

Effect of tissue culture cells in promoting prolonged survival of *N. gonorrhoeae* in artificial subcutaneous cavities of mice

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Arko (1972) and Flynn and Waitkins (1973) reported that gonococci survived for varying times, in artificial subcutaneous chambers in mice and other animals, but there was a discrepancy in the survival times observed. Flynn and Waitkins (1973) found that the gonococci survived for 2 days, whereas Arko (1972) noted survival for periods up to several months. The reasons for these differences may be that Arko employed the synthetic steroid Dexamethasone (Assem Schering) and massive doses of gonococci approx. 0.25×10^8 per ml. as opposed to the 0.5×10^4 gonococci per ml. used by Flynn and Waitkins.

Phagocytosis of gonococci by tissue culture mouse fibroblast cells and their subsequent protection from the bactericidal action of rat serum was noted in the same year (Waitkins and Flynn, 1973). Clearly, if ingested gonococci were also protected in the mucous membrane of the genital tract, such cells containing surviving gonococci might be the most effective inoculum in the transmission of the disease.

In order to explore this possibility it was decided to try to establish a prolonged infection within the *in vivo* situation of a subcutaneous cavity of the mouse. These cavities gave free access to the mouse's immunological defence mechanisms, both humoral and cellular, and because of this it seemed an ideal system to investigate the fate of the gonococcus.

Material and methods

ANTIBIOTICS

These were added to the tissue growth medium in the following concentrations: Vancomycin HCl (Lilly) 5 µg./ml., penicillin (Vetric) 100 units/ml., streptomycin 100 µg./ml., and nystatin 25 i.u./ml.

MEDIA

5 per cent. lysed horse-blood agar, prepared using Columbia agar (Oxoid) 39 g. distilled water 1,000 ml. The agar

was soaked for 15 min., mixed, and autoclaved at 121°C for 15 min. When it had cooled to 55°C, 50 ml. sterile horse blood (Wellcome) lysed by repeated freezing and thawing was added.

Difco G.C. Medium Base plus Defined Supplement (GCMB), prepared as described by White and Kellogg (1965). 1 per cent. *Glycerol peptone broth*. 10 ml. of a 1 per cent. peptone broth was added to 10 ml. of 8 per cent. glycerol.

Tissue culture media

Eagle's Minimum Essential Medium (MEM) was obtained as a ten times concentrated stock solution from Wellcome Reagents Ltd. (Eagle, 1959).

Eagle's Growth Medium (EGM). 100 ml. MEM, 100 ml. calf serum (Biocult), and 20 ml. of 4.4 per cent. sodium bicarbonate were added to 780 ml. deionized water.

Eagle's Maintenance Medium (EMM). As for EGM, but 20 ml. calf serum were added instead of 100 ml. *Medium '199'* (Wellcome). Stock solution diluted 1 in 10 with distilled water, plus 0.85 mg./litre sodium bicarbonate (Morgan, Morton, and Parker 1950).

Dulbecco solution, pH 7.3. Tablets for preparing Dulbecco A (Oxoid Ltd.) were dissolved in 100 ml. distilled water, autoclaved at 115°C for 10 min. Full Dulbecco Saline (PBS) was prepared as described by Dulbecco and Vogt (1954).

Versene solution. 0.2 g. Ethylene diamine tetra-acetic acid was added to 1 litre of Dulbecco A, and the pH adjusted to 7.4. It was sterilized by autoclaving at 115°C for 10 min., and dispensed aseptically into sterile bottles.

Electron microscope solutions. 3 per cent. glutaraldehyde solution was prepared from 25 per cent. stock solution diluted in phosphate buffer pH 7.3.

Sucrose phosphate buffer (0.2 molar). 13.68 g. sucrose was added to 100 ml. phosphate buffer pH 7.3. It was dissolved and sterilized by Seitz filtration.

TEST ORGANISM

The strain of *N. gonorrhoeae* (82406) used was isolated from a patient attending the Sheffield Venereal Disease Clinic. It was initially grown on 5 per cent. lysed horse-blood agar with 1 per cent. vancomycin (5 µg./ml.). It was identified by Gram's stain, a positive oxidase reaction, and fermentation of glucose but not maltose or sucrose.

Fermentations were tested in a modified Difco GC medium base, with 1 per cent. of the sugar (Flynn and Waitkins, 1972). After one subculture it was preserved by 'snap freezing' in liquid N₂ with 1 per cent. glycerol peptone broth as suspending medium. Kellogg's Type I colonies were used in these experiments (Kellogg, Peacock, Deacon, Brown, and Pakle, 1963).

MICE

Inbred strains of Balb/C mice (Sheffield strain), varying in weight from 15 to 20 g., were used. They were fed a standard diet and water was given *ad lib*.

SUBCUTANEOUS CAVITIES

Artificial chambers were made in the subcutaneous tissues of the mice as described by Flynn and Waitkins (1973) (Figs 1 and 2).

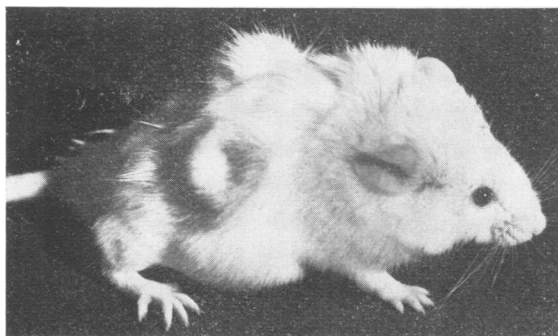


FIG. 1 Mouse with two vinyl rings in position

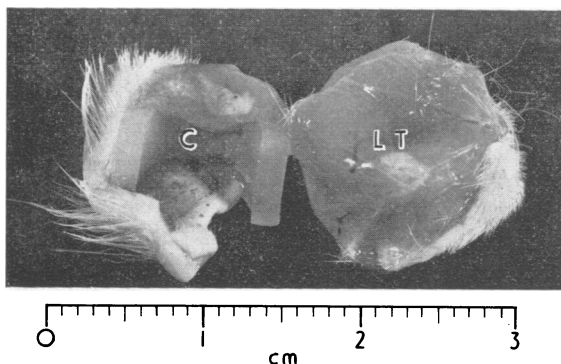


FIG. 2 Cross-section of subcutaneous cavity. Note layer of tissue (LT) on inside of cavity forming a complete cavity (C)

TISSUE CULTURES OF BALB/C INBRED MOUSE CELLS

Primary cell cultures were prepared from 14 to 16-day-old embryos. These were removed aseptically from the pregnant animal and skinned; the feet, head, and tail were removed and the remaining tissue chopped into fine pieces with scalpels in '199' medium. The fragments were then trypsinized with 0.25 per cent. trypsin in phosphate buffered saline pH 7.3, and left for 30 min. at 37°C. After this time, the trypsin was discarded completely, and the trypsination repeated three times, the discarded

trypsin supernatants were collected and 10 per cent. serum added to stop the action of the trypsin. The final solution was then centrifuged at 800 r.p.m. for 5 min, and the deposit washed in '199' medium. This procedure was repeated once. The resulting deposit of 'cells' was then re-suspended in EGM containing antibiotics. The cell suspension was then diluted with EGM and dispensed into 20 oz. bottles at a concentration of approximately 10⁶ cells/ml. A confluent cell sheet was normally obtained in 3 to 4 days.

Just before inoculation with *N. gonorrhoeae*, the overlay was poured off and the monolayer was thoroughly washed several times with warmed Dulbecco saline to remove all antibiotics. Finally, fresh Eagle's medium *without* antibiotics was added.

Previous observations by our group (Waitkins and Flynn, 1973) indicated that the greatest engulfment of gonococci occurred after 8 hrs' incubation. For this reason the tissue culture cells were inoculated with 5 × 10⁸ gonococci/ml. and incubated at 37°C for 8 hrs. The overlay was then replaced with fresh Eagle's medium containing 20 per cent. neat rat serum (which is bactericidal for the gonococcus), and the monolayer exposed to this environment for 30 min. The rat serum kills off all extracellular gonococci (Flynn, 1972). The monolayer was then washed thoroughly with PBS to remove all rat serum and broken up with a rubber-tipped glass rod. These tissue culture cells containing gonococci were then centrifuged at 800 r.p.m. for 5 min, to pack down the cells. The packed pellet was then re-suspended in 5 ml. PBS solution and the number of gonococci/ml. estimated by the technique of Miles, Misra, and Irwin (1938). 0.2 ml. volumes of 5 × 10⁴ gonococci/ml. in tissue culture cells were inoculated into the right chamber of each mouse. Into the left chamber 0.2 ml. volume of gonococci (5 × 10⁴ per ml.) without tissue culture were inoculated. A control of tissue culture cells alone (1 × 10⁶ cells/ml.) was inoculated into the right and left chambers of a further three mice.

To prove that the gonococci were truly intracellular within the tissue culture cells, thin sections of these cultures which had been exposed for 8 hrs to *N. gonorrhoeae* were cut and examined under the electron microscope as described below (Fig. 3, overleaf).

ELECTRON MICROSCOPY

A preparation that had been incubating for 8 hrs was washed twice with warmed PBS, and versene was added to break up the monolayer of cells. The dispersed cells were gently centrifuged at 400 r.p.m. for 4 hrs and fixed with 3 per cent. glutaraldehyde at 4°C for 2 hrs. The resulting pellet was washed gently three times in sucrose-phosphate buffer over a period of 24 hrs. The pellet was finally fixed in 2 per cent. unbuffered osmium tetra-oxide, block stained with 0.5 per cent. uranyl acetate (pH 5.0), dehydrated with increasing concentrations of alcohol, and impregnated with Araldite. Sections were cut by an ultramicrotome and stained with Reynolds' lead citrate. An A.E.I. EM/81 electron microscope was used for viewing.

HISTOLOGICAL PREPARATIONS

Haematoxylin and eosin stained thin sections of the walls of the subcutaneous cavities after inoculation were

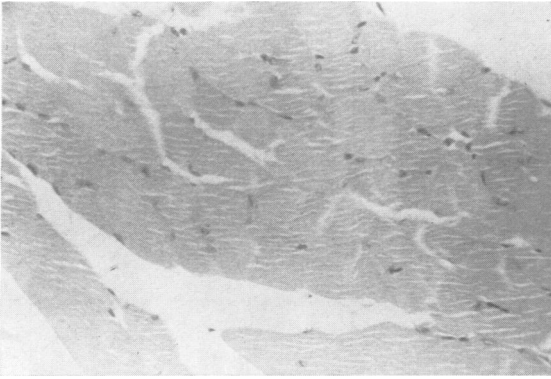


FIG. 4 Histological section of a cavity on Day 1 after inoculation. Very few polymorphs can be seen. Haematoxylin and eosin. $\times 270$

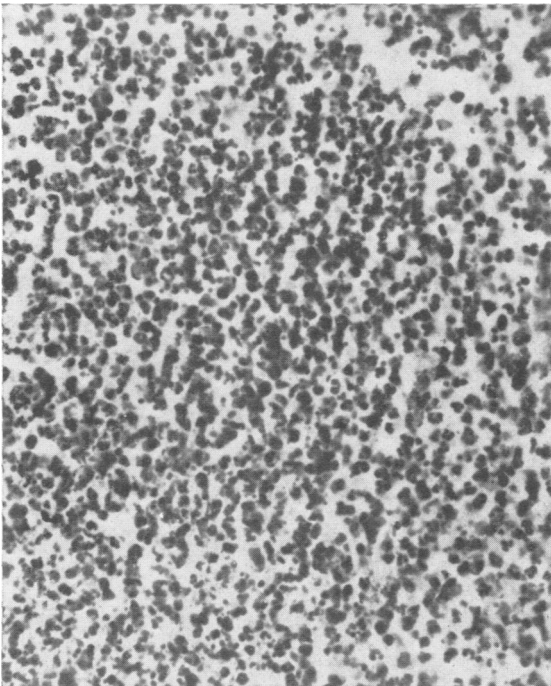


FIG. 5 Histological section of cavity 4 days after inoculation with gonococci alone. Large numbers of polymorphs and lymphocytes can be seen invading the tissues. Haematoxylin and eosin. $\times 460$

containing gonococci showed little polymorph invasion, while viable gonococci could still be cultured (Fig. 6). By day 10 there was a polymorph and lymphocyte invasion into the cavity containing gonococci inside tissue culture cells (Fig. 7), similar to that seen at day 4 in the gonococci alone cavity indicating that the gonococcus was now being destroyed by the cellular response of the mouse;

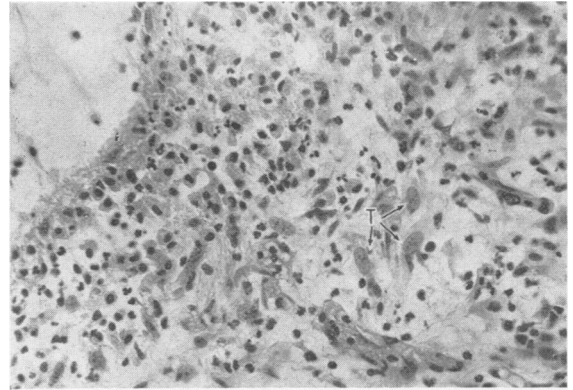


FIG. 6 Histological section of cavity 4 days after inoculation with gonococci inside tissue culture cells. Much smaller numbers of polymorphs can be seen. Note tissue culture cells (T) still adhering to cavity. Haematoxylin and eosin. $\times 460$

this was substantiated by the complete failure to recover live, viable gonococci after day 10 from cavities which had been inoculated with tissue culture cells containing gonococci.

Histological sections from control mice, which had not been inoculated with any form of gonococci or tissue culture cells, showed little evidence of immunological cellular reaction. However, sections from mice which had tissue culture cells inoculated into them again revealed polymorph and lymphocyte invasion by the 10th day (Fig. 8), but little polymorph invasion on the 4th day; these results were almost identical to those of the cavities containing tissue cells and gonococci.

Discussion

It has been observed previously that gonococci are engulfed and protected within tissue culture cells (Waitkins and Flynn, 1973) and in human epithelial cells from urethral pus (Waitkins, Flynn, and Carr, 1973). We suggested that these epithelial cells containing gonococci might be significant, particularly in the transmission of the disease. From the present series of experiments, it is clear that gonococci which have not been protected from the host defence mechanism are more rapidly destroyed than gonococci protected within tissue culture cells, probably because they can establish themselves and survive the immunological attack by the host. Fig. 6 shows clearly that, after 4 days, tissue culture cells were still intact within the cavities, perhaps even attached to them. The failure of the gonococcus to survive beyond 10 days was probably due to graft rejection, which caused the destruction of the tissue culture cells and the death of the gonococci they contained.

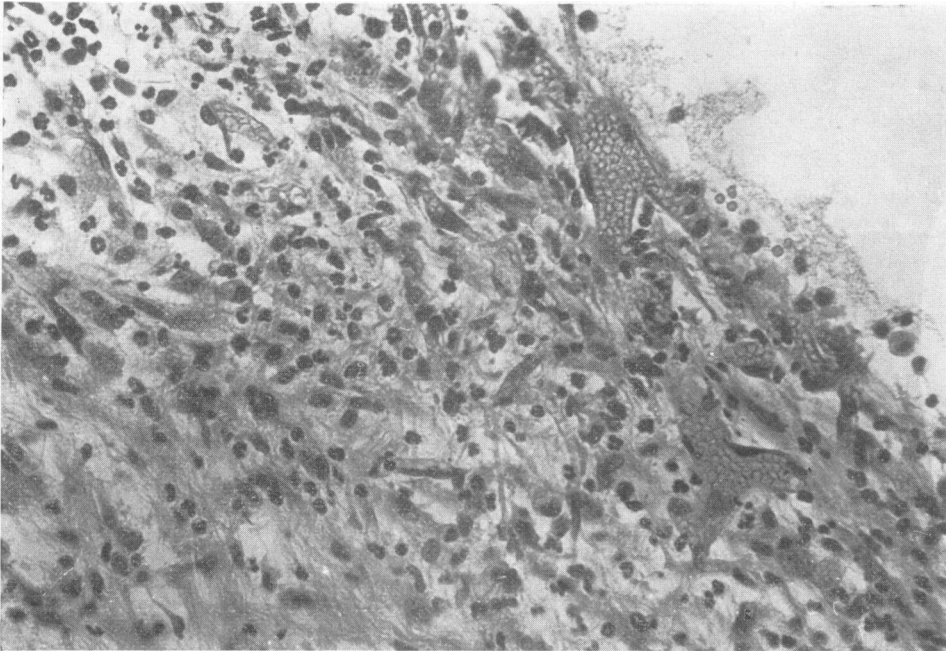


FIG. 7 *Histological section of cavity 10 days after inoculation with gonococci inside tissue culture cells. Large numbers of polymorphs and some lymphocytes can be seen. Haematoxylin and eosin. $\times 460$*

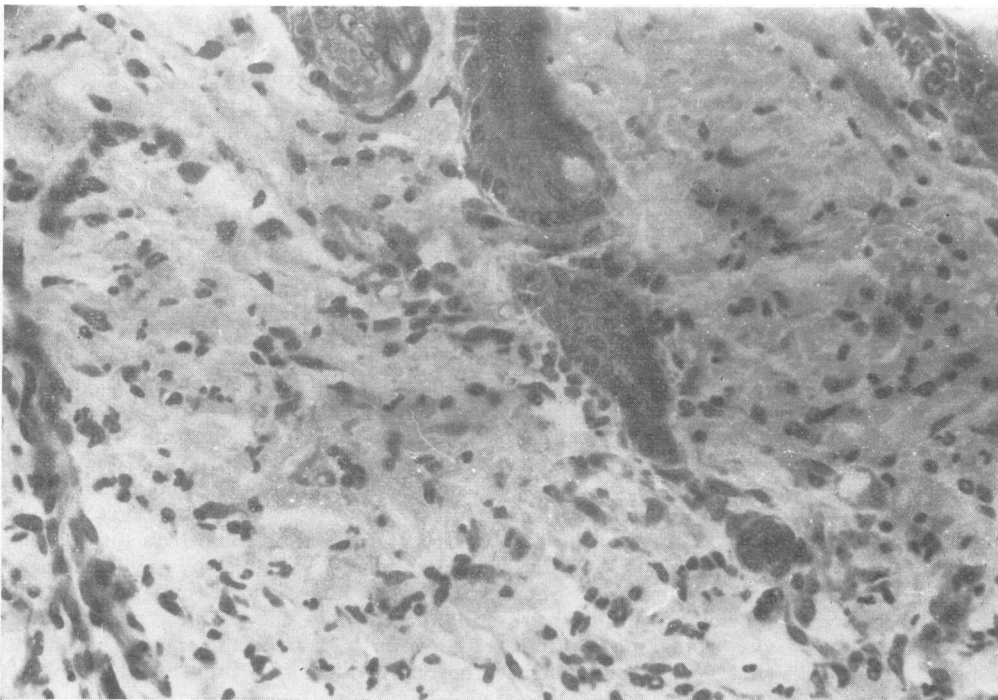


FIG. 8 *Histological section of cavity 10 days after inoculation with tissue culture cells alone. Polymorph and lymphocyte invasion suggests immunological rejection of tissue cells. Haematoxylin and eosin. $\times 460$*

Summary

Both tissue culture cells and human epithelial cells ingest gonococci, and subsequently protect them from the lethal action of various bactericidal agents. Using the convenient chamber produced by the subcutaneous insertion of a polyvinyl ring, the fate of the gonococci in mice was studied *in vivo*. Both humoral and cellular factors have free access to the organisms, and under normal circumstances these do not survive longer than 3 days. However, when gonococci were protected within tissue culture cells, their survival was prolonged. Only eventual graft rejection caused the death of the tissue culture cells and gonococci contained within them.

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