

A REPORT ON THE ATTEMPTED ISOLATION OF THE VIRUS OF TRACHOMA*†

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THE consensus of opinion at the present time is that trachoma is a virus disease, being due to the agent first described by Halberstaedter and Prowazek (1907), who produced an experimental infection by inoculating the conjunctivae of apes with material from a human case of trachoma. Both the apes and the human cases from which the material came showed characteristic inclusions in the cytoplasm of conjunctival cells.

Support for the work of Halberstaedter and Prowazek came from the experiments of Thygeson (1934), Thygeson and Proctor (1938), and Thygeson and Richards (1939), who produced infection in monkeys and in man, and also made estimates of viral size by filtration. Unfortunately they were unable to find a simple means of viral cultivation and, as with other workers, the disease they produced in monkeys was unlike the human infection.

Poleff (1939) stated that he had been able to cultivate the virus in the fertile chick embryo, as did Macchiavello (1944), and Arakawa, Kitamura, Mitsui, and Tanaka (1953) claimed to have isolated a virus in mouse brain. None of these reports has been confirmed and Macchiavello's account of the experimental infection in a volunteer suggests that he was dealing with an adenovirus rather than trachoma.

There is general agreement that no animal other than the monkey can be infected by the conjunctival route, and even the monkey infection is irregular and not typical of the human disease. Furthermore, the identification of inclusions in monkey infections has been made in only a minority of experiments.

Several workers, such as Thygeson (1939), and Murray, Chang, Bell, Tarizzo, and Snyder (1957), have tried to isolate the virus in tissue culture, but have apparently failed to obtain a transmissible agent which will fulfil the criteria necessary for the identification of a new virus.

In the following report an account is given of various attempts made to isolate the virus in three different areas of the world, namely in Jordan and in Gambia where the disease is endemic and a large proportion of the population is infected, and in London, where occasional cases were seen in the out-patient departments of Moorfields Hospital. These latter patients were old cases who had been infected before arriving in England.

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† Since this paper was submitted for publication the successful isolation of strains of trachoma virus has been accomplished by T'ang Fei-Fan and his co-workers (1957) and by Collier and Sowa (1958). Strains of virus isolated are sensitive to penicillin which may account for the failure of isolation reported here.

Material

Material for isolation of the virus was taken from the conjunctiva of infected persons by means of a spatula made from aluminium wire (throat swab wire is adequate for this purpose). The wire is hammered flat until a spatula about 5 mm. in diameter is obtained; all the edges are carefully smoothed with an Arkansas stone and the scraper is sterilized by heat. To obtain conjunctival cells, the conjunctiva is anaesthetized with pantocaine or cocaine and the scraper is then drawn firmly over the conjunctiva, taking care not to cause bleeding. From each patient smears of cells were made on slides for examination to determine the presence of inclusions, and the remaining cells were washed off the spatula into physiological saline or into 5 per cent. peptone water and kept as near to 4° C. as possible for virus isolation. Unless otherwise stated, all material stored for virus isolation had penicillin 50 units and streptomycin 100 u/g. per ml. added to the suspending fluids to inhibit bacterial growth.

Infection of eggs or of tissue cultures was performed as soon as material was shown to contain inclusions. In Jordan and in London this might take from a few minutes up to some 18 hrs, with material that required overnight staining, to identify inclusions. The Gambian material was flown to London for virus isolation and this entailed a flight of 36 hrs; whenever possible the material was freeze-dried before dispatch and was packed in ice so that on arrival in England the temperature was from 5–7° C. Freeze-drying followed the method of Collier (1955). Before use the freeze-dried material was reconstituted with distilled water.

At first, smears for the identification of inclusions were fixed in methyl alcohol for from 2 to 5 min. and then stained in a mixture of 1 per cent. Giemsa and 0.5 per cent. May Grunwald stain in phosphate buffer at pH 6.8 for 16 to 18 hrs; after staining, the slides were rinsed rapidly in 95 per cent. alcohol and then in phosphate buffer and allowed to dry. The dry film was examined for trachoma inclusions.

It was found that the Giemsa staining method was too slow and laborious, and to speed up diagnosis the iodine staining method of Rice (1936) was used. By this method cells were allowed to dry on the slide and then treated with 2.5 per cent. iodine in 5 per cent. potassium iodide for 2 to 5 min., and then blotted dry. The dry film is covered with immersion oil and examined with a 2/3 objective when the inclusions stand out a mahogany brown against the background of pale yellow cells; confirmation of an inclusion can be made with an oil immersion objective when the typical granular nature of the inclusion becomes evident. (A more detailed account of the method is to be published.)

Methods of Attempted Isolation

Fertile chick embryos were inoculated by the following routes:

(a) 11 to 13-day-old embryos were inoculated on the chorio-allantoic membrane, using the false air sac technique.

(b) Amniotic inoculation was performed in 11 to 12-day embryos.

(c) Using 13-day embryos, injection was made by means of a tuberculin syringe directly into the brain or eye of the embryo, under direct observation. As the mortality of the method is high all deaths within 36 hrs were considered to be non-specific.

(d) Yolk-sac inoculation was used because of the morphological similarity of the inclusions of trachoma and viruses of the Psittacosis group. Embryos were incubated for from 3 to 9 days before infection. In younger embryos, of from 3 to 7 days' incubation, inoculations were made blindly into the yolk sac. With older embryos a false air sac was produced and inoculation made directly into the yolk sac.

Eggs were observed for 7 days and, if at the end of that time no lesions were found or death had occurred, the material was ground in saline and passed to a further batch of eggs. It was customary to make either two or three blind passages in batches of three or four embryos before a specimen was considered to be negative.

Adult mice of a "virus-free" albino strain were inoculated intracerebrally under ether anaesthesia (vol. 0.02 ml.). The mice were watched for 14 days, after which the brains were removed aseptically and a 10 per cent. brain suspension passed intracerebrally to more mice. Only two blind passages were made in mice.

As suckling mice are more susceptible to virus infections, groups of mice from a few hours up to 7 days old have been inoculated either by the subcutaneous route (using 0.2 ml.) or intracerebrally (using 0.01 ml.). Intracerebral inoculation entails some mortality and all deaths within 24 hours were considered to be traumatic.

In a further attempt to increase the susceptibility to the virus, suckling mice were given 2 mg. cortisone, which was found to be the maximum dose tolerated, at the time of inoculation. In all these experiments a paired litter of mice was given cortisone alone and the mortalities in the two groups were compared, as an occasional death sometimes occurred as a result of the cortisone alone.

Tissue Culture

(a) Epithelial cells removed by scrapings were taken up in Earle's saline; on arrival at the laboratory they were centrifuged at 1,000 r.p.m. for 1 to 2 min., until a small pellet of cells was obtained. The pellet was removed and placed on a sterile coverslip and then embedded in a clot of either human or chick plasma. The culture was set up in depression slides. Using this technique, cells appeared to remain unchanged for up to 7 days.

(b) HeLa cells were cultivated in a medium of the following composition:

Lactalbumin hydrolysate	5 ml. of 5 per cent. solution
Human serum	20 ml.
Eagle's amino-acid mixture in Earle's saline	75 ml.

The pH was adjusted to 7.4 with sodium bicarbonate containing phenol red, and antibiotics were added. The antibiotics were penicillin 100 units, streptomycin 100 u/g. and mycostatin 25 units, to each ml. medium; in later experiments Neomycin was added, as this inhibits the pleuropneumonia-like organisms that have been demonstrated by Collier (1957) in HeLa cell cultures.

In maintenance medium for HeLa cell cultures, human serum was replaced by horse serum and lactalbumin was omitted.

Material was inoculated into three tubes and cells were observed daily for cellular changes; one tube was sacrificed at weekly intervals and fixed and stained by

Giemsa stain to attempt to demonstrate inclusions. As it was felt that the antibiotics might inhibit viral growth, several cultures were set up in their absence, but difficulty with bacterial and fungal contamination was encountered.

In addition to simple addition of the inoculum to the culture of HeLa cells, a more intimate mingling of cells was attempted by culturing the conjunctival cells in a suspension of HeLa cells.

In a few instances serial blind passage of material was attempted in HeLa cells.

(c) A strain of Chang's conjunctival cells obtained from Dr. J. C. Snyder was cultured in the following medium:

Human serum	20 per cent.
Chick extract	5 per cent.
Earle's amino-acid saline	75 per cent.

The pH was adjusted to 7.4 and antibiotics added as for HeLa cells. It was found that these cells degenerated in the absence of human serum and hence it was necessary to add 5 per cent. human serum to the maintenance fluid.

The inoculation procedures were the same as for HeLa cells.

(d) In certain cases of trachoma, the tarsal plate and overlying conjunctiva are removed at operation and use was made of this material to attempt to grow the trachomatous epithelium. Two methods were used:

(i) The fragment removed at operation was received in Earle's saline, this was cut into pieces of about 2 mm. square and imbedded in a plasma clot in a test tube. After overnight incubation to harden the clot, growth fluid as used for conjunctival cells was added. Tubes were incubated without rolling and the fluids were changed every 48 hrs.

(ii) The method of Medawar and Billingham (1951) for the removal of fibrous tissues from skin grafts was used. Tarsal fibrous tissue was digested until it could be stripped from the overlying epithelial sheet, which was then cut into fragments and treated in the same way as the indigested fragments. Unfortunately, it proved impossible to carry digestion to a stage where only epithelium remained, as we found that no cellular multiplication occurred in such explants, trypsin apparently having damaged the epithelial cells.

(e) On two occasions, inclusion-rich material was inoculated into the conjunctiva of a human volunteer. The technique was to cocaine the eye and rub the inoculum into a previously scarified epithelium of the upper tarsal conjunctiva. The conjunctiva was examined for changes using the slit lamp, and scrapings of epithelium were examined for the development of inclusions.

Clinical Material

One of the problems of trachoma is the diverse clinical conditions to which the name trachoma is applied. Because of this we limited our investigations to cases which fulfilled the criteria laid down by the WHO Expert Committee on Trachoma (1955). This committee stated that two of the following four features must be present before the diagnosis can be made:

- (1) Tarsal or limbal follicles
- (2) Epithelial keratitis
- (3) Pannus
- (4) Typical scars

However, it appeared to us that, even using these clinical features, more than one condition might be included, and for our isolation studies we also demanded the presence of typical trachomatous inclusions in the epithelial cells.

Since the areas in which this work was performed are widely separated, it is well to point out some of the differences in the disease in these areas. The cases seen in London were all old cases, and where inclusions were found they were almost all recrudescences of an old infection, the longest period between primary infection and relapse being 20 years, although it is possible this may have been a re-infection.

In Jordan, estimates of the trachoma incidence have been as high as 85 per cent. (Shusha, 1956), but by our standards of diagnosis we found the incidence much less than we had expected; the difference in estimates may be attributable partly to early treatment, but probably a more important factor is the common practice of designating all cases of chronic conjunctivitis as trachoma. Typical clinical trachoma was found in only 2 per cent. of hospital outpatients, and in only 20 per cent. of these was it possible to demonstrate inclusions in scrapings. Another impression that we gained from Jordan was that any form of antibiotic treatment greatly reduced the incidence of inclusions; thus active trachoma was much less common in refugee camps, where penicillin and streptomycin ointment was available free, than in villages where no free medical aid was available.

The clinical picture in Gambia showed some differences from those in Jordan, and the most striking of these was the relative absence of pannus from many of the cases which showed gross lid involvement. In the areas examined it was found that about 45 per cent. of the population had the WHO criteria of trachoma, and in about 20 to 25 per cent. of these it was possible to find cytoplasmic inclusions.

Results

The results of attempts at isolation are considered under each of the routes used:

(1) Chick Embryo

(a) *Chorio-allantoic Membrane*.—Six inclusion-positive scrapings were set up, each in batches of four eggs. An occasional lesion was seen in the primary culture but proved to be non-transmissible, and smears from the lesion stained by Giemsa stain failed to show the presence of inclusions.

(b) *Amniotic Inoculation*.—Four scrapings from cases showing inclusions were inoculated by this route; deaths were not more numerous than in control groups of eggs, and examination of embryos at 5 to 7 days failed to reveal any specific lesion.

(c) *Allantoic*.—Scrapings from three cases of trachoma were put into the allantoic cavity of batches of six embryos. No deaths occurred and tests for haemagglutination were negative, while stained smears did not show the presence of inclusions.

(d) *Visceral*.—The results of attempts to demonstrate virus by inoculation into the eye or brain of an embryo were negative.

(e) *Yolk Sac*.—In all, twenty positive scrapings were inoculated by this route in batches of four or six embryos. No greater death rate occurred than in saline controls, and yolk sac smears stained by Giemsa-May Grunwald stain and by Casteneda's stain did not show the presence of viral inclusions.

(2) **Mice.**—In neither adult nor in suckling mice could evidence of infection be found. No latent virus was unmasked by passage into a second group of animals.

In three of four groups of suckling mice in which cortisone was given with the inoculum, the mice receiving the trachomatous material died, while those receiving cortisone alone did not. Unfortunately, the carcasses were usually eaten by the mother, but those in one litter were killed when moribund, and in these no lesions which could be attributed to trachoma were found; the *post-mortem* appearances being similar to those seen by Antopol (1950). The carcasses of these mice were ground up in saline, and penicillin and streptomycin added, so that 10 per cent. emulsion of the mouse was made; inoculation of this suspension into a further batch of cortisone-treated mice gave a mortality similar to that in the control group receiving cortisone alone. It seems, therefore, that the initial deaths were due to some toxic factor acting synergistically with the cortisone rather than to any viral multiplication.

(3) Tissue Culture

(a) *Plasma Clot.*—Material from 10 cases with inclusions and 100 without inclusions was kept under observation by this method. In no instance was cellular multiplication observed, and neither was it possible to demonstrate the development of fresh inclusions.

In a few cases, preparations were also made on human serum agar, and watched by phase-contrast microscopy. No cellular change was seen that could be recognized as inclusion formation, but, owing to the lack of a hot box ciné-micrography apparatus, it was not possible to watch a single inclusion-containing cell over a period of time.

(b) *HeLa Cells.*—Material inoculated was all inclusion-containing, and with the exception of six batches from Gambia had all been inoculated with antibiotics:

- 25 batches inoculated from trachoma cases seen in Jerusalem
- 70 batches in which material was received freeze-dried from Gambia
- 40 batches in which material was received in Earle's saline from Gambia.

In three instances in Jerusalem, cytopathic degeneration suggestive of an adenovirus was seen, but owing to the interruption of work at the time of the Suez crisis the strains were lost and identification was not possible.

With the Gambian material it was striking that no cytopathogenic agents were isolated, as it was expected that both adenoviruses and herpes simplex which cause these changes would be encountered.

(c) *Conjunctival Cells.*—In view of the report of Murray and others (1957), in which isolation had been attempted from 600 cases of trachoma, only six positive scrapings were inoculated in these cells. Our findings like those of

Murray were negative. In our hands these conjunctival cells look most unlike the conjunctival cells in which trachomatous inclusions are normally found.

(d) *Tarsal Plate Cultures*.—Our experience with scrapings suggested that the superficial epithelial cells were incapable of multiplication, and also, judging by pH change in tissue culture media, had very little metabolism. Hence it was decided to try to culture the deeper layers of cells from the infected material found in tarsal plates removed at operation. It was hoped that, if epithelium grew out from such material, the fresh cells would become infected, and this would be made manifest either by cellular necrosis as was found by Rowe, Huebner, Hartley, Ward, and Parrott (1953) with outgrowths of tonsillar epithelium and adenoviruses, or by the appearance of inclusions in the cells. Unfortunately, most of the cellular proliferations from the explants were of fibroblasts, and in only six fragments did epithelium proliferate. In these six, the epithelial outgrowth occurred between the fifth and tenth day after the culture was set up and appeared as a fringe around the implant. Four of the explants showing epithelium were set up by the trypsin technique, and two were fragments directly implanted in plasma clots.

In all cases in which epithelium appeared to proliferate, growth was maintained for about 5 to 10 more days when, quite abruptly, the new cells were shed off the parent explant and underwent necrosis; no specific cellular degeneration appeared to precede the necrosis, although frequently the plasma clot in which the epithelium was growing commenced to undergo digestion at this time. Repair of the plasma clot or re-implantation of the fragment usually led to a secondary multiplication of fibroblasts.

It is essential to differentiate between the true outgrowth of epithelium and the large numbers of free rounded epithelial cells which appear round the explant during the first 72 hours. These latter cells are derived from the superficial layers of the epithelium, and like those removed by scraping do not appear to be able to multiply.

Two types of fibroblastic cells grow from the explants which do not form epithelium. The first to appear are small elongated cells which radiate from the explant like spines of a porcupine. Later, both within the area covered by these cells and in that peripheral to them, a larger fibroblastic cell appears, which shows a more random arrangement and often possesses three processes as against two in the smaller cells. Both these types of cells were kept alive in culture for up to 10 weeks, but during that time no evidence of damage (due to virus infection) appeared.

Attempts to obtain cultures of fibroblasts and of epithelium from conjunctiva that could be grown in serial culture were unsuccessful.

In order to demonstrate the absence of inclusions, tube cultures were washed in saline and then fixed in equal parts of absolute alcohol and ether, after which they were stained by the Giemsa-May Grunwald stain. Cultures

stained in this manner could be examined wet, or dried and examined after the tube had been filled with liquid paraffin; although tube preparations can only be examined by low-power objectives the typical trachomatous inclusion can readily be recognized at this magnification.

(e) *Complement-Fixation with Tissue Culture Fluids.*—Serum was obtained from a number of patients with clinical Stage II or III trachoma; these sera were pooled and inactivated at 56° C., and used to test tissue culture fluids for the presence of complement-fixing antigens.

In many instances tissue culture fluids proved highly anticomplementary, and it became necessary to dilute the antigen 1:5 before it could be used.

The following fluids were tested:

- 20 from HeLa cell cultures
- 6 from tarsal plate culture in which epithelium was necrosing
- 2 from conjunctival cell cultures.

In none was it possible to demonstrate significant evidence of complement-fixing antigens, although with one fluid from a tarsal plate there was a suggestion of partial fixation two tubes higher than the control; in view of the known anticomplementary nature of the tissue culture fluid, this was not considered to be significant.

(3) **Human Inoculation.**—On two occasions a volunteer was inoculated with material from patients containing many inclusions, which had been received freeze-dried. The subject was repeatedly examined for evidence of infection, but the only change noted was some discharge for 48 hours after inoculation which could be accounted for by the trauma of the procedure. There was no development of follicles and no corneal involvement, while scrapings failed to show the presence of trachoma inclusions.

Discussion

Despite our failure to isolate the trachoma virus, many points of interest arise from our results. The first is the confirmation of the many negative findings recorded in the literature, and the inability to obtain results similar to those of workers who have claimed to isolate the trachoma virus. In general, it seems that neither chick embryos nor mice are capable of supporting the multiplication of the virus, although it is possible that it may remain viable in the tissues without multiplying as has been suggested by the work of Stewart and Badir (1950), who maintained the virus by alternate passage in monkeys and eggs.

The claims to have isolated the virus by egg or mouse inoculation could be explained by this persistence of non-multiplying virus, or by the presence of another virus in the inoculum; this could well be the case with an adenovirus which one of us has found to persist for up to three passages in the

chick embryo. It is well to remember that to prove a virus to be the cause of a disease it is necessary to fulfil certain criteria which are as demanding as "Koch's postulates". These have been summarized by Jawetz, Thygeson, Hanna, Nicholas, and Kimura (1957), in their discussion on the aetiology of epidemic kerato-conjunctivitis, and so far no agent which will satisfy the requirements has been isolated from a case of trachoma; for example, none of the agents isolated has been made available to other laboratories so that the original findings can be confirmed by other workers.

With the material flown to London from Gambia, there is the criticism that the virus might not be viable after a 36-hr plane journey, and in favour of this is the inability to produce infection in a human volunteer. On the other hand it was possible to cultivate both epithelial and fibroblastic cells from the material, and it is difficult to imagine that the virus within a cell is more susceptible to trauma than the cell which it parasitizes. Work is still necessary to clarify this point, as the human experiments could have failed through an inadequate technique of infection or a natural resistance to infection of the individual; conversely, it could be argued that intracellular virus was non-infective and that extracellular particles were killed in transit. In favour of this latter point is the original observation of Halberstaedter and Prowazek (1907), that infection of apes was more readily accomplished with material from trachoma patients with discharging eyes than from those with relatively clean eyes.

These points emphasize one of the fundamental difficulties in the investigation of trachoma, namely, the lack of any suitable means of identification of the presence of the virus. We have relied largely on cytopathology and the development of inclusions, but it is quite possible that neither of these may occur, as there are frequently wide differences between the behaviour of a virus in its natural host and in tissue culture. Thus varicella fails to produce free virus in tissue culture, and measles virus produces giant cells only when bovine amniotic fluid or glutamine are added to the culture media. The most satisfactory way of demonstrating virus in tissue culture would be the use of human volunteers, who would not be exposed to a great risk as the early stages of the disease can be so effectively treated by tetracycline antibiotics.

Considering the problem of trachoma in Jordan and in Gambia, it is apparent that bacterial infections are a much greater problem in Jordan than in Gambia. Thus, with the greater incidence of active trachoma in Gambia, the late complications such as trichiasis should be much more numerous, but in fact this is not the case. In Jordan there appeared to be also more viral infections other than trachoma than were found in Gambia. (One of us, M.J.G., however, did see a number of cases of clinical herpes simplex infections in the latter territory.) It seems to us that these differences are rather a reflection of the climatic and social conditions than any intrinsic modification of the disease. Thus the lesser bacterial infection may well be

due to the static agricultural life of the Gambians, as opposed to the nomadic life of the Arabs. Furthermore, the strict division of the Gambian village into compounds may also be important in this respect, as it was not unusual to find two compounds in the same village with widely differing incidences of infection; the lower rate of infection being found nearly always in the compound with the higher social status. Alimuddin (1958) has drawn attention to the much greater incidence of pannus in the dry areas of Pakistan as compared with the wetter areas. Our experience would agree with his findings, in that pannus is not a feature of trachoma in Gambia, where the rainfall is much higher than in Jordan. Another interesting observation is that the incidence of trachoma dropped as soon as piped water supplies became available. This applied in both Gambia and Jordan, and it was only while writing this paper that we became aware that Victoria (1947) had made a similar observation in Argentina.

The failure to isolate trachoma from operative material was a disappointment, as it was hoped that the necrosis of epithelial outgrowths might be due to viral action; unfortunately it proved impossible to demonstrate the presence of virus by staining, by complement-fixation tests, or by the passage of material to other cell lines. Although, in the absence of attempts to infect humans, this does not preclude the presence of a virus, it does suggest that it was unlikely that any appreciable quantity of virus was present.

Our results show a complete inability to isolate the virus, and it may be of interest to others attempting isolations to draw attention to some of the possible reasons for this failure:

(1) *Failure to Survive Transportation.*—Snyder (1956) found difficulty in transporting virus and it may be that in all our experiments there was too great a delay between the taking of scrapings and the inoculation into animals or tissue cultures—particularly with the material from Gambia, but this point can be settled only by investigation in volunteers. In this connexion it should be noted that Julianelle and Harrison (1938) found that the virus would remain viable for from 1 to 7 days in saline kept at 4° C., whereas at room temperature (20–24° C.) the virus remained active only for from a few hours up to 1 day. It seems unlikely therefore, that time alone was sufficient to inactivate our inocula.

(2) *Antibiotics.*—Both Bietti (1954) and one of us (M.J.G.) observed that the systemic administration of penicillin may bring about a clinical cure of trachoma, and it is generally acknowledged that the tetracycline antibiotics are effective against the disease; it seems therefore that all attempts at isolation may have to be made in the absence of antibiotics. This greatly increases the difficulties of isolation in tissue culture, and it might be rewarding to make careful trials of the effects of other antibiotics on patients with trachoma inclusions to discover a suitable combination of antibiotics which would suppress bacterial growth but not the growth of the virus, and so be suitable for use in tissue culture.

Julianelle and Smith (1942) found that sulphonamides did not inactivate the virus *in vitro*, and it is probable that other antibiotics might not inactivate the virus

except when it was multiplying intracellularly. If this were so, it might be possible to sterilize with antibiotics the conjunctival secretions collected, and then to remove the cells and virus from the antibiotics by centrifugalization so that no inhibitory material would be carried over into the tissue culture.

(3) *The Virus may not be Cytopathogenic.*—It is possible that the virus is not cytopathogenic or inclusion-producing in tissue culture, or that it is incapable of entry into the cells of the culture. In this connexion it might be worth investigating the action of lysozyme, which is normally present in high concentration in the conjunctival sac, and might facilitate the entry of virus into the conjunctival cells. Alternately, the cells may fail to release infective virus, as has been found with varicella virus in tissue culture.

(4) *Role of Inclusions.*—Although there is little doubt in our minds of the association of cytoplasmic inclusions with trachoma, there is a possibility that they may not represent the infective agent. In this connexion it should be remembered that inclusions are found in superficial cells that are relatively inert and, on the whole, normal in appearance, whereas the main pathological changes of the disease occur in the subepithelial layers in which follicle formation and intense cellular infiltration occur. To explain this it was suggested that a viral toxin might be formed. Toxins have been described in certain viral infections such as lymphogranuloma inguinale (Rake and Jones, 1943), but even in these cases the toxin could not readily be separated from the virus particle, and it is usual to suppose that the toxic reactions to a virus disease are due to viral growth damaging cells rather than to any hypothetical toxin. It seemed to us more possible that the inclusions represented an "incomplete" viral form, produced as through the limited metabolism of these superficial cells preventing complete development of the virus. If this were the case, it would be essential to obtain material from the deeper layers of the conjunctiva for virus isolation. It might imply also that cells which would enable the virus to multiply in tissue culture would not show viral inclusions.

(5) *Cell Type.*—Throughout the previous discussion it has been assumed that the cells used can support the multiplication of trachoma virus. Quite possibly this may not be the case, as viruses do not always multiply in the tissue apparently most closely resembling that from which they are isolated; thus cytomegalic inclusion virus grows in fibroblasts, and certain ECHO viruses from human sources will multiply in monkey kidney but not in human cells. Hence it may be necessary to obtain completely fresh cell lines, and this would seem to be one of the most fruitful approaches to the problem of isolating the trachoma virus.

Summary

It has proved impossible to isolate trachoma virus in HeLa cells, conjunctival cells, mice or chick embryos. Some of the possible reasons for failure are discussed.

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REFERENCES

- ALIMUDDIN, M. (1958). *Brit. J. Ophthalm.*, **42**, 360.
- ANTOPOL, W. (1950). *Proc. Soc. exp. Biol. (N.Y.)*, **73**, 262.
- ARAKAWA, S., KITAMURA, O., MITSUI, Y., and TANAKA, C. (1953). *Arch. ges. Virusforsch.*, **5**, 208.
- BIETTI, G. B. (1955). "XVII Concilium Ophthalmologicum 1954, Canada, U.S.A. Acta", vol. 1, p. 160.
- BILLINGHAM, R. E., and MEDAWAR, P. B. (1951). *J. exp. Biol.*, **28**, 385.
- COLLIER, L. H. (1955). *J. Hyg. (Camb.)*, **53**, 76.
- (1957). *Nature (Lond.)*, **180**, 757.
- and SOWA, J. (1958). *Lancet*, **1**, 993.
- HALBERSTAEDTER, L., and PROWAZEK, S. VON (1907). *Arb. Gesundh. Amte*, **26**, 44.
- JAWETZ, E., THYGESON, P., HANNA, L., NICHOLAS, A., and KIMURA, S. J. (1957). *Amer. J. Ophthalm.*, **43**, No. 4, pt. 2, p. 79.
- JULIANELLE, L. A., and HARRISON, R. W. (1938). *Ibid.*, **21**, 529.
- and SMITH, J. E. (1942). *Ibid.*, **25**, 317.
- MACCHIAVELLO, A. (1944). *Rev. Ecuat. Hig.*, **1**, 33.
- MURRAY, E. S., CHANG, R. S., BELL, S. D., TARIZZO, M. L., and SNYDER, J. C. (1957). *Amer. J. Ophthalm.*, **43**, no. 4, pt. 2, p. 32.
- POLEFF, L. (1939). *Brit. J. Ophthalm.*, **23**, 738.
- RAKE, G., and JONES, H. (1943). *Proc. Soc. exp. Biol. (N.Y.)*, **53**, 86.
- RICE, C. E. (1936). *Amer. J. Ophthalm.*, **19**, 1.
- ROWE, W. P., HUEBNER, R. J., GILMORE, L. K., PARROTT, R. H., and WARD, T. G. (1953). *Proc. Soc. exp. Biol. (N.Y.)*, **84**, 570.
- SCOTT, J. G. (1945). *Brit. J. Ophthalm.*, **29**, 244.
- SHUSHA (1956). WHO. Special Report on Trachoma in Jordan (Tech. Rep. Ser. No. 106).
- SMITH, C. H. (Unpublished observations).
- SNYDER, J. C. (1956). Personal communication to Sir Stewart Duke-Elder.
- STEWART, F. H., and BADIR, G. (1950). *J. Path. Bact.*, **62**, 457.
- T'ANG FEI-FAN, CHANG HSIAO-LOU, HUANG YUAN-T'UNG, and WANG K'o CH'YEN (1957). *Chinese Med. J.*, **75**, 429.
- THYGESON, P. (1934). *Arch. Ophthalm. (Chicago)*, **12**, 307.
- (1939). *Ibid.*, **21**, 229.
- and PROCTER, F. I. (1935). *Ibid.*, **13**, 1018.
- and RICHARDS, P. (1938). *Ibid.*, **20**, 569.
- VICTORIA, V. (1947). "El tracoma". El Ateneo, Buenos Aires.