adjuvant vaccine prepared from batch 1 into each buttock.

On day 8 a fresh lot of adjuvant vaccine was prepared by the syringe method from batch 2 of the aqueous suspension; this time a stable water-in-oil (W/O) emulsion resulted, and was inoculated intramuscularly ($2 \times 0{\cdot}5$ ml.) into six more baboons.

Controls. On day 8 six baboons were each given $2 \times 0{\cdot}5$ ml. of a dummy W/O vaccine made from uninfected HeLa cells, containing 145 mg. total nitrogen per 100 ml.

Challenge. On day 40 all animals were challenged in their right eyes with MRC–4 in its 3rd yolk-sac passage; the infectivity titre was $10^{4\cdot1}$ EID50/ml.

Table 4. *Aqueous vaccines used for Experiment 7: prepared from live MRC–4 f grown in HeLa cells*

	Batch no.		
	1	2	3
Infectivity titre			
(*a*) $\log_{10}$EID50/ml.	9·0	9·3	8·6
(*b*) $\log_{10}$IFU/ml.	8·7	9·7	9·1
Total particle count ($\log_{10}$/ml.)	10·1	10·3	10·2
Reciprocal titre of complement-fixing group antigen	2560	2560	ND
Total nitrogen (mg./100 ml.)	267	834	334

EID50 = 50 % egg infective dose.
IFU = inclusion-forming units.
ND = not done.

Table 5. *Experiment 7: vaccination with aqueous and mineral oil suspensions of live MRC–4 f grown in HeLa cells; challenge with MRC–4*

No. of baboons	Vaccine	Mean score (√) at 28 days after challenge	Difference from mean score (√) of control group	L.S.D.† (P = 0·05)	No. protected* / No. vaccinated
6	Aqueous	2·26	−3·61	1·60	6/6
6	Adjuvant (oil-in-water)	4·00	−1·87	1·60	2/6
6	Adjuvant (water-in-oil)	4·21	−1·66	1·60	3/6
6	No vaccine	5·87	—	—	—

95 % confidence limits on scores for individual vaccinated animals: upper, 68; lower, 12.
* That is, with individual scores of 12 or less.
† L.S.D. = least significant difference.

Results

All the baboons given aqueous vaccine were protected to a significant extent against conjunctival challenge with MRC–4, but the adjuvant vaccines were less effective (Table 5). The oil-in-water suspension protected only two of six animals, and the correctly prepared water-in-oil emulsion was little better, since it failed to protect half the baboons receiving it.

Rechallenge after 11 months

Eleven months after the 1st challenge, the left eyes of all surviving animals were inoculated with the 3rd yolk-sac passage of MRC–4; the infectivity titre was $10^{4\cdot2}$ EID50/ml. None of the vaccinated animals resisted the second challenge and immunity was not prolonged by using a mineral oil adjuvant (Table 6).

Table 6. *Experiment 7: rechallenge with MRC–4, 11 months after vaccination*

No. of baboons	Vaccine	Mean score (√) at 28 days after challenge	Difference from mean score (√) of control group	L.S.D.† ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
5	Aqueous	4·84	+0·25	1·68	0/5
4	Adjuvant (oil-in-water)	4·50	−0·09	1·79	0/4
6	Adjuvant (water-in-oil)	5·42	+0·83	1·60	0/6
6	No vaccine	4·59	—	—	—

95% confidence limits on scores for individual vaccinated animals: upper, 49; lower, 4.
* That is, with individual scores of 4 or less.
† L.S.D. = least significant difference.

Table 7. *Experiments with mineral oil adjuvants: titres of complement-fixing antibody at times of challenge*

Expt. no.	Vaccine	Strain	Mean CF titre* at time of: 1st challenge	2nd challenge
4	Aqueous	MRC–187	< 5	—
	Adjuvant	MRC–187	15	—
	Aqueous	Normal yolk sac	< 5	—
	Adjuvant	Normal yolk sac	< 5	—
5	Aqueous	MRC–187	39	—
	Adjuvant	MRC–187	36	—
	Aqueous	Normal yolk sac	0	—
	Adjuvant	Normal yolk sac	3	—
6	Aqueous	MRC–062	2	ND
	Adjuvant (1 dose)	MRC–062	26	ND
	Adjuvant (2 doses)	MRC–062	92	67
	None	MRC–062	< 5	2
7	Aqueous	MRC–4 *f*	2370	113
	Adjuvant (oil-in-water)	MRC–4 *f*	4560	640
	Adjuvant (water-in-oil)	MRC–4 *f*	3225	508
	None	MRC–4 *f*	7	58

* Reciprocal of geometric mean titre of antibody fixing complement with group antigen.
ND = not done.

Serological findings in Experiments 4–7

Tests for psittacosis–lymphogranuloma–trachoma (PLT) group complement-fixing (CF) antibody were made as described by Collier & Blyth (1966). The sera of animals immunized with yolk sac vaccines were tested with antigen prepared in HeLa cells, and yolk sac antigen was used for testing sera from animals immunized with HeLa cell vaccine. Table 7 gives the geometric mean titres of sera from vaccinated and control groups at the time of challenge. None of the aqueous yolk

sac vaccines prepared from strains MRC–187 or MRC–062 induced high titres of CF antibody, and little advantage was gained by using mineral oil adjuvant, although a second dose of adjuvant vaccine (Expt. 6) resulted in a somewhat higher mean titre.

The HeLa cell vaccines prepared from MRC–4 *f* (Expt. 7) induced circulating CF antibody in high titres. The mineral oil suspensions performed somewhat better than the aqueous vaccine, but the difference was not pronounced; it is interesting that the physical state of the emulsion had little influence in this respect.

Sera from the baboons receiving two doses of adjuvant vaccine (Expt. 6) were again tested 11 months after vaccination, and still contained small amounts of antibody. Over a similar period there were pronounced falls in titre in the baboons given HeLa cell vaccine (Expt. 7); and whereas the mean titre in the group given aqueous vaccine fell by a factor of 20, there was a 65- to 70-fold fall in both the groups vaccinated with mineral oil suspensions.

In none of these experiments was there any relation between the CF titres in individual baboons at the time of challenge and their conjunctival responses.

TESTS OF CROSS-PROTECTION, AND USE OF PRECIPITATED ANTIGENS

Experiments 8 and 9 were made primarily to determine whether two more trachoma agents, MRC–17 and MRC–1/OT, would protect against challenge with the inclusion conjunctivitis strain MRC–4. The opportunity was also taken to test the immunogenicity of TRIC agents precipitated either by protamine (Chambers & Henle, 1941) or by calcium phosphate.

Experiment 8: vaccination with live MRC–17 grown in yolk sac: challenge with MRC–4

Aqueous vaccine. Twenty-four grams (wet weight) of heavily infected yolk sacs were shaken in 320 ml. of PBS for 30 min. at 4° C. The suspension was strained through several layers of surgical gauze to remove gross solid material and divided into two equal parts, one of which was partially purified and concentrated by differential centrifugation to give a final volume of 30 ml. aqueous vaccine in SPG.

Protamine-precipitated (Pr-P) vaccine. The remaining 160 ml. of crude suspension was mixed with an equal volume of Seitz-filtered 2% (w/v) protamine sulphate, and stood at 4° C. for 10 min. The resulting precipitate was deposited by horizontal centrifugation at 170 *g.* for 10 min., and resuspended in 30 ml. SPG

Both vaccines were sealed in 1·2 ml. amounts in ampoules and stored at −70° C. until required. The infectivity titre of the aqueous vaccine ($10^{3·6}$ EID 50/ml.) was lower than usual in these experiments, and a further loss in titre took place as a result of protamine treatment (Table 8).

Vaccination. Two groups each of six baboons were vaccinated respectively with aqueous and Pr–P vaccine; subcutaneous and intravenous injections were given according to the schedule for aqueous vaccine in Expt. 4. Six unvaccinated baboons served as controls.

Challenge. The inoculum was a pool of 2nd and 4th passage MRC–4 grown in the yolk sac, with an infectivity titre of $10^{4\cdot9}$ EID 50/ml. It was administered on day 24, i.e. 10 days after the final dose of vaccine.

Table 8. *Vaccines used for Experiments* 8 *and* 9

Experiment no.	8		9	
Strain	MRC–17		MRC–1/OT	
Chick embryo passage	48		32	
Vaccine	Aqueous	Protamine	Aqueous	Phosphate
Infectivity titre ($\log_{10}$ EID50/ml)*	3·6	3·0	5·8	4·9

* EID50 = 50 % egg infective dose.

Table 9. *Experiment* 8*: vaccination with suspensions of live MRC–*17 *grown in yolk sac: challenge with MRC–*4

No. of baboons	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
6	Aqueous suspension	3·98	− 1·83	1·28	2/6
6	Protamine-precipitated antigen	4·44	− 1·37	1·28	2/6
6	No vaccine	5·81	—	—	—

95 % confidence limits on scores for individual vaccinated animals: upper, 67; lower, 11.
* That is, with individual scores of 11 or less.
† L.S.D. = least significant difference.

Results

There was no difference between the aqueous and Pr–P vaccines in terms of immunogenicity (Table 9). Both were partially effective in that they each protected 2/6 baboons against challenge with MRC–4.

Experiment 9*: vaccination with live MRC–*1*/OT grown in yolk sac: challenge with MRC–*4

Aqueous vaccine. Forty-nine grams (wet weight) of infected yolk sacs were shaken with 120 ml. of PBS and strained through gauze as described for Expt. 8. The crude suspension was centrifuged at 140 ***g***. for 10 min. to remove coarse particles; the elementary bodies were then deposited by angle centrifugation at 11,000 ***g*** for 30 min, at 4° C. Half the deposit resuspended in 50 ml. SPG constituted the aqueous vaccine, which was stored at − 70° C. in 1·2 ml. amounts.

Phosphate-precipitated (Ph-P) vaccine. The use of calcium-phosphate columns for purifying viruses was suggested by Taverne, Marshall & Fulton (1958). In our experiments, the brushite form of the salt ($CaHPO_4 . 2H_2O$) was prepared by the method of Tiselius, Hjertén & Levin (1956) and washed in 0·2 M phosphate buffer, pH 7·0. The remaining half of the angle deposit was suspended in 15 ml. of the

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same buffer, to which were added 15 ml. freshly precipitated calcium phosphate. After standing at room temperature for 15 min., with stirring at 5 min. intervals, the mixture was centrifuged at 170 *g* for 5 min.; the deposit was retained, and the supernatant was treated twice more with calcium phosphate; the total amount used was 44 g., equivalent to 4 g. per yolk sac. The final supernatant was discarded; the pooled deposits (44 ml.) were made up to 60 ml. with SPG, and stored in 1·2 ml. amounts at −70° C. During purification, progressive removal of elementary bodies from successive supernatant fluids was verified by examining Castañeda-stained smears.

The infectivity titre of the Ph-P vaccine was about 10 times less than that of the aqueous material (Table 8).

Vaccination. The aqueous vaccine was given to six baboons according to the schedule used for Expt. 4, except that the final intravenous injection was given on day 21. The Ph-P vaccine was also given to six baboons on days 0, 7 and 21, but as three subcutaneous doses each of 2·0 ml. (equivalent to 1·0 ml. of aqueous vaccine). The six control animals received no vaccine.

Challenge. The inoculum was a pooled suspension of the 2nd and 3rd yolk sac passages of MRC-4, with an infectivity titre of $10^{5\cdot0}$ EID 50/ml.; it was inoculated on day 31, 10 days after the final dose of vaccine.

Results

Neither vaccine protected against challenge with MRC–4. One animal receiving the aqueous suspension appeared to have an enhanced response to challenge (Table 10).

Table 10. *Experiment* 9*: vaccination with suspensions of live MRC–*1*/OT grown in yolk sac; challenge with MRC–*4

No. of baboons	Vaccine	Mean score (√) at 20 days after challenge	Difference from mean score (√) of control group	L.S.D.‡ ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
6	Aqueous suspension	4·52	+0·55	1·27	0/6†
6	Phosphate-precipitated antigen	5·04	+1·07	1·27	0/6
6	No vaccine	3·97	—	—	—

95 % confidence limits on scores for individual vaccinated animals: upper, 41; lower, 2.
* That is, with individual scores of 2 or less.
† One animal had a significantly high score (43).
‡ L.S.D. = least significant difference.

Experiment 10*: cross-protection test with live MRC–*4 f *and MRC–*187 *grown in the yolk sac*

The live aqueous vaccines were partially purified by centrifugation, and further purified by treatment with molar KCl as described by Collier & Blyth (1966) for the preparation of group antigen. The infectivity titre of the MRC–4 *f* suspension

was originally $10^{6.9}$ EID50/ml.; it was diluted 20-fold to make it comparable in this respect with the MRC–187 vaccine (Table 11). After dilution, its total particle count was 100 times less than the MRC–187 suspension.

Vaccination. Both vaccines were given subcutaneously to groups of baboons on days 0 and 7, and intravenously on day 14 by the method described in Expt. 4. Groups A and B received MRC–4*f*, and groups C and D were given MRC–187 vaccine. Two control groups (E and F) were left unvaccinated.

Challenge. The MRC–4 inoculum was prepared from the 5th yolk sac passage, and contained $10^{4.3}$ EID50/ml. The MRC–187 challenge consisted of the vaccine itself diluted to this infectivity titre. On day 24 groups A, C and E were challenged with MRC–4, and groups B, D and F with MRC–187; all inoculations were made into the right eyes.

Table 11. *Vaccines used for Experiment* 10

	Strain	
	MRC–4*f*	MRC–187
Chick embryo passage	19	12
Infectivity titre ($\log_{10}$ EID50/ml.)	5·6	5·7
Total particle count ($\log_{10}$/ml.)	8·1	10·2

EID50 = 50% egg infective dose.

Table 12. *Experiment* 10*: cross-protection test with live MRC–*4 f *and MRC–*187 *grown in yolk sac*

Group	No. of baboons	Vaccine strain	Mean score (√) at 28 days after challenge	Difference from mean score (√) of control group	L.S.D.† ($P = 0{\cdot}05$)	No. protected* / No. vaccinated
		(*a*) Challenge with MRC–4				
A	5	MRC–4*f*	3·48	−2·21	1·65	3/5
C	5	MRC–187	4·63	−1·06	1·65	0/5
E	5	No vaccine	5·69	—	—	—
		(*b*) Challenge with MRC–187				
B	5	MRC–4*f*	3·99	−1·64	1·75	1/5
D	5	MRC–187	3·71	−1·92	1·75	2/5
F	4	No vaccine	5·63	—	—	—

95% confidence limits on scores for individual vaccinated animals (*a* and *b*): upper, 66; lower, 10.

* That is, with individual scores of 10 or less.

† L.S.D. = least significant difference.

Results

In terms of the mean group scores, the severity of infection induced in the controls by both challenges was very similar (Table 12). MRC–4*f* protected 3 of 5 baboons challenged with MRC–4, but only 1 of 5 challenged with MRC–187; and whereas MRC–187 vaccine immunized 2 of 5 baboons against challenge with the homologous strain, it failed completely against challenge with MRC–4.

Rechallenge after 10 *months*

All surviving animals were challenged in their left eyes with the appropriate TRIC agents; the MRC–4 inoculum was made from the 6th yolk-sac passage, and had an infectivity titre of $10^{5\cdot0}$ EID50/ml.; the MRC–187 challenge was prepared from the 13th yolk-sac passage, and contained $10^{4\cdot3}$ EID50/ml.

Results

As in previous experiments, any immunity originally induced by either vaccine was no longer demonstrable (Table 13).

Table 13. *Experiment* 10*: rechallenge* 10 *months after vaccination*

Group	No. of baboons	Vaccine strain	Mean score (√) at 28 days after challenge	Difference from mean score (√) of control group	L.S.D.† (P = 0·05)	No. protected* / No. vaccinated
		(*a*) Challenge with MRC–4				
A	5	MRC–4*f*	5·61	+0·77	1·17	0/5
C	5	MRC–187	5·75	+0·91	1·17	0/5
E	3	No vaccine	4·84	—	—	—
		(*b*) Challenge with MRC–187				
B	5	MRC–4*f*	5·27	−0·14	1·17	0/5
D	5	MRC–187	5·88	+0·47	1·17	0/5
F	4	No vaccine	5·41	—	—	—

95 % confidence limits on scores for individual vaccinated animals. (*a*) Upper, 58; lower, 4. (*b*) Upper, 67; lower 7.

* That is, with individual scores of (*a*) 4 or less; (*b*) 7 or less.

† L.S.D. = least significant difference.

Serological results in Experiments 8–10

Table 14 gives the mean serum titres of CF antibody in vaccinated and control groups, tested on the day of challenge. None of the aqueous yolk-sac vaccines prepared from strains MRC–17, MRC–1/OT, MRC–4*f* or MRC–187 induced high titres of CF antibody. In Expt. 10 there was a pronounced difference between groups A and B in terms of mean serum titre; the reason is not clear, since both groups received the same vaccine in identical dosage. Eleven months later, however, the titres in both groups had fallen to the same low values.

The amounts of CF antibody elicited by protamine-precipitated and by untreated aqueous MRC–17 vaccines were similar; but the phosphate-precipitated MRC–1/OT vaccine induced higher CF titres than did its aqueous counterpart. Again, there was no relation between individual CF antibody response and the course of conjunctival infection after challenge.

DISCUSSION

Whereas successive subcutaneous and intravenous doses of an aqueous suspension of MRC–4*f* protected all of six baboons against challenge with MRC–4, 1·0 ml. of adjuvant vaccine given intramuscularly was less effective, and a water-

in-oil emulsion performed little better than an oil-in-water preparation (Expt. 7). By contrast with MRC–4 (Collier & Blyth, 1966), strain MRC–187 proved to be a comparatively poor immunogen, even when given as an aqueous suspension and tested against challenge with the homologous strain; and combination with oil adjuvant failed to improve it. Oil adjuvant also failed to render MRC–062 immunogenic against challenge with MRC–4.

Table 14. *Tests of cross-protection, and of precipitated antigens; titres of complement-fixing antibody at times of challenge*

Expt. no.	Vaccine	Strain	Mean CF titre* at time of: 1st challenge	2nd challenge
8	Aqueous	MRC–17	28	—
	Protamine-precipitated		34	—
	None		1	—
9	Aqueous	MRC–1/OT	10	—
	Phosphate-precipitated		160	—
	None		2	—
10	Group A	MRC–4 *f*	14	7
	Group B		147	8
	Group C	MRC–187	2	4
	Group D		3	5
	Group E	None	< 5	16
	Group F		< 5	2

* Reciprocal of geometric mean titre of antibody fixing complement with group antigen.

In the absence of any adjuvant effect, the comparatively poor performance of the mineral oil vaccines was probably due to their being given in lower dosage than the aqueous suspensions, and by a different route. Emulsification with oil and Arlacel did not seem to affect the antigen itself, at least in terms of viability (Expt. 6). There was no evidence that the poor results with oil adjuvant were due to premature challenge; and, as judged by a second challenge 11 months later, there was no indication that baboons successfully immunized with adjuvant vaccine retained their immunity longer than those protected by aqueous suspensions.

As well as being a poor immunogen, MRC–187 induced little or no circulating CF antibody; MRC–062 also failed in this respect. Since the vaccines were comparable in infectivity titre with a yolk sac vaccine made from MRC–4 which induced high CF titres (Collier & Blyth, 1966), it seems that MRC–062 and MRC–187 are inherently incompetent at inducing formation of PLT group CF antibody. The addition of mineral oil adjuvant was of little value in this respect, and did not prolong the persistence of circulating antibody. The MRC–4 *f* vaccine prepared in HeLa cells induced higher titres than the MRC–4 yolk sac vaccine previously described; this was probably due to its greater content of antigen.

The lack of relationship between CF antibody response and the course of infection following challenge confirms our previous findings (Collier & Blyth, 1966).

Our observation that mineral oil adjuvant failed to increase the immunogenicity of TRIC antigens agrees with that of Snyder *et al.* (1964), who found that whereas an aqueous vaccine inactivated with formalin significantly diminished the trachoma attack rate in Saudi Arabian children, the same batch of suspension emulsified with Drakeol 6 and Arlacel A did not. By contrast, Grayston, Wang, Woolridge & Alexander (1964) found that mineral oil adjuvant improved the immunogenicity of a trachoma vaccine tested in monkeys against challenge with the homologous strain. They maintain that mineral oil behaves as an adjuvant only if the vaccine contains at least 10^8–10^9 elementary bodies, a condition that was met in our own experiments. Khaw *et al.* (1963) contend that mineral oil adjuvant prolonged the CF antibody response to trachoma vaccine in human volunteers, but they made no direct comparison with aqueous vaccine prepared from the same suspension. They also stated that adjuvant vaccine modified the response of volunteers to conjunctival challenge, without however reducing the infection rate. Like us, they found no relationship between CF antibody titre and severity of response to conjunctival challenge. From the results of field trials in Ethiopia, Bietti (1964) claims that a single dose of oil adjuvant vaccine had no effect, but that an additional dose of aqueous vaccine 45 days later diminished the trachoma attack rate in healthy subjects, and favourably modified the course of established trachoma. There is therefore no general agreement about the value of oil adjuvants for trachoma vaccines. The conflicting reports suggest that much more remains to be learnt about the methods of making and administering preparations of this nature; and in particular, the mass of antigen required, its degree of purity and the influence of particle size remain to be determined.

In the cross-protection tests, MRC–17 afforded better protection against conjunctival challenge with MRC–4 than did an MRC–1/OT vaccine with a higher infectivity titre; the challenge dose of MRC–4 used in both experiments (nos. 8 and 9) was similar. In these experiments, both protamine sulphate and calcium phosphate were used primarily in attempts to purify and concentrate the antigen; it was hoped that calcium phosphate might also act as an adjuvant, but it proved disappointing in this respect.

In the cross-protection experiment with MRC–4 *f* and MRC–187 (Expt. 10) the vaccines were made comparable in terms of infectivity titre, but it should be noted that the MRC–187 vaccine contained 100 times more elementary bodies. The challenge inocula prepared respectively from MRC–4 and MRC–187 had the same infectivity titres and induced infections of comparable severity in the control animals. MRC–4 *f* protected 3 of 5 animals against challenge with MRC–4, but only 1 of 5 against MRC–187; and whereas MRC–187 vaccine conferred partial protection against challenge with the homologous strain, it failed completely to protect against MRC–4. These findings may be supplemented with data from the other experiments. For example, the failure of MRC–4 *f* to protect all the animals challenged with MRC–4 was probably due to insufficient dosage (cf. Expt. 7). This also seems to explain the failure of aqueous MRC–187 vaccines to protect against homologous challenge in Expts. 4 and 5, since a vaccine with a rather higher infectivity titre and total particle count protected 2 of 5 baboons in Expt. 10.

It is not yet clear whether the infectivity titre or the total particle count is the more important factor in determining the critical dose of live vaccine necessary to confer protection. Apart from considerations of dosage, however, there are clear indications of antigenic differences between the TRIC agents used in these tests. Thus the Gambian trachoma strains MRC–1/OT and MRC–17 differed in their ability to protect against a strain of inclusion blennorrhoea (MRC–4); for a given dose of live vaccine, MRC–1/OT was less effective than the homologous strain in protecting against MRC–4 (cf. expt. 2, Collier and Blyth, 1966); and the findings in Expt. 10 suggest that there is an antigenic difference between MRC–4 *f* and MRC–187.

Antigenic differences between PLT agents can be demonstrated by the mouse toxin protection test (Bell & Theobald, 1962; Wang & Grayston, 1963) and by immunofluorescence (Hanna & Bernkopf, 1964; Nichols & McComb, 1964). None of the combinations of strains used in our experiments have been tested by these methods; but Grayston *et al.* (1964) obtained evidence suggesting that the serological grouping elicited by the mouse toxin test is reflected by the results of cross-protection tests in the monkey conjunctiva.

The results of the second challenges in Expts. 7 and 10 confirmed our previous observation that the immunity induced in baboons by aqueous vaccines is of comparatively short duration.

The serological findings in Expts. 8 and 10 confirmed that MRC–187 does not readily call forth group complement-fixing antibodies, and showed that two more Gambian strains, MRC–17 and MRC–1/OT, resemble it in this respect; but the addition of calcium phosphate to the MRC–1/OT vaccine resulted in rather higher antibody titres. In Expt. 10, the antibody response to aqueous MRC–4 *f* vaccine was much less than in Expt. 7, probably because of the smaller dose of antigen. Again, there was no relation between individual antibody titres at the time of challenge and response to conjunctival inoculation.

In Expts. 4, 6 and 9, three baboons receiving mineral oil adjuvant vaccines and one given aqueous vaccine had significantly enhanced responses to conjunctival challenge administered comparatively soon after vaccination. This reaction seems to be induced only by relatively ineffective vaccines; it has not been observed in baboons challenged a second time a year or so after vaccination. It appears that vaccines that fail to confer solid protection may induce instead a short-lived state of hypersensitivity; and that, like immunity, this state is unrelated to the titre of circulating CF antibody. Similar findings are reported by Grayston, Woolridge & Wang (1962) who found that in monkeys large single doses of a trachoma vaccine combined with aluminium hydroxide adjuvant resulted in an enhanced response to conjunctival challenge, whereas repeated small doses without adjuvant afforded partial protection. This hypersensitivity was ascribed to the TRIC agent rather than to egg material in the vaccine. By contrast, no such reaction was seen in vaccinated children who subsequently acquired trachoma. Grayston (1963) also reported that pannus developed in a proportion of monkeys after conjunctival inoculation with TRIC agents. This lesion was observed only in animals previously inoculated with TRIC or psittacosis agents by the parenteral or conjunctival

routes, and was also ascribed to hypersensitivity. To the best of our knowledge, no corneal lesions developed in any of our baboons, but minor degrees of pannus might well have escaped detection under the low magnification used for the clinical examinations.

SUMMARY

A mineral oil adjuvant failed to enhance or to prolong the somewhat short-lived immunity induced in baboons by trachoma/inclusion conjunctivitis (TRIC) vaccines, and was of little or no value in increasing or prolonging the formation of group complement-fixing (CF) antibody. Vaccines prepared with protamine sulphate or with calcium phosphate were no more immunogenic than the untreated parent suspensions.

Cross tests with aqueous vaccines revealed antigenic differences in the TRIC agents examined, in terms of their ability to protect against conjunctival challenge. Strain MRC–1/OT differed from MRC–17 and from MRC–4, and MRC–187 from MRC–4*f*.

The four Gambian trachoma agents tested were much less effective than the MRC–4 strain of inclusion conjunctivitis in inducing group CF antibody in baboons. The titre of circulating antibody bore no relation to the state of immunity to conjunctival challenge.

In three baboons given mineral oil adjuvant vaccine and in one given aqueous vaccine the conjunctival responses to subsequent challenge were significantly enhanced. This reaction may be an expression of a hypersensitive state induced by relatively ineffective vaccines; like immunity, it is unrelated to the titre of CF antibody in the serum.

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