INFECTION OF ARTIFICIAL AIR POUCHES IN THE CONNECTIVE TISSUE OF MICE WITH NEISSERIA GONORRHOEAE

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PLATES X-XIV

Numerous animal models have been employed for the study of Neisseria gonorrhoeae infection (Arko, 1972 and 1974; Buchanan and Gotschlich, 1973; Flynn and Waitkins, 1973; Diena et al., 1975; Veale et al., 1975; Arko et al., 1976; Chandler, Kraus and Watts, 1976; Johnson, Taylor-Robinson and Slavin, 1977; Novotny et al., 1977). The methods have included subcutaneous implantation in rabbits, guinea-pigs and mice of artificial chambers made from polyethylene or other materials, infection of chick embryos, intracerebral infection of mice, and urethral infection of chimpanzees.

Each of these animal models has certain drawbacks. Chamber implants involve extensive manipulation of the animals and the infectious process may not be similar to that occurring in normal tissues. The high cost and scarcity of the animal limits the usefulness of the chimpanzee model, and in some instances, e.g., mouse intracerebral infections, the model does not resemble the human disease.

The mouse connective-tissue air pouch (Higginbotham, Doughterty and Jee, 1956) has been used extensively for the study of mast-cell responses (Clark and Higginbotham, 1968) and has recently been suggested as a possible means for the study of cell-mediated immune reactions in the mouse (Clark, Menduke and Wheelock, 1975). The mouse air pouch is easily manipulated and allows the study of inflammatory reactions such as that seen in most gonococcal infections. We have therefore investigated its use as a possible means of studying N. gonorrhoeae infections.

MATERIALS AND METHODS

Organisms. Neisseria gonorrhoeae was isolated from primary cultures of clinical cases of gonorrhoea. Kellogg's type-1 colonies (Kellogg et al., 1963 and 1968) were selected from the primary isolates, subcultured on chocolate-agar plates overnight in an atmosphere containing 5% CO₂ and then inoculated in Schaedler's Broth (Baltimore Biological Laboratories) supplemented with 1% IsoVitaleX (Baltimore Biological Laboratories). The broth was incubated in a tissue-culture incubator for 24 h before use, to saturate the medium with CO₂. Broth cultures were incubated overnight in 5% CO₂ and the type-1 N. gonorrhoeae cells were centrifuged from the cultures and washed with pyrogen-free physiological saline before use. Three separate isolates of N. gonorrhoeae were used in this study. No detectable differences were observed between the responses of the experimental animals to inoculation with each isolate.

Infection and examination of mice. Connective-tissue air pouches were prepared in random-bred Swiss Webster mice by injecting 1.0 ml of air subcutaneously on the dorsal surface to form an air bubble in the loose subcutaneous connective tissue (Higginbotham et al., 1956) as illustrated in fig. 1. N. gonorrhoeae cells (10⁵) in 0.2 ml of saline were then injected into each pouch. At appropriate intervals, the animals were killed and each connective-tissue bubble was dissected free from the surrounding tissues (fig. 1). A portion of the tissue at the ventral surface of the bubble was removed and either spread on a glass

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microscope slide, rapidly air dried and stained with May-Gruenwald Giemsa for light microscopy or fixed with 3% glutaraldehyde in cacodylate buffer for electron microscopy.

Recovery of the gonococci from an air pouch was attempted either by swabbing the interior of the pouch with a sterile swab, which was then cultured on chocolate agar or GC Agar (Difco) supplemented with 1% IsoVitaleX in an atmosphere of 5% CO₂, or by homogenising a portion of the connective tissue from the pouch in a tissue homogeniser and culturing the homogenate on chocolate or GC agar.

Connective-tissue samples fixed in glutaraldehyde for electron microscopy were post-fixed in 1% OsO₄, dehydrated in graded ethanols and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined in a Hitachi HS-8 electron microscope.

RESULTS

Connective-tissue samples were obtained from inoculated animals at various intervals after challenge. Samples removed from animals inoculated 6 h earlier with 10⁵ colony-forming units of N. gonorrhoeae exhibited a typical acute-inflammatory response in the connective tissue (fig. 2a). The fibroblasts of the connective tissue were seen together with an infiltrate consisting mainly of polymorphonuclear leucocytes. The cellular response 24 h after infection was still predominantly polymorphonuclear with some apparent localisation of gonococci around fibroblasts (fig. 2b, arrow). The infiltrating leucocytes were actively engaged in phagocytosis of the gonococci 6 h after infection.

Connective-tissue spreads obtained from animals 7 days after infection exhibited localised areas of heavy polymorphonuclear infiltrate (fig. 3a). These areas did not extend over the entire connective-tissue spread as did the infiltrate seen 6 and 24 h after infection; 10 days after infection the leucocyte infiltration was still less intense and this allowed the localisation of gonococci within fibroblasts (fig. 3b) to become apparent. The leucocyte infiltration was absent 21 days after infection except in very localised areas. In samples of connective tissue obtained at this time, cytoplasmic inclusions resembling gonococci were evident within fibroblasts. In samples obtained from animals 35 days after infection, intracytoplasmic inclusions were still evident within mononuclear cells of the connective tissue. Animals inoculated with either saline or Schaedler's broth alone demonstrated a transient polymorphonuclear infiltrate and a return to normal connective tissue in 72–96 h. No cytoplasmic inclusions such as those seen in the fibroblasts of the infected mice were noted in the control animals.

Recovery of viable N. gonorrhoeae from the air pouches was attempted either by swabbing the interior of the pouch or by culturing homogenised tissue. Viable organisms producing Kellogg's type-1 colonies on appropriate media could be recovered from the infected animals.

<table>
<thead>
<tr>
<th>Interval (days) after infection</th>
<th>Number of mice in groups of five that yielded N. gonorrhoeae from swabs</th>
<th>homogenised tissue</th>
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<td>&lt;1</td>
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<td>3</td>
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* Group contained six mice.
for as long as 10 days after infection (table). Because the connective-tissue samples from
different animals varied greatly in size, no attempt was made to assess precisely the content
of viable organisms. However, more than 300 colonies of type-1 \textit{N. gonorrhoeae} were
obtained from single swabs of infected pouches for up to 10 days after infection. After 10
days no viable organisms could be recovered.

Connective-tissue samples were also taken for electron microscopy. Fig. 4 demonstrates
the apparent phagocytosis and degradation of gonococci by connective-tissue fibroblasts
within 6 h of infection. Intracellular gonococci were also evident in fibroblasts from animals
21 and 35 days after infection (fig. 5) although viable gonococci could not be recovered; as
no degradative processes could be seen in the intracellular organisms, it seems that gonococci
may be able to withstand an intracellular environment for extended periods of time.

\textbf{DISCUSSION}

We have shown that \textit{N. gonorrhoeae} will set up an infectious process in connective-tissue
air pouches in mice. The method should provide a convenient and useful means of studying
gonococcal infections.

The infiltration of polymorphonuclear leucocytes into the connective tissue of mice
inoculated with viable gonococci was very striking, and more pronounced than that which
usually follows the injection of non-infectious agents or of organisms such as \textit{Staphylococcus
aureus} (unpublished data). The infiltration persisted for considerable periods and a more
chronic type of inflammation with a mononuclear component did not develop. The areas
of leucocyte infiltration were fairly well localised, suggesting a local release of a chemotactic
substance. It is possible that intracellular gonococci eventually destroy the fibroblasts
containing them, thereby releasing viable organisms and cellular debris into the ground
substance of the connective tissue and attracting leucocytes. This observation is supported
by the findings of Novotny, Short and Walker (1975) who observed that the gonococcal
infectious unit was a cluster of bacteria surrounded by remnants of phagocytic cells; these
observations have led to a proposed explanation of the infectious process in gonorrhoea
(Novotny \textit{et al.}, 1977) in which multiplication in macrophages is an essential part. Our
present observations suggest that the fibroblast might take the place of the macrophage in
such a scheme.

The uptake of gonococci by connective-tissue fibroblasts observed in the current study is
not surprising as there have been numerous reports in the literature concerning the uptake of
these organisms by different types of cell (Ward and Watt, 1972; Swanson, 1973; Waitkins
and Flynn, 1973; Garcia-Kutzbach \textit{et al.}, 1974; Stone, Vernon and Warren, 1974; Novotny
\textit{et al.}, 1975 and 1977; Waitkins, 1975). Electron micrographs of intracellular gonococci in
neutrophils (Ovchinnikov, Delektorskij and Dmitriev, 1976) as well as in tissue-culture and
other cells resemble those of the intracellular organisms in our study. Proof that these
intracellular inclusions are gonococci requires additional studies now in progress.

Our inability to recover viable gonococci after 10 days from the air pouches may have
been due to the techniques employed, because in electron micrographs gonococci appeared
to remain within fibroblasts for up to 35 days. The homogenisation method employed may
not readily have released the organisms from the fibroblasts and it is possible that the
organisms recovered within 10 days of infection were extracellular. In view of the substantial
number of colony-forming units recovered for up to 10 days, the organisms were probably
multiplying in the connective tissue of the air pouch. It does not seem likely that any of the
inoculated organisms would have survived for 10 days in the face of the massive polymorpho-
nuclear infiltrate that they induced.

Recent studies by Payne and Finkelstein (1975) have shown the role of iron in infection
with \textit{N. gonorrhoeae}. The Schaedler's broth employed for the growth of the infecting
organisms in the present study contains haemin, 0·01 g/litre; this would allow the organisms
to maintain their virulence.

The use of connective-tissue air pouches in mice should lend itself to immunological
studies in relation to gonococcal and possibly other infections.
ARTIFICIAL AIR POUCHES IN THE CONNECTIVE TISSUE OF MICE AS A MEANS OF STUDYING NEISSERIA GONORRHOEAE INFECTIONS

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SUMMARY

Artificial air pouches in the connective tissue of mice were evaluated as a means of studying Neisseria gonorrhoeae infections. Animals inoculated with type-1 N. gonorrhoeae cells developed an infection characterized by infiltration of polymorphonuclear leucocytes. Viable cocci could be recovered from the air pouches for up to 10 days after infection and intracellular cocci were evident in electronmicrographs within connective-tissue fibroblasts for at least 35 days, indicating that a persistent infection had been established. The mouse air pouch should be of value in the study of gonococcal and other infections.

We wish to acknowledge the technical assistance of Katherine Zachariasewycz and Stanford Ratner.

REFERENCES

INFECTION OF MICE WITH Neisseria gonorrhoeae

Fig. 1.—(Above). Mouse inoculated with 1 ml of air to form a bubble (arrow) in the subcutaneous connective tissue. (Below). The skin has been retracted to expose the connective-tissue bubble (arrow).
INFECTION OF MICE WITH Neisseria gonorrhoeae

Fig. 2.—Connective tissue "spreads" from animals inoculated with Neisseria gonorrhoeae. May-Gruenwald Giemsa (MGG). × 500. (a) At 6 h after infection. Fibroblasts (F) present with a striking polymorphonuclear infiltrate. (b) At 24 h after infection. There is apparent localisation of gonococci around fibroblasts (arrow).
Infection of mice with *Neisseria gonorrhoeae*

**Fig. 3.**—Connective-tissue "spreads" from animals inoculated with *N. gonorrhoeae*. MGG. (a) At 7 days after infection. ×500. (b) At 10 days after infection. Gonococci have localised around fibroblasts (arrow). ×2500.
Fig. 4.—Connective tissue from animals infected with *N. gonorrhoeae*. Electronmicrograph (EM). ×10,000. (a) At 2 h after infection. Gonococcus (arrow) in close proximity to fibroblast (F). (b) At 6 h after infection. Intracellular gonococci present in fibroblast with possible degraded organism (arrow x) and organisms in vacuoles possibly undergoing degradative changes (arrows y and z).
Fig. 5.—Connective tissue from animals infected with *N. gonorrhoeae*. EM. ×20,000. (a) At 21 days after infection. Note relatively normal appearance of intracellular organisms (arrow). (b) At 35 days after infection. Gonococcus within fibroblast (arrow).


