LABORATORY STUDIES OF EYE INFECTIONS IN SOUTH AFRICA

THE PROPERTIES OF THE VIRUS OF TRACHOMA

E. CUTHBERTSON, D. M. SMITH, and JAMES GEAR

South African Institute for Medical Research, Johannesburg

As described by Whitney and Gear,[1] a virus morphologically resembling the virus of trachoma was isolated in yolk-sac culture in chick embryos, the first of which was inoculated with a suspension prepared from a swab taken from the eyes of a 7-year-old African girl, Dorothea Phogele. This patient presented clinically with the typical picture of trachoma and she was admitted to the Jane Furse Hospital, Sekukuniland, North Eastern Transvaal, for further investigation.

The virus so isolated, named the Jane Furse strain, has been successfully established in serial passage in yolk-sac culture of chick embryos. Its properties have been studied and the findings are briefly reported in this paper.

Yolk-sac Culture

From the 1st to the 3rd passage, virus particles were scanty and were found only after prolonged search. In the 4th passage elementary bodies were numerous. The virus since its 5th passage has been passaged weekly. In these passages the yolk-sac membrane was harvested and the yolk allowed to drain. The membrane was then ground with glass and a 20% suspension prepared in nutrient broth. Of this suspension 0·2 c.c. was then inoculated through a hole punched in the blunt end of the egg into the yolk sac. This amount was used until the 27th passage. Most of the inoculated eggs were dead on the 4th or 5th day. From the 27th to the 30th passage the inoculum was reduced to 0·1 ml. and it was found that 25% of the eggs died on the 6th day. The remainder were harvested on the 7th day. Smears were made by snipping off a small piece of yolk-sac membrane washed free of yolk, and then dabbing it on to the centre of a clean glass slide. Other smears were made by spreading a fragment of yolk-sac membrane on a clean glass-slide covering with filter paper and then placing another slide over the filter paper and clipping the two slides together with paper clips. After warming the slide by passing through a bunsen flame and then allowing it to stand for 10 minutes, the slides were separated, and the filter paper gently lifted and the yolk sac peeled off the surface of the slide leaving a thin, often one-cell-thick impression smear. This was then either fixed in methyl alcohol and stained with Giemsa stain or stained by Machiavello's method.

These smears showed numerous elementary bodies in a large proportion of the eggs of each passage. Most were clearly defined, usually round, sometimes elongate or oval particles, many staining reddish pink with Machiavello's method and purple with Giemsa's stain, usually occurring singly, often in pairs and occasionally in larger clumps. These particles were usually found lying free, but in the impression smears compact masses were seen in the cytoplasm of some cells.

In the appearance in yolk-sac culture this virus resembles those isolated respectively in China by Tang *et al.*[2] and in West Africa by Collier and Sowa.[3]

Titration of Virus

The amount of virus in the yolk-sac membranes was determined by titration at intervals. Beginning with an original 20% suspension in broth, tenfold dilutions to 10^{-5} were made. Of each dilution 0·2 ml. was inoculated into the yolk sac of each of 4 7th-day embryonated eggs. Eggs dying before the 7th day of subsequent inoculation, and those still alive on the 7th day, were harvested. Smears were prepared from the yolk sacs and stained by Machiavello's method. By this method only those eggs in which the virus had grown profusely would give positive results. However, one set of eggs would be directly comparable with another. The results of this study show a progressive increase in the amount of virus present from the 6th to the 27th passage when the titre was 10^{-4}

Thermal Stability

In tests for thermal stability 20% yolk-sac suspensions were exposed in a water bath to temperatures of 37°, 56°, 60°C and +4° and −20°C respectively for varying periods. The results found are summarized as follows:

Temperature	*Survival period*
+4°	7 days+
37°	48 hours+
56°	30 minutes
60°	5 minutes
−20°	15 weeks+
−20° freeze-dried	6 months+
−70°CO_2 box	3 months+

EXPERIMENTAL ANIMAL PATHOGENICITY

The pathogenicity of virus suspensions for a number of experimental animals was investigated. The protocols of these experiments are summarized as follows:

White mice. In 3 separate experiments, 2 litters each of 6 7-day-old white mice were inoculated intraperitoneally and intracerebrally with 20% yolk-sac suspension containing large numbers of elementary bodies. These mice remained healthy and developed normally.

At the same time, on each of the 3 occasions, a batch of 12 adult white mice were inoculated intraperitoneally and intracerebrally with 20% infected yolk-sac suspension. They also remained well.

Guinea-pigs. A 30% suspension of yolk-sac membrane containing numerous elementary bodies was rubbed with a swab into the right eyes of 6 guinea-pigs. Smears and swabs

were taken at weekly intervals for 1 month and examined for the presence of virus. None was detected.

Rabbits. Two rabbits were similarly inoculated with a 30% suspension of yolk sac. Smears and swabs were taken from their eyes at weekly intervals for 1 month and examined for the presence of virus with negative results.

Calf. On 18 August 1959 a 30% suspension of yolk sac was inoculated into the left eye by rubbing it with a bacteriological cotton-wool swab soaked in the suspension. Smears and swabs were taken from both eyes weekly and examined for the presence of virus. None was detected.

Ram. On 18 August 1959 a ram was similarly inoculated into the left eye with 30% infected yolk sac suspension. Smears and swabs taken at weekly intervals thereafter and examined for the presence of virus gave negative results.

Gerbils, Tatera afra. After exposure to X-rays for 20 minutes giving a dose of approximately 600 R, 3 gerbils *Tatera afra* were inoculated intraperitoneally with a 20% yolk-sac suspension heavily infected with trachoma virus.

One was sacrificed on the 4th day and 1 on the 7th. The peritoneal fluid was inoculated into 6-day-old embryonated eggs and smears were made from the surface of the spleen and peritoneum and, after staining with Machiavello and Giemsa stains respectively, were examined for the presence of elementary and inclusion bodies. None were detected. The egg culture also yielded negative results.

These findings were of particular interest because gerbils were found to be unusually susceptible to infection with the rickettsiae of the typhus fever *R. prowazeki, R. mooseri*, and of tickbite fever *R. conori var. pijperi.*

Vervet monkeys, Cercopithecus aethiops pygerythrus. 25 May 1959. In an experiment carried out by Miss E. Whitney after taking smears and swabs from both eyes, 2 immature vervet monkeys were inoculated in the right eye with infected yolk-sac suspension by rubbing a cotton-wool swab soaked in 20% suspension of infected yolk sac on the conjunctiva. Smears and swabs were then taken weekly for 16 weeks and examined for virus. None was detected.

Rhesus monkeys. On 6 November 1959 an adult male rhesus monkey, *Macacus rhesus* was inoculated with the virus of trachoma by rubbing a cotton-wool swab soaked in a yolk-sac suspension containing numerous elementary bodies of trachoma virus. Smears and swabs were then taken at weekly intervals.

As before the smears were stained with either Macchiavello's or Giemsa stains and examined for the presence of elementary and inclusion bodies. No virus particles or inclusion bodies were detected.

A suspension was prepared from the swab in nutrient broth and then inoculated into the yolk sacs of 6-day-old embryonated eggs. Three passages of the eggs inoculated with the suspension prepared from the 1st swab were made, but with negative results.

On 8 January 1960 a baby rhesus monkey was inoculated, first into the right eye by rubbing with a swab soaked with yolk-sac suspension. Smears and swabs were taken weekly. The smears were stained and examined as before and a suspension prepared from the swabs was inoculated into yolk sacs of 6th day embryos. No virus was detected.

On 29 January 1960, three weeks later, this monkey's left eye was similarly inoculated and smears and cultures taken as before. Again the results were negative.

Baboons. These have not yet been tested, but it is planned to do this in the near future when the susceptibility of the South African species *Papio ursinus* will be investigated.

From the experimental studies so far carried out it is apparent that this virus is relatively non-pathogenic for common experimental animals, and also for some less commonly used rodents and for the rhesus and vervet monkeys.

TISSUE CULTURE

A comprehensive and determined effort was made to establish the trachoma virus in tissue culture.

Tissue cultures were prepared for inoculation from many different tissues, namely:

1. *The human tissues* used included human amnion, human amnion line, human conjunctiva lines 1 and 2, human embryonic eye line, human eye line, Hela cells and K.B. cells.

2. The following *animal tissues* were used: Vervet monkey kidney, vervet monkey embryonic kidney line, vervet monkey testis, bone marrow and lymphoid tissue, white mouse lung, *Mystromys albicaudatus* kindey, guinea-pig heart, chick embryo, and dog kidney.

HISTORY OF CELL CULTURES

The following are brief accounts of the history of the cell cultures used in this work:

Human Tissues

Human amnion cultures are prepared as a routine from placentae received every week from the Queen Victoria Hospital, Johannesburg. A cell suspension sufficient for the preparation of about 50 tissue-culture tubes is received by the Trachoma Unit each week.

Human amnion line. The human amnion line was established in permanent line culture by Mrs. F. le Roux On 13 November 1959 it was received and replanted after trypsinization in Eagle's medium +10% human serum. The growth was unsatisfactory. The tissue was again trypsinized and the suspension replanted in Eagle's medium +10% fowl serum. The growth was more satisfactory and now the culture grows well and is in its 68th passage. The medium used for maintenance is Connaught 199 medium+5% fowl serum.

Human conjunctiva 1. On 1 July 1959 tissue from a naevus of the eye was received in nutrient fluid from Dr. Graham Scott, who had removed it at operation that day. This was cut into small fragments which were planted in clot culture prepared with fowl plasma and chick embryo extract. On 17 July 1959 a good growth was apparent and the culture was trypsinized and subcultures prepared. These also grew well and since then weekly passages have been made. The culture is now in its 27th passage.

Human conjunctiva 2. On 15 July 1959 this tissue, a pterguim, removed at operation that day by Dr. Graham Scott, was received in nutrient medium. It was minced and planted in clot culture with fowl plasma and chick embryo extract. A good outgrowth was observed on 1 August 1959 and the culture was trypsinized and, after washing the resulting suspension of cells, was reseeded into a fresh set of tubes. Good growths were obtained and now this culture line is in its 22nd passage.

Human embryo eye. On 12 June 1959 an eye from a human embryo was received from Dr. H. H. Malherbe. It was minced and planted in clot culture prepared with fowl-plasma and chick-embryo extract.

On 18 June 1959 a good outgrowth of cells was apparent. The culture was then trypsinized and planted on fresh tubes. Since then weekly trypsinizations and subcultures have been prepared from the resulting suspension of cells. The planting and outgrowth medium used is Connaught 199 plus 20% human serum. The maintenance medium is Connaught 199+5% bovine or horse serum. This line of cells is now in its 32nd passage. In appearance the cells resemble fibroblasts.

Human eye cell culture. On 24 September 1959 this eye was enucleated from an adult patient by Dr. E. Epstein, and was collected at the operating theatre and taken to the laboratory. Here it was minced and the fragments planted in clot cultures prepared with fowl-plasma and chick-embryo extract. On 19 October 1959 a good outgrowth was obtained and the culture was trypsinized and subcultures prepared from the cell suspension. Subcultures have been made at weekly intervals since then and the line of cells so established is now in its 17th passage. In appearance these cells resemble fibroblasts.

Hela cells line culture. This culture was originally received from Dr. G. C. Gye in 1954 and has been maintained in culture since then.

K.B. cells. The K.B. culture line was received from Dr. P. Sureau of Madagascar.

Animal Tissues

Vervet monkey kidney-cultures. These cultures are prepared each week from kidneys removed the same day from wild caught vervet monkeys *Cercopithecus aethiops pygerythrus.*

Monkey-embryo kidney line. A line of cells was successfully established from the kidneys of an embryo monkey by Dr. H. H. Malherbe. This was received from him on 20 November 1959 and has been maintained in continuous culture since then during which time it has been passaged 10 times.

Vervet monkey bone marrow. 7 January 1960. Bone marrow removed from the ribs was prepared in clot-tissue cultures with fowl-plasma and chick-embryo extract. It is now in its 4th passage. The growth shows a mixture of epithelial and fibroblast-like cells.

Monkey lymphoid tissue. 7 January 1960. A lymph gland removed from the groin of a vervet monkey was minced and clot cultures prepared with fowl-plasma and chick-embryo extract. The growth resembles fibroblasts.

Mouse lung line 1. On 30 November 1959 fragments from a lung of an adult white mouse were planted in clot culture prepared with fowl-plasma and chick-embryo extract.

On 6 November 1959 a good outgrowth of cells was apparent and the culture was trypsinized and subcultures were prepared. This culture was successfully maintained for 6 passages when it became contaminated and was discarded.

Mouse lung line 2. A similar line was established from a primary culture planted on 4 January 1960. It has now been successfully passaged 4 times.

Mystromys albicaudatus kidney line. This line of cells was successfully established by Mrs. I. Spence from the kidneys of *Mystromys albicaudatus*, a rodent of the South African veld which breeds well in captivity and has been of value as an experimental animal.

Guinea-pig heart line. This line of cells was established by Mrs. Le Roux from the heart of a young guinea-pig. It has been successfully maintained since and was received for trachoma virus studies on 13 November 1959. It is now in its 45th passage.

Dog kidney line 1. 18 January 1960. A 5-day-old puppy, supplied by Dr. J. H. Mason of this Institute, was sacrificed and the kidneys removed. These were minced and clot cultures prepared. A good outgrowth was apparent on the 4th day and the culture was trypsinized on the 6th day, and subcultures were prepared. It is now in passage 3.

Dog kidney line 2. 28 January 1960. A 1-day-old puppy was sacrificed and the kidneys aseptically removed. Clot cultures were prepared and from them subcultures as described above.

Guinea-pig kidney. 28 January 1960. Kidneys removed from the 3-week-old guinea-pig and cultures and subcultures have been prepared.

All tissue-culture tubes were actively growing homogenous sheets of cells at the time of their inoculation. Before inoculation they were washed 3 times with Connaught 199 medium containing streptomycin.

In the 1st series of experiments the virus suspension was prepared from yolk sacs of infected chick embryos. A 20% suspension was made in nutrient broth and this was inoculated in 0·5 ml. amounts directly on to the cell sheet and allowed to stand for 10 minutes before adding 2 ml. of nutrient fluid consisting of Connaught medium 199+5% horse serum with only streptomycin added. The tubes were rolled and incubated at 37°C.

In the 2nd series of experiments the inoculum was prepared as before, but was left on the tissues of the culture tubes for periods of 30 minutes to 2 hours before the nutrient medium was added. It was then centrifuged at 3,000 r.p.m. for 1 hour.

In the 3rd series the inoculum was centrifuged at 10,000 r.p.m. for 1 hour and the pellet resuspended in bovine plasma albumen before inoculating directly on to the tissue before addition of the nutrient medium. The experiments, using the 3rd method, were repeated 4 times.

In a 3rd series of experiments the inoculum was frozen and thawed 3 times before being used to inoculate the tissue-culture tubes.

In none of these experiments was growth of the trachoma virus observed.

In one series of experiments the temperature of incubation was 37°C, in another 34°C, and in yet another 32°C.

The same tissues were then used, but they were inoculated with a concentrated inoculum.

In the 1st of this series the infected yolk sacs were harvested and a 20% suspension prepared in bovine plasma albumin. The suspension was then centrifuged at 3,000 r.p.m. for 30 minutes and 0·5 ml. inoculated directly on to the washed tissue before the addition of medium.

In a 4th series of experiments several tissues were inoculated while in suspension following trypsinization of their seed cultures and then planted in tubes. The tissues used were the lines derived from human conjunctiva, human embryonic eye, guinea-pig heart, monkey embryo kidney, and Hela cells. The cells were washed well in Hank's balanced salt solution and then suspended in Connaught medium 199+10% bovine serum. A 20% suspension of infected yolk sacs was added in the proportionate amounts of 0·2 ml. to 2 ml. of nutrient medium. The cells were then planted in tubes and left stationary at 37°C for 48 hours before being placed in roller drums. Only the Hela and human conjuntival cells grew.

The Hela cells show no degeneration. The human conjunctival cells showed rounding on the 6th day after inoculation. The fluid was harvested and passed to fresh tissue-culture tubes. Rounding of the cells occurred in some tubes of each passage for 5 passages, after which no further degeneration was seen.

Fluid from each passage was inoculated into the yolk sacs of 6-day-old embryonated cells. The eggs were harvested after 7 days. Films were prepared from the yolk-sac membrane and stained with Macchiavello stains. In none were elementary bodies detected.

In experiment 38 guinea-pig heart tissue was washed 3 times with Connaught medium and then 0·1 ml. of infected yolk-sac suspension was inoculated on to the tissue sheet and left for 10 minutes before adding the nutrient medium of Connaught medium + bovine serum.

Marked degeneration of the cells was observed on the 8th day after inoculation. The fluid was harvested and passaged to fresh tissue-culture tubes of the guinea-pig heart line. Seven passages were made in series. In each degeneration of the culture cells was observed. The control tubes for each of these passages remained normal.

At each passage the harvested fluid was inoculated into the yolk sacs of 6-day-old embryonated eggs. On the 7th day after inoculation the yolk sacs were harvested, ground, and suspended in nutrient broth and passed back into tissue culture tubes. Degeneration was observed for 2 passages but not after the 3rd passage. Films were also prepared from the yolk sacs and stained with Macchiavello's stain. No elementary bodies resembling those of trachoma virus were observed.

The harvested fluid from the tissue culture tubes was also inoculated into tissue culture tubes of other tissues, but these showed no degeneration.

From these findings it seems that the agent which was passed in series causing degeneration of the guinea-pig heart cultures was a virus, but was probably not trachoma virus. Its origin is not known.

Monkey testes. On 16 February 1960 the testes of a vervet monkey, *Cercopithecus aethiops pygerythrus* were removed and planted. Good growth occurred and the culture was trypsinized, and the cells resuspended and planted. A good growth again occurred and this was again trypsinized on 29 February 1960, and culture tubes prepared and incubated. A confluent sheet of cells was obtained and this was inoculated on 11 March 1960 with a rich suspension of trachoma virus. This was prepared by centrifuging a yolk suspension at 3,000 r.p.m. for 2 hours at 4°C. The supernatant was removed, and the pellet resuspended in bovine plasma albumen and inoculated directly on to the tissue in 0·1 ml. amounts. The tubes were then left for 30 minutes and LY medium without serum and without phenol red was added.

On 17 March 1960 very little degeneration of cells was noted. The cells were scraped from the tube and the suspension centrifuged at 1,800 r.p.m. for 5 minutes. The pellet was resuspended and inoculated into 6-day-old embryonated eggs.

The eggs were harvested on the 7th day and smears prepared from the yolk-sac membrane showed granules staining pink with Macchiavello's stain and resembling the elementary bodies of trachoma virus.

This experiment was then repeated. Smears made from the tissue culture showed numerous elementary bodies resembling those of trachoma virus. Many cells also showed masses of elementary bodies within the cytoplasm staining pink with Macchiavello's stain. These resembled trachoma virus and were so numerous that it was apparent that growth of the virus had occurred in these cultures of testicular tissue from the vervet monkey.

DISCUSSION

In all over 70 different experiments were carried out in attempts to culture the trachoma virus. In these experiments well-tried techniques eminently successful in growing other viruses were used, but without success. Various modifications were then tried including lower temperatures of incubation, different methods of inoculation, and different concentrations of virus in the inoculum and various media, and differing proportions of the constituents of the medium. Streptomycin was the only antibiotic used. Media without phenol red pH indicator was also tried.

All these attempts to culture the trachoma virus gave negative results.

It seemed unlikely that a virus which grows so well in the yolk sacs of chick embryonated eggs would not have some tissue culture preparation favourable for its growth, and so our search for it was continued. The desired preparation has now apparently been found in tissue cultures prepared from from the testes of vervet monkeys *Cercopithecus aethiops pygerythrus*. In the smears of cells from these cultures inoculated with a rich suspension of trachoma virus prepared from yolk-sac cultures, large numbers of elementary bodies staining pink with Macchiavello's stain were seen. Smears made from the yolk sacs of eggs inoculated with these cultures showed granules staining pink with Macchiavello's stain and resembling the elementary bodies of trachoma virus. Further studies of this virus are under way to confirm its identity.

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