Pulmonary Infection of Adult White Mice with the TE 55 Strain of Trachoma Virus

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SUMMARY

The effects of intranasal inoculation of mice more than 6 weeks old with the TE55 strain of trachoma virus were studied. The histology and course of the pulmonary lesions produced are described. Infected mice showed a poor response of complement-fixing antibodies after infection. Precipitating antigens were detected in infected mouse lungs.

INTRODUCTION

Thygeson & Nataf (1958) stated, 'It is now generally agreed that only primates are susceptible to experimental infection with trachoma virus'. Since then Giroud, Renoux & Nataf (1958) have reported pulmonary hepatization in mice after intranasal inoculation of material from trachomatous conjunctivae; Hurst & Reeve (1960) reported passage of the TE55 strain in mouse brain; and Bernkopf (1959) reported the susceptibility of young mice to infection after intranasal inoculation with the TE55 and Dari strains of trachoma virus. Bell, Snyder & Murray (1959) showed a rapid toxic effect of certain strains after intravenous injection into adult mice, but this phenomenon did not depend upon the growth of virus. These workers were chiefly interested in the mouse either as a means of growing the virus or as an experimental system for neutralization tests. For the second purpose a lethal effect of intranasal inoculation was required. Bernkopf (1959) found that 9-day-old mice were highly susceptible, three-week old mice much less susceptible, and six-week old mice insusceptible to intranasal inoculation with the TE55 strain. Insusceptibility in this sense means that the mice did not die.

The experiments reported here were made to examine the effects of intranasal inoculation of older, and therefore 'insusceptible' mice with the TE55 strain of trachoma virus. It was considered that these mice would either be totally insusceptible, in which case the system could be studied as an example of host resistance developing with age, or partly susceptible, in which case lesions would develop which would not lead to death, and would presumably resolve. Preliminary observations showed that older mice were, in fact, partly susceptible, and that they would provide a system in which the pathogenesis and immunology of trachomatous infection could be studied in a non-primate host.

METHODS

Virus. The TE55 strain of trachoma virus (T'ang *et al.* 1957), propagated in chick embryo yolk sac, was provided by Dr L. H. Collier (The Lister Institute, London). At the beginning of these experiments the virus was in its 37th passage,

at the end in its 48th. The methods of propagation and storage were described previously (Watkins, 1961). Infected yolk sac homogenates were inoculated on to blood agar and into thioglycollate medium (Brewer, 1940) to examine for bacterial contamination.

Estimation of infectivity. The infectivity of virus suspensions is expressed as the Mean Survival Time (MST), in days, of four to six chick embryos after injection of 0.2 ml. of suspension into each yolk sac at 7 or 8 days of incubation. The MST is approximately inversely proportional to the infectivity of the suspension inoculated (Watkins, 1961). The most infective suspensions gave an MST of 4–5 days, the least an MST of 13–14 days, by this technique.

Mice. An inbred strain of Swiss albino mice was used. All mice weighed 20 to 30 g., and were over 6 weeks old. Two or three drops of infected yolk sac homogenate (0.05–0.075 ml.) were inoculated intranasally under ether anaesthesia. Mice were killed by dislocation of the cervical vertebrae.

Mouse lung homogenates were prepared by grinding the lungs in 2 ml. of sucrose + potassium glutamate (SPG) (Bovarnick, Miller & Snyder, 1950) containing 50,000 μ g. streptomycin/ml. in a Griffiths tube. The homogenate was centrifuged at 400 g for 10 min. and the supernatant fluid used in experiments. This supernatant fluid will be referred to as MLH (mouse lung homogenate). 0.2 ml. of MLH was injected into four to six 7 day yolk sacs for infectivity studies. Eggs were candled daily for 14 days.

Histology. Tissues for histological examination were fixed in Zenker-formol solution or 5% (v/v) formol-saline and stained with Giemsa's stain or haematoxylin and eosin. Homogenates examined for elementary bodies were stained with Giemsa stain (G. T. Gurr, London).

Antisera. The antiserum designated R1/3 was prepared by five weekly intramuscular injections of 1 ml. of stock infected yolk sac homogenate (MST 4–5 days) into a rabbit. A booster dose was given 5 months later and blood was taken 1 week after the booster dose. The serum was stored at -20° .

Mouse sera were obtained by bleeding from the tail or the jugular vein under light ether anaesthesia. After separation the pooled sera were preserved by the addition of thiomersalate to 1/10,000 (w/v), or sodium azide to 0.1% (w/v) or by freezing at -20° . All sera were inactivated for 30 min. at 56° .

Preparation of antigens. Antigens were prepared in one of two ways.

(1) Yolk sacs rich in elementary bodies were homogenized in 10 ml. phosphate-buffered saline (PBS, Dulbecco & Vogt, 1954) per yolk-sac. The homogenate was shaken with an equal volume of ether and the mixture placed at 4° overnight. The lower red layer was centrifuged at 400 g for 10 min., the deposit washed three times in 2 ml. of PBS and the washings added to the supernatant fluid, which was centrifuged at 10,000 g for 30 min. This deposit was resuspended to the original volume in PBS and centrifuged at 400 g for 10 min. and the supernatant from this at 10,000 g for 30 min. After a third cycle of centrifugation at 400 g for 10 min. and 10,000 g for 30 min. the final deposit was resuspended in CFT diluent (referred to below) at 8 ml./original yolk sac. Smears of this antigen preparation showed more than 100 elementary bodies/microscope field at a magnification of $\times 1700$. Electron microscopy of a suspension prepared in this way was kindly undertaken by Dr D. Kay (Sir William Dunn School of Pathology). A drop of virus suspension

was placed on a formvar coated grid, fixed in formaldehyde vapour, washed in water and examined after shadowing with palladium. Plate 1, fig. 1, shows intact virus particles.

(2) Some antigens were prepared by the method of Nigg, Hilleman & Bowser (1946) which involves phenolizing an infected yolk sac suspension with 0.5% phenol for four weeks at 37°. In our experiments the antigen was shaken with phenol at 37° for 2 weeks.

Control antigens for each type of virus antigen were prepared from normal yolk sacs of the same age. It is known that sera from cases of lymphogranuloma venereum will fix complement with phenolized trachoma antigen (Woolridge, Jackson & Grayston, 1960). Two LGV sera (kindly provided by Professor C. F. Barwell, The London Hospital) gave titres of 1/256 and 1/512 with phenolized antigen in the CF test described below. They did not fix complement with controls made from normal yolk sacs.

Complement-fixation tests. These were performed in cups in Perspex trays (Salk pattern haemagglutination trays). The unit volume was 0.02 ml. delivered from a calibrated dropping pipette. The minimal haemolytic dose (MHD) of complement was determined for 100% haemolysis in the presence of antigen. Twofold serial dilutions of antiserum were mixed with undiluted antigen and 2 MHD of complement. Fixation took place at 4° for 18 hr. followed by 20 min. at room temperature. After addition of the haemolytic system (2.5% (v/v) sheep red cells and 3-4 MHD of haemolytic serum) incubation was continued at 37° until haemolysis was complete which took 1½-2 hr. Controls of ½ MHD, 1 MHD and 2 MHD of complement and serum and antigen controls were included in each batch of tests. The guinea-pig complement had been preserved by Richardson's method (1941) (Burroughs Wellcome & Co., London). Horse anti-sheep cell serum was preserved with glycerol (Burroughs Wellcome & Co., London). All dilutions were made in barbitone CFT diluent made up from commercially obtained tablets (Oxoid, England).

Agar gel precipitation (Ouchterlony, 1948). Perspex moulds were made consisting of six pegs arranged around a central peg. Holes were bored through the centres of the pegs. Each peg was 1.0 cm. in diameter, and the distance between any two adjacent pegs was 0.5 cm. The mould was placed in a flat-bottomed Petri dish 10 cm. in diameter and 10 ml. of 2% (w/v) agar in 0.9% (w/v) NaCl with 1/10,000 thiomersalate were poured in. The mould was removed when the agar had set, 0.1 ml. of antiserum or antigen solution was placed in the cups and the plate incubated at 37° in a humid atmosphere. Lines of precipitation developed in the agar within 2 days; further incubation produced no change in the number of lines.

RESULTS

Absence of infectivity and antigens in lungs of control mice

It is well known that latent infection with several viruses may occur in laboratory mice. To test for the presence of such viruses normal yolk sac homogenate was instilled intranasally into normal mice; the lung homogenate from these mice was then instilled into fresh mice and so on for five passages. The homogenates from control mice were examined for infectivity by inoculation into yolk sacs and for the presence of antigen precipitating with serum R1/3 in agar. Neither infectivity

nor precipitating antigen was detected in these homogenates after various intervals between intranasal inoculation and removal of the lungs (Table 1). Homogenates of normal mouse lungs, normal mouse lungs after intranasal inoculation of normal yolk sac homogenate, and normal mouse lung after intranasal inoculation of normal mouse lung homogenate were examined on several other occasions for yolk sac infectivity and the presence of precipitating antigen. The results were uniformly negative. It was concluded that any 'elementary body virus' and precipitating antigen recovered from lungs after intranasal inoculation of yolk sac homogenate containing the TE 55 strain of trachoma virus must have been derived from inoculated virus and not from an awakened latent infection.

Table 1. *Presence or absence of lines in agar produced by mouse lung homogenate and rabbit antiserum R1/3 after various inocula intranasally*

Inoculum	Time between inoculation and removal of lungs (days)	No. of mice	No. of lungs positive	No. of lungs negative
Normal yolk sac homogenate	7	6	0	6
	11	2	0	2
	14	6	0	6
Virus TE 55 infected yolk sac homogenate (MST* between 4 and 5 days)	1	7	3	4
	2	1	1	0
	3	1	1	0
	4	1	1	0
	7	4	1	3
	11	2	1	1
	14	2	2	0
	28	1	1	0
Normal mouse lung homogenate (passage)	7	5	0	5
	14	6	0	6
	20	6	0	6
	52	4	0	4
Passage infected (virus TE 55) mouse lung homogenate	4	3	1	2
	10	4	4	0
	14	4	4	0
	18	1	1	0
	22	1	1	0
No inoculum	0	10	0	10

* Mean survival time.

Table 2. *Relation between lethality of virus TE 55 for chick embryos after yolk sac inoculation, and mice after intranasal inoculation*

MST of inoculum in yolk sac (days)	No. of mice inoculated	No. and percentage of mice dying of pneumonitis
4-4	27	14 (51.8 %)
4-6	140	3 (2.14 %)
4-7	60	0
> 5	> 400*	0

* At various times throughout these experiments.

Lethality of virus for mice after intranasal inoculation

Table 2 shows the relation between the infectivity of TE 55 virus for the yolk sac, expressed as the mean survival time (MST) of chick embryos, and the percentage of mice dying of pneumonitis after intranasal inoculation. It is apparent that deaths occurred only after the administration of inocula with high infectivity for chick embryos. All the fatally infected mice died between 2 and 4 days after infection. Post-mortem examination showed massive consolidation of both lungs, and virus was recovered from several lungs by inoculation into the yolk sac. The mice which survived inoculation showed for the most part no signs of illness; some of those which received higher doses had staring fur for a few days, and sometimes râles could be heard by holding the mouse close to the ear.

Histology of lesions

The massively consolidated lungs in fatally infected mice showed acute inflammation (Pl. 1, figs. 2 & 3). Typical inclusion bodies were seen occasionally. It was impossible to discern which cells contained these bodies, although in some cases they were situated in the alveolar wall (Pl. 2, fig. 4*a, b*). The inclusion shown was photographed at two levels to show the elementary bodies which it contained. At the periphery of this inclusion larger bodies can be seen, conforming to the description of trachomatous initial bodies first given by Lindner (1910). The inclusion bodies did not differ morphologically or in staining properties from trachoma inclusions in yolk sac entodermal cells and HeLa cells.

In mice which received a sublethal dose the size of the lesion varied with the infectivity of the inoculum. Inocula with a chick embryo MST of 5-6 days produced local consolidations sometimes a few millimetres in diameter, sometimes affecting a single lobe in its entirety. The numbers and sizes of the lesions were too variable to be used for virus titration in the way psittacosis virus has been titrated in mouse lung (Rudd & Burnet, 1941). After inocula having chick embryo MST values greater than 7 or 8 days, lesions visible to the naked eye were generally not seen; when they were seen they were small. The earliest histological lesions were seen 2 days after infection in an experiment with an inoculum whose MST value for chick embryos was 5 days; they consisted of areas about 70 μ in diameter of infiltration with polymorphonuclear leucocytes. There was also some generalized capillary dilation. By the 3rd day the areas had become confluent, and gave the low-power appearance shown in Pl. 2, fig. 5. This photograph shows peribronchial infiltration, and is taken from a set of serial sections which demonstrated that the bronchioles in the centre of the inflammatory area opened into normal alveoli. In this case, therefore, the picture is that of a bronchiolitis; in other mice the lesions affected only the alveoli. The difference is presumably due to variation in the distance to which the inoculum penetrated as a result of inspiration. On the 4th day the vessels in the areas of inflammation were surrounded by zones of mononuclear cells (Pl. 2, fig. 6). Resolution of the lesions was completed between the seventh and ninth days, but some perivascular cuffing with mononuclear cells persisted until at least the 28th day. After resolution of the acute lesions there was no evidence of tissue destruction or fibrosis or lymphocytic infiltration characteristic

of ophthalmic trachoma. Apart from enlargement of hilar lymph nodes, which persisted for 2-3 weeks after infection no other organs showed any abnormalities.

Recovery of virus from mouse lung after intranasal inoculation of infected yolk sac homogenate

Infective virus, indistinguishable morphologically from trachoma virus, was recovered from lungs by yolk sac inoculation several times at intervals from 1 hr. to 7 days after intranasal inoculation of yolk sac homogenates of infectivities ranging from MST 4.4 days to MST 8.0 days. The infectivities of MLH ranged from MST 7.5 days to MST 12.5 days in all these experiments. The infectivities of lungs from mice killed at intervals up to 7 days after receiving the same inoculum showed no significant increase, which suggested that virus was either remaining static in the lung, or was being removed at a rate which kept pace with its replication. No infectivity was detected 14 days or more after inoculation and recovery of virus was not uniformly successful in the period up to 7 days after inoculation.

Presence of viral antigen in lungs

Soluble antigens were detected in infected lungs by diffusion in an Ouchterlony plate against a rabbit antiserum (R1/3) prepared by repeated intramuscular injections of infected yolk sac homogenate. Homogenates prepared from normal mouse lungs after instillation of normal yolk sac or normal lung homogenates never gave precipitation lines with this or any other anti-trachoma virus serum. Lines were not seen after inoculation of virus suspensions whose MST was greater than 6 or 7 days, and even after the inoculation of suspensions of higher infectivity their appearance was not constant (as Table 1). They were most frequent when homogenates were made from lungs showing massive consolidation; lines were given by a homogenate prepared 14 days after infection in an experiment in which half the mice died of pneumonitis. The antigen persisted for several weeks; lines were produced with a homogenate prepared 4 weeks after inoculation of virus with an MST of 4 days. The pattern of lines was constantly a single line near the antigen cup with a less well-defined complex of one to three lines near the antiserum cup. The lines were also produced with a second rabbit antiserum prepared by injection of infected yolk sac homogenate.

Serial passage of virus in mouse lung

Two passage series were attempted. The first was started by intranasal inoculation of six mice with virus of MST 5 days. Passage of infected lung homogenate was undertaken at intervals of 7-14 days except for an interval of 22 days between the eighth and ninth passages. Six mice were inoculated at each passage, and homogenates for further passage were made from two to six mice in each passage. The mice of all passages remained clinically well, except for one mouse left for observation out of the fifth passage, which died 18 days after inoculation with patchy pneumonitis of both lungs. Apart from the homogenates of the first two passages virus was not recovered from the lungs by a single passage of the homogenate in yolk sacs, which meant that there was no direct evidence that virus was being passed. There was, however, some indirect evidence. First, antigens were detected

in homogenates of all passages after the first four (which were not examined), by precipitation in Ouchterlony plates. The precipitation lines were identical with those described in the preceding section. Secondly, patches of pneumonitis were observed in mice of the second, fifth and ninth passages, and one mouse of the sixth passage showed moderate perivascular and peribronchial accumulations of cells of the kind described in the section on histology.

The second series was carried on for five passages. The first passage, of virus suspension with an MST of 4.4 days, killed half the mice in 8 days, and the second passage was made with homogenate of consolidated lungs. Precipitation lines identical with those of the first series were given by homogenates of lungs of all the passages. Virus was recovered from the first passage homogenate, by single passage yolk sac inoculation, but not from the subsequent passages. A control passage series started with homogenate of normal mouse lung, and continued for five passages, was negative in each passage for yolk sac infectivity (determined by a single passage) and the presence of precipitating antigens.

Table 3. *Complement-fixation titres of mouse sera after intranasal inoculation of trachoma virus TE55*

MST of inoculum (days)	No. of mice	No. dead with pneumonitis	Pulmonary consolidation in survivors	No. of mice in pool	Titre of serum	Time after inoculation (days)	Antigen*
4.6	140	3	+	7	< 1/8	3, 6, 9, 21	P
				3*	1/8	12, 15, 18, 28, 30	
					< 1/8	6, 9	P
					1/8	3, 12, 15	
Not determined	24	3	+	5	< 1/8 1/64	7, 14, 28 21	E
5.2	54	0	±	3	< 1/8	2, 4, 6, 8, 10, 12, 14	P
4.7	60	0	+	4	< 1/8	14	E

* Sera taken from mice which were clinically ill 8 days after inoculation. P, phenolized antigen; E, etherized antigen.

Complement-fixation tests on sera from infected mice

Table 3 shows that pooled sera gave low titres of complement-fixing antibody against the two antigens used. Higher titres were obtained from mice surviving an inoculum which killed some of their fellows, but only in one pool (taken 21 days after infection) was the titre greater than 1 in 8. Pooled sera with titres of 1 in 8 occurred 12 days or more after infection, with the exception of one pool which gave a titre of 1 in 8, 3 days after infection. The significance of this is uncertain, since pools made 6 and 9 days after infection in the same experiment were negative.

DISCUSSION

It is clear that the TE 55 strain of trachoma virus will grow in the lungs of mice older than 6 weeks, although the type of cell in which growth occurs is at present unknown. After intranasal inoculation of Indian ink all the ink is taken up by

macrophages within 24 hr. (Watkins, unpublished). If trachoma virus is taken up in the same way it is possible that growth occurs within these macrophages. The inclusion bodies seen in mouse lung resembled trachoma inclusion bodies in conjunctival epithelium, so that the method of virus replication in a host far removed taxonomically from the natural host is, morphologically at least, of the same kind. It was surprising that inocula containing about 10^9 elementary bodies produced so few inclusion bodies, even in massively consolidated lungs. This may have been due to absence of infectivity of most of the particles, since the LD₅₀ of this strain for chick embryos by the yolk sac route was of the order of 10^3 to 10^4 elementary bodies. An alternative explanation, that the particles successful in producing inclusion bodies were genetically different from the unsuccessful ones, is made unlikely by the failure of repeated passage to produce an increase in virulence. Material which killed chick embryos in more than 6 days rarely produced naked-eye or histological lesions. This suggests that the production of lesions depends on a balance between destruction of virus and its successful establishment within a cell.

The pathogenesis of the acute inflammation, which resembles that occurring in the early stages of human conjunctival infection, is still obscure. The extent of the reaction seemed out of proportion to the small numbers of inclusion bodies present. A simple reaction to inert foreign material can be excluded, since nothing similar is seen after heavy inocula of Indian ink. Three explanations are possible. The reaction may be provoked by the release of toxic elementary bodies from cells in which the virus is growing. Growth of virus may be accompanied by the production of a soluble toxin, as suggested by Mitsui (1954) and others. The detection of diffusible antigens by gel precipitation gives some support to this idea, since it shows that some kind of soluble material is associated with the growth of virus. The third explanation is based on the finding by Bell *et al.* (1959) that some strains of trachoma virus produce a rapid toxic death in mice after intravenous injection of washed elementary bodies. It is possible that intranasal inoculation of a large number of elementary bodies may itself produce an inflammatory reaction. On this view the growth of virus alone would be responsible at the most for only a small proportion of the reaction. At present it is impossible to say which of these three explanations is correct, or whether more than one mechanism may be involved.

The low titres of complement-fixing antibody against particulate (etherized) and phenolized antigen, together with the late appearance of detectable antibody, suggest that this type of antibody may have little to do with the process of resolution of the inflammation. Mice are capable of producing high titres of complement-fixing antibody (up to 1/5120) after intranasal infection with organisms of the pleuropneumonia group (Lemcke, 1961), so the low titres obtained after trachomatous infection may mean that trachoma virus is a relatively poor antigen for mice. Low titres of complement-fixing antibody are usual after infection of human beings with members of the psittacosis-lymphogranuloma group of viruses; it is difficult to ascertain whether this is due to a poor host response, or to deficiencies in the antigens used in complement-fixation tests.

The failure to recover infective virus from the lungs of passage mice when there was circumstantial evidence that their lungs were infected may perhaps be explained by the fact that two completely different criteria of infectivity were being compared. On the one hand, in trying to detect virus by yolk sac inoculation in these experi-

ments enough virus had to be present to kill the embryo within 14 days; on the other hand, to infect mouse lungs with mouse-lung passaged material enough virus had to be present to give rise to detectable diffusible antigen and to occasional small patches of pneumonitis. If the second method was more sensitive than the first the failure to recover virus from passage lungs can be explained. However, the possibility cannot be excluded that continued passage in mouse lung produced a strain of virus with altered properties of growth in yolk sac endoderm.

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EXPLANATION OF PLATES 1 & 2

Fig. 1. Electron micrograph of etherized suspension of trachoma virus TE55. Palladium-shadowed ($\times 50,000$).

Fig. 2. Area of inflammation in mouse lung 3 days after intranasal inoculation with trachoma virus TE55 ($\times 450$).

Fig. 3. Area of inflammation in mouse of fifth passage series ($\times 450$).

Fig. 4. Trachoma inclusion body in same lung as that shown in Fig. 2, photographed at two different levels ($\times 1500$).

Fig. 5. Mouse lung showing foci of inflammation 3 days after intranasal inoculation of trachoma virus TE55 ($\times 40$).

Fig. 6. Perivascular infiltration of mononuclear cells into pneumonitic area in mouse lung 4 days after intranasal inoculation of trachoma virus TE55 ($\times 200$).