J. Hyg., Camb. (1967), **65**, 79
Printed in Great Britain

Immunogenicity of experimental trachoma vaccines in baboons

III. Experiments with inactivated vaccines

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(*Received* 19 *November* 1966)

INTRODUCTION

In preceding papers (Collier, 1961; Collier & Blyth, 1966*a*, *b*) it was demonstrated that baboons can be immunized against ophthalmic infection with trachoma/inclusion conjunctivitis (TRIC) agents by parenteral injection of live vaccines. Compared with inactivated vaccines, such preparations have disadvantages; the control of contaminants is more difficult, and unless kept at very low temperatures they rapidly lose viability, a serious drawback under field conditions. Live vaccines have been administered without ill effects to numerous baboons in the laboratory, and to some hundreds of children in pilot field trials; but the recent finding by Collier & Smith (1967) that at least one strain of TRIC agent can disseminate and multiply in primates after parenteral injection dictates caution in the further use of live trachoma vaccine, at least until more is known about the nature of such artificially induced infections. In the present paper we describe experiments with vaccines inactivated by heat, formalin and ultraviolet light, made in an attempt to avoid the disadvantages of live preparations. Experiments P 1, P 2 and P 3 were done at the Pfizer Laboratories, except that the ultraviolet irradiation in Expt P 3 was undertaken by one of us (L.H.C.) at the Lister Institute, Elstree, in collaboration with Mr L. Vallet. Experiment 11 is the last of the Trachoma Research Unit series to be described in these three papers.

MATERIALS AND METHODS

Except where otherwise stated, the materials and experimental procedures are those of Collier & Blyth (1966*a*).

TRIC agent. Strain TRIC/ /GB/MRC-4/ON (Jones, 1961; Jones & Collier, 1962), referred to for brevity as MRC-4, was used throughout. All vaccines were prepared from its 'fast-killing' variant MRC-4*f* (Reeve & Taverne, 1963); their characteristics and those of the challenge inocula are given in Table 1. MRC-4 and

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MRC-4*f* used for vaccines and challenge inocula were propagated exclusively in the chick embryo yolk sac. In the first two papers of this series, titres were expressed in terms of 50% egg infective doses (EID 50). Here they are given as 50% egg lethal doses (ELD 50), which for MRC-4*f* approximates closely to the EID 50, and for MRC-4 is usually half to one $\log_{10}$ unit lower.

Table 1. *Characteristics of vaccines prepared from MRC-4f and challenge inocula*

	Experiment no.				
Vaccines	P 1	11	P 2	P 2 (re-challenge)	P 3
Chick embryo passages ...	6	6	8	—	11
Titre ($\log_{10}$ ELD 50/ml.)	3·8	7·0*	5·8	—	5·3
Challenge inocula					
Strain	MRC-4	MRC-4	MRC-4*f*	MRC-4*f*	MRC-4*f*
Chick embryo passage	2+5 pooled	2+4 pooled	4	3	7
Titre ($\log_{10}$ ELD 50/ml.)	N.D.	3·8	N.D.	2·8	3·8

* Estimated by extrapolation; highest dilution tested killed all chick embryos.
N.D. = not done.

Table 2. *Experiment P 1: vaccination with live or heat-inactivated MRC-4f; 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
8	Live	2·95	−2·67	1·04	4/8
8	Heated	5·08	−0·54	1·04	0/8
7	No vaccine	5·62	—	—	—

95% confidence limits on scores for individual vaccinated animals: upper = 67; lower = 9.
L.S.D. = least significant difference.
* That is, with individual scores of 9 or less.

HEAT- AND FORMALIN-INACTIVATED VACCINES

Experiment P 1: vaccination with live or heat-inactivated MRC-4f; challenge with MRC-4

Vaccine was prepared by differential centrifugation of heavily infected yolk sacs. Part of the suspension was used as a live vaccine, and was kept at −70° C. until use. The remainder was inactivated at 37° C. for 5 days; absence of residual live TRIC agent was confirmed by two negative blind passages in chick embryo yolk sacs.

Vaccinations. Live vaccine was given to eight baboons, each of which received 1·0 ml. subcutaneously on days 0 and 6, and 1·0 ml. intravenously on day 13. An identical schedule was used for vaccinating a second group of eight baboons with inactivated suspension. Seven control animals received no vaccine.

Challenge. All animals were challenged in their right eyes on day 55, i.e. 42 days after the final intravenous dose.

https://doi.org/10.1017/S0022172400045575 Published online by Cambridge University Press

Results

Whereas the live vaccines protected 4 out of 8 animals, heating at 37° C. for 5 days completely destroyed immunogenicity (Table 2).

Rechallenge after 18 *months.* The animals given live vaccine were rechallenged with a suspension of MRC-4*f* containing $10^{2\cdot8}$ ELD 50/ml.; as in previous similar experiments, they were no longer immune to conjunctival infection.

Experiment 11: *vaccination with formalin-inactivated MRC-4f; challenge with MRC-4*

Vaccine was prepared from chick embryo yolk sacs by differential centrifugation. The final suspension was treated with ultrasonic vibrations to ensure even dispersion; formalin was added to a final concentration of 0·1 % (v/v) (0·04 % formaldehyde) and the vaccine was kept at 4° C. for 46 hr., after which undiluted 0·3 ml. samples were inoculated into five chick embryos. No TRIC agent was detected in them, or in two subsequent blind yolk-sac passages. The inactivated vaccine was held at 4° C. until use; there was an interval of 34 days between the addition of formalin and the start of immunization. The titre of an untreated sample kept at 4° C. for 46 hr. was approximately 10^7 ELD 50/ml. (Table 1).

Vaccination. Six baboons each received 1·0 ml. subcutaneously on days 0 and 7, and 1·0 ml. intravenously on day 14. The six control animals received no vaccine.

Challenge with a suspension of MRC-4 (Table 1) was given 10 days after the final intravenous dose of vaccine.

Results

The mean score for the vaccinated group was somewhat higher than that for the control animals, but not significantly so; in terms of individual scores none of the vaccinated baboons gave evidence of immunity.

By contrast with live vaccines prepared from MRC-4*f* (Collier & Blyth, 1966*a*, *b*), the formalin-inactivated material induced little or no group complement-fixing (CF) antibody.

Experiment P 2: *vaccination with live or formalin-inactivated MRC-4f; challenge with MRC-4f*

Vaccine was prepared by differential centrifugation of yolk sacs infected with MRC-4*f* (Table 1); it contained 16 mg. total nitrogen per 100 ml. Half the suspension was used as live vaccine, and was stored in ampoules at −70° C. until use. To the remainder, formalin was added to give a final concentration of 0·2 % formaldehyde; inactivation was allowed to proceed at 4° C. for 5 days, during which period the suspension was twice treated with ultrasonic vibrations to facilitate penetration of formalin. Residual free formaldehyde was then titrated by a method similar to that of MacFadyen (1945); about half the quantity originally added was detectable, and was neutralized with an appropriate quantity of sodium bisulphite. The absence of live TRIC agent was confirmed by three

negative blind passages in chick embryos. The inactivated vaccine was stored at −70° C. until use.

Vaccinations. Five baboons each received 1 ml. of live vaccine subcutaneously on days 0 and 7, and 1 ml. intravenously on day 14. The same schedule was used to vaccinate six more animals with inactivated vaccine; and a third group of six baboons was given three 1·0 ml. doses of inactivated vaccine intramuscularly at weekly intervals. Seven unvaccinated baboons were used as controls.

Challenge. Seven days after the final dose of vaccine, all animals were challenged in their right eyes with MRC-4 in its third yolk-sac passage, but this inoculum failed to induce infection. Fourteen days later (21 days after the final dose) the animals were successfully challenged with a suspension of MRC-4*f* (Table 1).

Table 3. *Experiment P* 2: *vaccination with live or formalin-inactivated MRC-4f; challenge with MRC-4f*

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	Live	0·40	—†	—†	4/5
6	Formalin inactivated 2 × 1·0 ml. s.c., 1 × 1·0 ml. i.v.	2·43	−1·88	1·59	2/6
6	3 × 1·0 ml. i.m.	2·91	−1·40	1·59	0/6
7	Normal yolk sac	4·31	—	—	—

95 % confidence limits on scores for individual vaccinated animals: upper = 47, lower = 3.
L.S.D. = Least significant difference.
* That is, with individual scores of 3 or less.
† Four animals had zero scores, which are not included in the overall analysis of variance because of statistical difficulties. However, none of the controls had zero scores, and in terms of the χ^2 test this difference between the live vaccine group and the controls is significant at about the 1 % level of probability.

Results

In terms of group mean scores, the live vaccine was more immunogenic than the formalin-treated vaccine (Table 3); and the inactivated material was rather more effective by the subcutaneous and intravenous routes than by intramuscular injection. It should, however, be mentioned that of the six animals injected intramuscularly, three had low scores (4, 4 and 5) that just failed to attain the lower confidence limit for this experiment; this was also true of the single unprotected animal in the live vaccine group, and of one of the four unprotected baboons given inactivated vaccine by the subcutaneous and intravenous routes.

The live vaccine with a titre of $10^{5{\cdot}8}$ ELD 50/ml. performed noticeably better than that in Expt P 1, in which the titre was 100-fold lower.

Rechallenge after 10 *months.* The left eyes of all surviving animals were inoculated with the batch of MRC-4*f* used for rechallenge in Expt P 1. The upper and lower 95 % confidence limits on scores for animals rechallenged in Expt P 2 were 29 and 0. The scores for the five surviving animals originally given live vaccine were

4, 4, 1, 0 and 0. One of the two baboons originally protected by subcutaneous and intravenous injections of killed vaccine was still immune; and as might be expected, none of those given inactivated vaccine intramuscularly resisted rechallenge.

ULTRAVIOLET-INACTIVATED VACCINE

This experiment showed that ultraviolet (UV) light completely destroyed the immunogenicity of MRC-4*f*; but since there appear to be no other references to the dynamics of inactivation of TRIC agent by this method, a detailed account of the investigation is given.

Collier, McClean & Vallet (1955) described the use of the Habel & Sockrider (1947) apparatus for irradiating vaccinia virus. In brief, it consists of an inclined cylinder 5·1 cm. in diameter and 71 cm. long that rotates about its axis at 1000 r.p.m. A 24 in. Hanovia low-pressure mercury lamp is mounted axially; over 90% of the UV emission is at 2537 Å at about 3 W. The suspension to be irradiated is introduced into the upper end of the cylinder, forms a thin film that flows past the UV source, and is collected at the lower end. Exposure can be varied by altering the flow-rate and angle of inclination, and by screening off sections of lamp to reduce its effective output. Collier *et al.* (1955) determined the amount of irradiation that completely destroyed the infectivity of purified vaccinia without impairing its antigenicity; this was assigned an arbitrary value, termed 'Relative Exposure (R.E.) 1·0'; it was twice the amount of irradiation needed to reduce the infective titre from $10^{7·5}$ pock-forming units (PFU)/ml. to $10^{0·9}$ PFU/ml.

The TRIC suspension used to vaccinate baboons was irradiated in the Habel–Sockrider apparatus; but a pilot experiment was first done by a different method to determine the degree of irradiation required in comparison with that used for vaccinia.

Comparison of inactivation rates of TRIC agent and vaccinia

TRIC agent was MRC-4*f* purified from infected yolk sacs and suspended in 0·1 M phosphate buffer, pH 7·0. It contained $10^{8·3}$ elementary bodies and 30 mg. total nitrogen per 100 ml.

Vaccinia virus was the Lister Institute strain purified from sheep lymph; the final suspension was made in 0·004 M McIlvaine buffer pH 7·2. To make its UV absorption comparable with that of the TRIC suspension it was brought to the same turbidity by adding normal yolk-sac extract.

UV irradiation was done by exposing 10 ml. samples in 9 cm. diameter Petri dishes to a Hanovia bactericidal lamp, Model 12, mounted 66·5 cm. vertically above. The characteristics of the lamp were such that a 120 sec. exposure was approximately equivalent to R.E. 1·0 in the Habel–Sockrider apparatus. Samples were rocked gently during exposure to ensure uniform irradiation. After exposure to UV for varying periods, vaccinia suspensions were titrated in the chick embryo chorioallantoic membrane (Collier, 1955); TRIC suspensions were titrated in HeLa cells (Furness, Graham & Reeve, 1960).

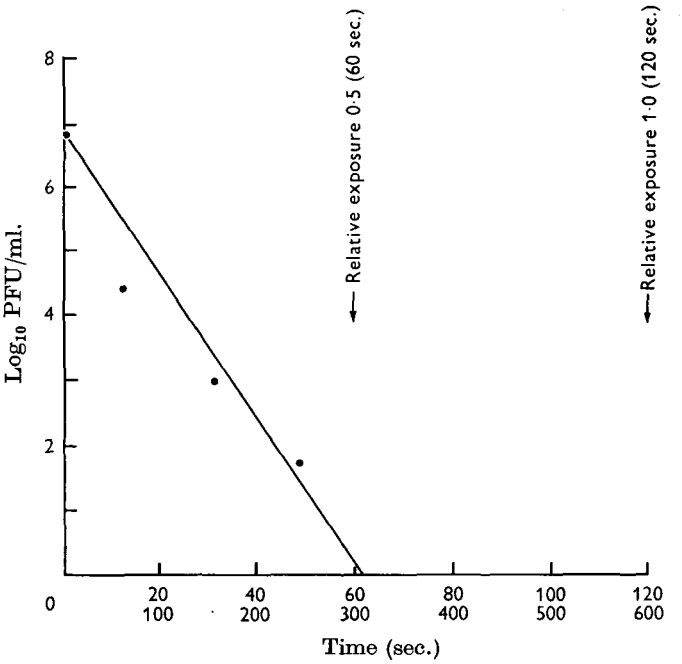

Fig. 1. Inactivation of vaccinia virus exposed in Petri dishes to ultraviolet light. The points represent titres of vaccinia virus suspension containing normal yolk-sac extract irradiated for various times on the scale 0–600 sec. The original titre was $10^{6.8}$ pock-forming units (PFU) per ml. The solid line is derived from the data of Collier *et al.* (1955), and represents the inactivation curve of a highly purified vaccinia suspension with this starting titre. (Time-scale 0–120 sec.)

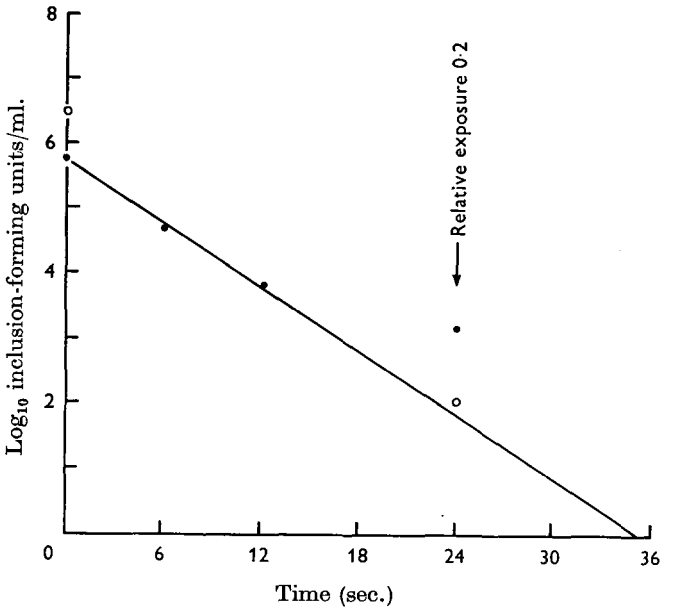

Fig. 2. Inactivation of MRC-4*f* by ultraviolet light. Closed circles, irradiated in Petri dishes. Open circles, irradiated in Habel–Sockrider apparatus.

https://doi.org/10.1017/S0022172400045575 Published online by Cambridge University Press

Results

Figure 1 shows that the rate of inactivation of vaccinia virus was exponential, but in this experiment was approximately 5 times slower than that calculated from previous work with highly purified suspensions; the difference is accounted for by the presence of normal yolk-sac extract.

Figure 2 shows that inactivation of MRC-4*f* also proceeded exponentially. The unexpectedly high titre recorded after irradiation for 24 sec. was probably inaccurate because of the scanty inclusions formed on titration of this sample in HeLa cells. The rate of inactivation was calculated to be 7·5 times faster than the vaccinia suspension of comparable turbidity, and 1·5 times faster than that of highly purified vaccinia. With a TRIC suspension comparable in purity to the vaccinia preparations of Collier *et al.* (1955), the inactivation rate would presumably have been 5 (7·5:1·5) times as fast.

Irradiation in the Habel–Sockrider apparatus

A preliminary test in the Habel–Sockrider apparatus confirmed that MRC-4*f* was inactivated at the rate predicted from experiments in Petri dishes. A suspension with an initial titre of $10^{6·5}$ IFU/ml. was irradiated at R.E. values of 0·2, 0·4, 0·6 and 0·8. At R.E. 0·2 (corresponding to a 24 sec. exposure in Petri dishes), the titre had fallen to $10^{2·0}$ IFU/ml., and was thus close to the expected value (see Fig. 2). It was estimated that the infectivity of a suspension with this starting titre would be completely abolished at R.E. 0·3, and twice this exposure (i.e. R.E. 0·6) was chosen to inactivate the vaccine used for immunizing baboons.

Experiment P 3: *vaccination with live or ultraviolet-inactivated MRC-4f; challenge with MRC-4f*

Vaccine was prepared by differential centrifugation of yolk sacs infected with MRC-4*f* (Table 1). Part was kept as a live vaccine in ampoules at −70° C. The remainder was irradiated by the Habel–Sockrider method at R.E. 0·6; this material was stored in the liquid state until use, except for a portion that was freeze-dried in 1·0 ml. amounts. Both liquid and dried suspensions were held at 4° C.

Vaccinations were done according to the schedule in Expt 11. Three groups each of six baboons were given respectively live vaccine, irradiated liquid vaccine and irradiated freeze-dried vaccine. Six control animals received a dummy vaccine made from extract of normal yolk sacs.

Challenge with MRC-4*f* (Table 1) was administered 10 days after the final intravenous dose of vaccine.

Results

Table 4 shows that live vaccine protected four out of six baboons; the scores of the two remaining animals were 1 and 4 respectively. By contrast, none of the animals given irradiated liquid vaccine was protected to a significant extent, and in only one of the group vaccinated with irradiated dried vaccine was there a significant reduction in score.

Serological findings in Experiments P 2 and P 3

The poor antibody response to formalin-treated vaccine in Expt 11 has already been noted. The serological results of the Pfizer experiments are described separately, since they were done by a method differing from that of Collier & Blyth (1966*a*), and as a routine sera were tested not at the time of challenge but at intervals before and afterwards.

Table 4. *Experiment P 3: vaccination with live or ultraviolet-inactivated MRC-4f; challenge with MRC-4f*

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
6	Live	0·50	—†	—†	4/6
6	UV irradiated				
	Liquid	3·32	+0·19	1·24	0/6
	Dried	2·41	−0·72	1·24	1/6
6	No vaccine	3·13	—	—	—

95 % confidence limits on scores for individual vaccinated animals: upper = 33, lower = 0.
L.S.D. = least significant difference.
* That is, with individual scores of 0.
† Four animals had zero scores, which are not included in the overall analysis of variance because of statistical difficulties. However, none of the controls had zero scores, and in terms of the χ^2 test this difference between the live vaccine group and the controls is significant at about the 3 % level of probability.

Table 5. *Experiments P 2 and P 3; mean titres of complement-fixing antibody 1 week after challenge*

Expt no.	Vaccine	Mean CF titre* at time of: 1st challenge	2nd challenge
P 2	Live	36	30
	Formalin-inactivated		
	2 × 1·0 ml. s.c., 1·0 ml. i.v.	8	< 5
	3 × 1·0 ml. i.m.	17	6
	No vaccine	< 5	< 5
P 3	Live	57	—
	UV inactivated		
	Liquid	26	—
	Dried	20	—
	Normal yolk sac	13	—

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

Complement fixation tests for antibody reacting with heated (group) antigen were done by the method of Bradstreet & Taylor (1962), using as antigen a boiled extract of yolk sacs infected with MRC-4, three minimal haemolytic doses of complement, and fixation overnight at 4° C.

Results

Table 5 gives only the results of tests made 1 week after challenge, since these are most nearly comparable with those described elsewhere in these papers. By this method of testing, the live MRC-4*f* vaccine appeared to induce lower titres than those obtained by the Trachoma Research Unit technique. In terms of group mean titres the vaccines inactivated with formalin or with ultraviolet light induced rather less antibody than did their live counterparts. The serological responses of the animals used in Expt P 1 were not determined.

DISCUSSION

The immunogenicity of a vaccine that protected baboons against conjunctival infection was destroyed at 37° C., suggesting that the protective antigen differs from the heat-stable complement-fixing antigen possessed by TRIC and related micro-organisms; this may explain why there is little or no relation between immunity and the serum titre of antibody reacting with complement and group antigen. The antigen protecting against infection seems also to differ in this respect from that which immunizes mice against TRIC toxin injected intravenously (Wang, Kenny & Grayston, 1967).

A recent review of the literature on trachoma vaccine (Collier, 1966) contains several references to the use of formalin-inactivated antigens. For example, Bietti, Guerra, Felici & Vozza (1962) maintained that this type of vaccine alone or with adjuvants protected volunteers against challenge by the conjunctival route; and Snyder *et al.* (1964) found that it significantly reduced the trachoma attack rate in Saudi Arabian children. Apart from our own experiments, however, there seem to have been no studies of the action of formalin on potency in terms of a comparison of formalin-treated preparations with equivalent doses of live vaccines. It is clear from a comparison of live and inactivated vaccines prepared from the same suspension that formalin impairs immunogenicity (Expt P 2); and although in Expt 11 the inactivated vaccine was not compared directly with a live counterpart, other experiments in this series showed that live vaccines with a similar or lower content of MRC-4*f* afforded substantial protection. In Expt P 2 the performance of the inactivated vaccine was superior to that in Expt 11 although the original titre was lower and a higher concentration of formalin was used. The technique of inactivation was, however, refined by neutralization of the residual formalin and subsequent storage at $-70°$ C.; and it may be that prolonged exposure of the antigen to residual formalin during storage at 4° C. before use in Expt 11 accounts for this finding.

Within the limits of our observations the rate of inactivation of MRC-4*f* by ultraviolet light was exponential, and was faster than that of vaccinia virus. Experiment P 3 showed unequivocally that about twice the dose of UV light needed to abolish infectivity destroyed immunogenicity. This finding contrasts with that of Grayston *et al.* (1963) who immunized 36 Taiwanese children with a UV inactivated trachoma vaccine. The attack rate and average duration of disease were significantly less than in 53 control children, but unfortunately the

preparation and characteristics of the vaccine were not described. Collier *et al.* (1955) showed that the margin between inactivation of vaccinia and loss of antigenicity is narrow, and that the dose of UV light must in consequence be carefully controlled. Our experiment suggests that with TRIC agent the margin must be narrower still or non-existent, and it is difficult to see how the amount of irradiation used could be much reduced without the risk of failing to achieve complete destruction of infectivity.

The abolition or impairment of immunogenicity by heat, formalin and ultraviolet light may be explained in terms of damage to a protective antigen. On the other hand, live TRIC agent may be more effective as a vaccine because it multiplies after injection and thus gives rise to a greater mass of antigen than is provided by a killed suspension. The experiments reported here do not enable us to choose between these alternatives. However, Collier & Smith (1967) recently showed that MRC-4*f* did in fact multiply within baboons after parenteral injection, and that the degree of immunity resulting depended on the route of inoculation. Intravenous injection was followed by a high level of multiplication in the spleen and firm immunity to conjunctival challenge administered shortly afterwards; live TRIC agent given subcutaneously multiplied mostly in the skin and regional lymph nodes and was not so effective in inducing immunity. In the Introduction, attention was drawn to the implication of these findings for further field work with live trachoma vaccines. Our results suggest that neither heat, formalin nor ultraviolet light are satisfactory inactivating agents, and that further research on this problem is necessary.

In conclusion, it is interesting that the 'fast-killing' variant of MRC-4 used for challenge in Expts P 2 and P 3 still induced characteristic follicular conjunctivitis and inclusion bodies; another strain of inclusion conjunctivitis, TRIC/ /GB/ MRC-1/G (formerly LB 1), lost pathogenicity for the baboon conjunctiva at about the time that it acquired the ability to kill chick embryos comparatively quickly (Collier, 1962; Reeve & Taverne, 1963).

SUMMARY

Heating at 37° C. for 5 days completely abolished the capacity of a trachoma/ inclusion conjunctivitis (TRIC) vaccine to protect baboons against conjunctival infection. Treatment with formalin also impaired or abolished immunogenicity.

The rate of inactivation of TRIC agent by ultraviolet light was exponential, and was about 7 times faster than that of a suspension of vaccinia virus of comparable turbidity. By contrast with vaccinia, irradiation of TRIC agent with twice the dose of ultraviolet light needed to destroy infectivity resulted in loss of immunogenicity.

The deleterious action of these inactivation procedures on the potency of TRIC vaccines may be explained in terms of damage to a protective antigen; alternatively, live vaccines may be more immunogenic because TRIC agents are capable of multiplying within primate hosts after parenteral injection.

Unlike the same type of variant of another strain of inclusion conjunctivitis, the 'fast-killing' variant of MRC-4 was still pathogenic for the baboon conjunctiva.

We are much indebted to Mr L. Vallet (Lister Institute of Preventive Medicine) for his helpful advice concerning the ultraviolet irradiation. We are most grateful to Mr P. Avis (Pfizer Ltd.) for his help with the statistical aspects of this paper.

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