THE SURVIVAL OF GONOCOCCI AND MENINGOCOCCI IN SUBCUTANEOUS DIFFUSION CHAMBERS IN MICE

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PLATE II

The use of a Millipore diffusion chamber technique for the study of antimicrobial systems in vivo was reported by Roantree and Collis (1960) and by Steward and Roantree (1961). The procedure was subsequently used for a variety of bacteriological investigations in small laboratory animals, including studies on staphylococci (Houser and Berry, 1961); streptococci (Tan, Hackel and Kaplan, 1961); salmonellae (Steward, Collis and Roantree, 1964; Osawa et al., 1967); Listeria (Osebold and DiCapua, 1968; Osebold, Pearson and Medin, 1974); and Trypanosoma cruzi (Logan and Hanson, 1974).

The work of Arko (1972) on subcutaneous chambers in small laboratory animals provided a new approach to the study of Neisseria gonorrhoeae in experimental animals. Flynn and Waitkins (1973) found that gonococci survived for only 1 day when held in simple open chambers implanted subcutaneously in mice. In subsequent experiments, Waitkins (1975) showed that when gonococci were taken into cells grown in tissue culture and then held in chambers in mice, the survival time of the intracellular bacteria was extended.

The present study was undertaken in an attempt to determine the extent to which the longer survival of gonococci within tissue-culture cells may simply have been due to protection of the organisms from the cellular defences of the host. Standardised suspensions of gonococci were placed in Millipore diffusion chambers which were implanted subcutaneously instead of intraperitoneally as in most previous reports. Our diffusion chambers allowed the diffusion of all humoral factors but excluded any cells and are described as "closed", whereas Arko's chambers allowed free access of both humoral and cellular elements and are described as "open".

Studies on the survival of gonococci in these Millipore chambers were made in groups of mice and the results obtained in normal mice compared with those in mice pre-immunised with whole gonococci. Similar studies were also carried out with meningococci (N. meningitidis) in the chambers in place of gonococci.

MATERIALS AND METHODS

Experimental animals. Swiss albino Webster mice of 18–22 g were used. They were fed a standard diet and water was given ad libitum.
Organisms. Freshly isolated strains of *N. gonorrhoeae* selectively subcultured for colonial types 1 and 4, and recently isolated strains of *N. meningitidis* of types A and B were used.

Culture media. The strains of gonococci and meningococci used to prepare inocula for chambers were first selectively subcultured on Difco G.C. Base with 2% defined supplement. After incubation for 18–20 h at 36°C in air with 10% carbon dioxide, the growth was harvested and emulsified in Phosphate Buffered Saline (PBS, Oxoid), pH 7.3 and standardised by readings on the nephelometer which had been calibrated to give estimated viable counts for these suspensions. Recorded counts were confirmed by plate counts from time to time.

The same medium was used for the isolation of organisms from chambers removed from test mice.

Immunisation of mice. Groups of mice were immunised by intramuscular injection of a suspension of live gonococci or meningococci standardised to a density of approximately $10^9$ organisms per ml. Three injections of 0.1 ml were given each week for 3 weeks.

Diffusion chambers. Initially the diffusion chambers were constructed by bonding 14-mm Millipore filters of pore size 0.45 μm to Plexiglass rings (14-mm outside diameter x 10-mm internal diameter x 2 mm deep) with a filling hole 0.59 mm in diameter through which the bacterial suspension was injected. Early in the investigation, a polymorphonuclear leucocyte was found in one sample of chamber fluid and so we decided to use filters of smaller pore size (0.22 μm) in all subsequent chambers. There was in effect no difference in the findings between the first group of 20 mice implanted with chambers made with 0.45-μm membranes and the remainder when 0.22-μm ones were used. In retrospect, it seems probable that the cell we found was evidence of a cracked membrane in that chamber. As the volume of bacterial suspension that could be recovered from conventional Millipore chambers was inadequate, we subsequently made a rather deeper ring by cutting 8-mm sections from PVC tubing of 12-mm outside diameter x 9-mm inside diameter (Baird and Tatlock, 275/0250).

![Graph](image-url)

**Fig. 2.—Survival of gonococci in Millipore chambers in mice; □—□ *Neisseria gonorrhoeae* in normal mice; ○—○ *N. gonorrhoeae* in pre-immunised mice; △—△ CF-antibody level in mice.
and assembling these in the same way as the standard Millipore chambers. A further advantage of these PVC rings was that chambers made from them could be sterilised by autoclaving at 115°C (10 lb/in²) for 10 min. whereas the Millipore chambers made from Plexiglass were not heat resistant and had to be sterilised by ultraviolet irradiation.

A major problem in making PVC chambers was to ensure a flat surface at the cut ends. This was achieved by fitting lengths of the tubing on to the shaft of a small mixer motor and cutting with a fixed scalpel blade whilst the tube revolved. Sections cut in this way gave perfect surfaces for the bonding of Millipore filters. Fig. 1 shows the two types of chamber.

Experimental design

Millipore chambers containing gonococci were implanted subcutaneously into 100 normal mice; 50 received type-1 gonococci and 50 received type-4. A further 10 mice, preimmunised with gonococci, had chambers implanted containing type-1 gonococci. 100 normal mice had chambers implanted containing meningococci.

0.1 ml of the test bacterial suspension was injected with a 23-gauge needle into the chamber and the hole was sealed with a plastic plug and Millipore cement. After sealing, sample chambers were tested by immersion in liquid culture medium; no positive cultures were observed with the test samples. Filled chambers were implanted subcutaneously over the back in groups of mice, each mouse received one chamber with the Millipore surfaces parallel to the skin. Half of the first group of mice received original chambers and half modified chambers, thereafter the larger chambers were used. Chambers were removed at intervals for sampling; the external surface was swabbed to remove tissue fluids, the upper filter was removed and the contents of the chamber mixed and taken up into 9.9 ml of PBS to give a standard dilution of 1 in 100. Viable counts were then done by the method of Miles, Misra and Irwin (1938). The identity of the organisms was confirmed by Gram's stain, oxidase and sugar fermentation tests, and by fluorescent antibody tests. The colonial types of the gonococci grown from the chambers were checked. After the chambers had been removed, the anaesthetised mice were bled out and the serum was separated. Serum samples were then tested for complement-fixing antibody in tests with Wellcome gonococcus antigen (Burroughs Wellcome lot no. K. 7230).

RESULTS

Composite "growth curves" derived from individual chamber counts show the behaviour of organisms in implanted chambers (figs. 2 and 3) and the amounts of antibody produced against gonococci (fig. 2).

Plot A, fig. 2, shows a sharp initial fall in the number of gonococci in the chamber up to the 3rd day after implantation, at which point the organisms began to grow, reaching a maximum count of $8.5 \times 10^7$ per ml by day 10; the numbers then declined fairly rapidly so that no organisms were isolated after 17 days. The probable reason for this is suggested by plot B of fig. 2 which shows the titre of (CF) antibody in the serum of these mice. As might be expected, the number of organisms present in the Millipore chambers declined as the amount of antibody increased. The assumed relationship of antibody to the decline was supported by the results obtained when chambers were put into a group of mice pre-immunised with gonococci (Plot C). The schedule of immunisation was arranged so that at the time of implantation the mice showed an antibody titre of c. 640 which was equal to that developed by about the 18th day in the first group of mice. In the pre-immunised mice the number of viable organisms declined at a rate similar to that seen in the latter part of
plot A, leading to the very rapid elimination of all the gonococci. In the first group of mice, in the main, the gonococci remained in the colonial type in which they were implanted. Colonial type 4 did not revert back to type 1, although occasional type-4 colonies were obtained from chambers inoculated with type-1 colonies.

In contrast to the findings obtained with gonococci (plot A), the behaviour of meningococci of types A and B in similar chambers was quite different (fig. 3). The total count (plot D) began to rise from the very earliest sample tested after implantation and continued to do so. Meningococci survived to the end of the longest period tested, which was 49 days, at which time local swelling developed around the implant and this led to induration and local necrosis around the upper edge of the chamber until finally the entire chamber was rejected. The rejected chamber still contained viable meningococci.

DISCUSSION

Our observations on the growth of neisseriae in implanted Millipore chambers show clearly that these organisms can survive for 18-49 days when
they are protected from cellular factors. This was not the case when chambers of the Arko type, in which the organisms were freely accessible to host cells, were used by Flynn and Waitkins (1973).

The differences that we observed in the behaviour of gonococci and meningococci are interesting but not entirely surprising. We know from experience that the meningococcus is a much less delicate organism than the gonococcus. The meningococci in our experiments might therefore have been more resistant to lysis on implantation so that there was no initial drop in the count and the early antigenic stimulus would be negligible. On the other hand, with the gonococci there was marked initial lysis, as shown by the fall in the initial count, and there would be a substantial antigenic stimulus leading to a brisk antibody response that eliminated the organism from the chambers. In the absence of such an antibody response the meningococci continued to grow. These differences are similar to those reported by other workers. For example, Roantree and Collis (1960) tested a series of enterobacteria and found their survival in Millipore chambers to be directly related to their sensitivity to complement-dependent serum factors. Osebold and DiCapua (1968) found *Listeria monocytogenes* within Millipore chambers to be insensitive to such serum factors and to survive for as long as 173 days.

If an effective immune response is to develop it is normally essential for the host cells, especially macrophages, to have access to the organisms. It is, therefore, most interesting that the diffusible products of lysis of the gonococcus were able to stimulate an antibody response in the test mice leading to the destruction of gonococci within Millipore chambers. These observations both on the effectiveness of immunisation with whole gonococci and on that occurring naturally in mice exposed to the diffusible products of lysis of gonococci are in marked contrast to those of Turner and Novotny (1976) who found that antibodies to gonococcal pili were without influence on the host's defence response to gonococci in open subcutaneous chambers in mice.

**Summary**

Studies are reported on the survival of gonococci and meningococci in Millipore diffusion chambers implanted subcutaneously in normal mice and in pre-immunised mice. The chambers allowed the passage of nutrients and humoral factors but excluded host cells.

After an initial fall in the viable count —attributed to lysis by non-specific serum factors—there was evidence of multiplication of *Neisseria gonorrhoeae*; the subsequent development of specific antibody led to the disappearance of gonococci 16 days after the chambers were implanted.

*N. meningitidis* behaved differently in the implanted chambers. Meningococci did not appear to be lysed by non-specific humoral factors and so the viable count showed no initial fall. The meningococci survived for 49 days, at which time the entire chamber was rejected.

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REFERENCES


STEWARD, J. P., COLLIS, L. R. AND ROANTREE, R. J. 1964. Effects of active immunization and of total body X-irradiation upon the humoral bactericidal system of the guinea pig as measured with strains of enteric bacilli.  J. Immun., 92, 616.


FIG. 1.—The two types of chamber used (×2).