DIFFERENTIAL ABILITY OF COLONIAL TYPES OF *NEISSERIA GONORRHOEAE* TO PRODUCE INFECTION AND AN INFLAMMATORY RESPONSE IN SUBCUTANEOUS PERFORATED PLASTIC CHAMBERS IN GUINEA-PIGS AND RABBITS

D. R. VEALE, H. SMITH, K. A. WITT AND R. B. MARSHALL

Department of Microbiology, University of Birmingham, Birmingham B15 2TT and Microbiological Research Establishment, Porton, Salisbury, Wiltshire

PLATE XVII

RESEARCH on gonorrhoea has been hampered by the lack of suitable animal models. Although human volunteers have been infected with urethral pus and with gonococci grown *in vitro* (Hill, 1943; Kellogg *et al.*, 1968) such experiments are limited by ethical considerations. An animal model would not only facilitate investigations of the pathogenicity of *Neisseria gonorrhoeae* but might also act as an assay system for testing the anti-gonococcal activity of drugs and vaccines. Recently, Brown, Lucas and Kuhn (1972) infected the urethra of male chimpanzees with *N. gonorrhoeae* isolated from human pus and from culture, and demonstrated transmission of infection from a male to a female chimpanzee by coitus. This system seems to parallel the human situation but is expensive. However, similar experiments might be accommodated by a model in small laboratory animals that has been developed by Arko (1972) from a method used in virology (Tosi *et al.*, 1970). Perforated polyethylene practice golf-balls were implanted subcutaneously into rabbits, and stainless-steel springs into guinea-pigs, rats, hamsters and mice. The chambers became encapsulated with connective tissue and filled with a sterile transudate. Gonococci were inoculated into the chambers and persistent infections established in all five species. An anti-inflammation agent (dexamethasone) was used in the rabbits, rats and guinea pigs but not in hamsters and mice. Rats were the most difficult animals to infect. Using a similar system, Flynn and Waitkins (1973) implanted annular plastic chambers subcutaneously in mice. They did not use anti-inflammation drugs and achieved only fleeting infections. Many years before, *N. gonorrhoeae* had been grown *in vivo* in collodion sacs (Harris, 1939) and in the anterior chamber of the rabbit eye (Miller, 1948). However, for use in studies of pathogenicity and immunity the latter system has technical drawbacks, and collodion sacs exclude host defence-mechanisms, especially the inflammatory response. The Arko system seemed convenient

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technically but the inflammatory response was not mentioned in the original description (Arko, 1972), and was referred to briefly and only with reference to mice in a more recent paper (Arko, 1974).

This paper describes the examination of a modified Arko technique in guinea-pigs and rabbits to see if it would provide a basis for future studies of the aggressive mechanisms of gonococci. There were two objectives. The first was to determine whether there is an inflammatory response to infection and, if so, whether infection can be sustained, preferably without the use of anti-inflammation agents. The second objective was to try to detect differences in infectivity between different colonial types of gonococci, and to determine whether such differences correspond with those detected in human infections (Kellogg *et al.*, 1968).

MATERIALS AND METHODS

Animals. The animals used were Hartley strain guinea-pigs (weight 500–600 g), New Zealand white rabbits and, occasionally, other rabbit strains bred at Allington Farm, near Salisbury, Wiltshire.

Strains of N. gonorrhoeae. Large- and small-colony types, strains AL and AS, were isolated by selective subculture from the urethral pus of a single patient. Strain BS (small-colony type) was selected from the urethral pus of another patient. Strains AS and BS grew as small shiny colonies, possessed pili and had other properties similar respectively to those of the types 1 and 2 of Kellog *et al.* (1963). Strain AL produced large colonies, was not pilated, and resembled the type-4 organism described by Kellog *et al.* (1963). The strains were preserved by snap-freezing in a solution of Proteose Peptone (Oxoid) 1% w/v and glycerol 8% v/v in water and stored in liquid nitrogen.

Culture media. Gonococci were selectively subcultured on a solid GC Basal Medium (Oxoid, CM 367) with a supplement similar to that described by Amies and Garabedian (1967) but not containing antibiotics. Small-colony types were stable on this medium. A medium containing the same supplement but a different base (Difco Bacto GC Medium Base, 0289-01) was used for viable counts since it gave a better plating efficiency. Plates were incubated in candle-cans for 24 h for the preparation of infection inocula and 48 h for the determination of viable counts. Trypticase Soy Broth (Baltimore Biological Laboratories, 11768) containing 10% of inactivated sheep serum (SS-TSB) was used for dilutions.

Plastic chambers. Guinea-pigs were implanted with two polyallomer 4·5-ml centrifuge tubes (Beckmann Ltd, Croydon) which had had 11 holes made in their sides and bottoms with a hot No. 2 cork borer and their mouths partially closed with Michel clips (fig. 1). Rabbits were implanted with two to four 12-ml tubes which had had 15 holes in their sides and bottoms made with a No. 3 cork borer and their mouths similarly closed (fig. 1). Rabbits were occasionally implanted with two plastic practice golf-balls as described by Arko (1972 and 1974). The chambers were sterilised by autoclaving (at 121°C for 15 min.), except the polyvinyl golf balls, which were sterilised by gentle boiling for 30 min.

Implantation of chambers. Animals were shaved in the lumbar region and anaesthetised, rabbits with Nembutal (Abbot Laboratories) and other animals with ether. The skin was swabbed with sterilising fluid (Penotrane, Ward Blenkinsop & Co., Wembley). After a small incision, the skin was separated from the underlying connective tissue and a plastic chamber pushed through the incision and under the skin. The wound was closed with Michel clips and sprayed with plastic dressing (Nobecutane, British Drug Houses). The chambers were left 30-60 days to encapsulate and fill with transudate.

Inoculation and sampling of chambers. Gonococcal colonies from a 24 h culture were washed from the solid medium with 2–5 ml of SS-TSB and the suspension was shaken with glass beads for 5 min. Total counts (in a Thoma chamber) and viable counts (60–100% of total counts in most experiments) were made and suitable dilutions prepared. Before

injections, the skin was swabbed with sterilising fluid. A sample of 0·2 ml of the transudate was removed for cell counts before inoculation. Various doses of gonococci, suspended in 0·2 ml of SS-TSB, were injected with a 25-gauge needle into the chambers through one of the perforations. For passaging organisms, the contents of an infected chamber were diluted with SS-TSB and injected in a similar manner. No attempt was made to dissociate or count the inoculum except for the determination of the minimal infectious dose on passaged material, when the organisms grown *in vivo* were treated as those grown *in vitro*.

Chambers were sampled by inserting a needle (as for inoculation) and withdrawing about 0·4 ml of fluid. This was diluted in SS-TSB for viable counts of gonococci and total counts or erythrocytes and leucocytes. In some experiments, a drop of transudate was smeared on a degreased slide and stained with Giemsa stain (Gurr's Improved, Searle Ltd, High Wycombe). Gonococci isolated from chambers were identified by colonial form, oxidase reaction and microscopic appearance after Gram-staining (Wilkinson, 1962).

Persistent infection and approximate MID50. A strain was said to produce persistent infection if gonococci could be cultured (not less than 500 organisms per ml) from the exudate for at least 4 days after inoculation. In most cases of persistent infection the numbers of gonococci were greater than 500 per ml. Approximate MID50s of the various types were obtained by noting the lowest inocula to produce a persistent infection, as defined above, in 50% of the chambers inoculated.

Counts of erythrocytes and differential counts of leucocytes. Total counts were done in a Neubauer chamber. Differential counts of polymorphonuclear leucocytes (PMN) and mononuclear phagocytes (MNP) were obtained by counting at least 200 cells in a stained smear (Gurr's Improved Giemsa stain). Some smears contained up to 90% of degenerate cells that were impossible to type.

Treatment of rabbits with betamethasone (Glaxo Laboratories, Greenford, Middlesex). Rabbits were given either a single dose of 1–4 mg soluble betamethasone injected into the chambers with the gonococci (day 0) or multiple doses of insoluble betamethasone (2 mg subcutaneously on days −2, −1, 0 and twice weekly thereafter) combined with a single dose of soluble betamethasone (1 mg per chamber) injected alongside the chambers on day 0.

TABLE I

Number of persistent infections produced by various strains of gonococci in guinea-pig chambers

Inoculum	Number of chambers persistently infected*/number tested, with the stated inoculum of (and approximate MID50 for)			
	AL	AS	BS	AS or BS passaged†
0–50	...	0/6	0/6	3/4
50–500	...	2/6	4/11	4/4
500–5×10^3	0/2	3/8	5/6	7/7
5×10^3–5×10^4	0/2	2/6	5/5	8/8
5×10^4–5×10^5	0/2	5/6 (10^4)	1/1 (10^3)	4/4 (5)
5×10^5–5×10^6	2/3‡	5/7	3/4	...
5×10^6–5×10^7	0/3	3/3	1/2	...
5×10^7–5×10^8	2/3‡	1/1	2/2	...
5×10^8–5×10^9	0/4	...	3/3	...

... = Tests not done.

* Not less than 500 gonococci per ml of transudate for at least 4 days.

† Three times in guinea-pig chambers.

‡ Three of these infections disappeared in 7 days and the other in 10 days.

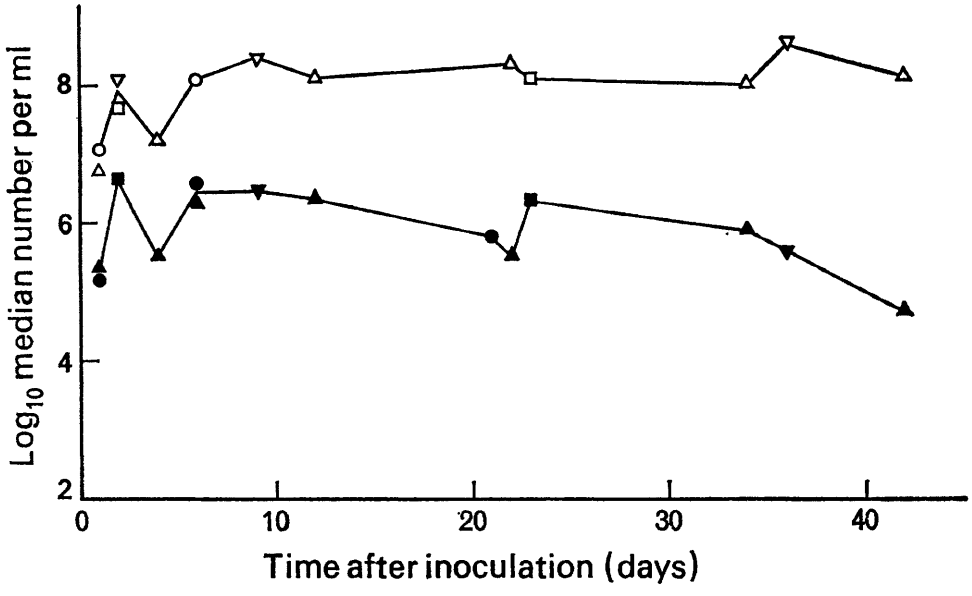

FIG. 2.—Concentration of gonococci (solid symbols) and leucocytes (open symbols) in guinea-pig chambers at the indicated times after inoculation. Each symbol represents the median concentration in 5 to 11 persistently infected chambers: ▲, △ = experiment 1; ●, ○ = experiment 2; ■, □ = experiment 3; ▼, ▽ = experiment 4. Variously sized doses of gonococcal strains AS and BS were given. Median concentration of leucocytes in the chambers before inoculation = 3×10^6 per ml.

TABLE II

Percentages of polymorphonuclear leucocytes (PMN) and mononuclear phagocytes (MNP) in guinea pig-chambers at various times after inoculation*

In guinea-pigs with	Percentage of							
	PMN on day†				MNP on day†			
	1	3	8	10	1	3	8	10
Persistent infection	76	90	83	89	21	6	12	8
Unsuccessful infection	89	80	51	60	14	12	38	30

* The corresponding percentages in uninoculated chambers were 53 and 36 (mean of observations on 35 chambers).

† Two guinea-pig experiments involving 21 chambers were done, one sampled on days 1 and 8 and the other sampled on days 3 and 10. The figures are the mean percentages of PMN and MNP in all the chambers when various inocula of strains AS, BS and AL were used.

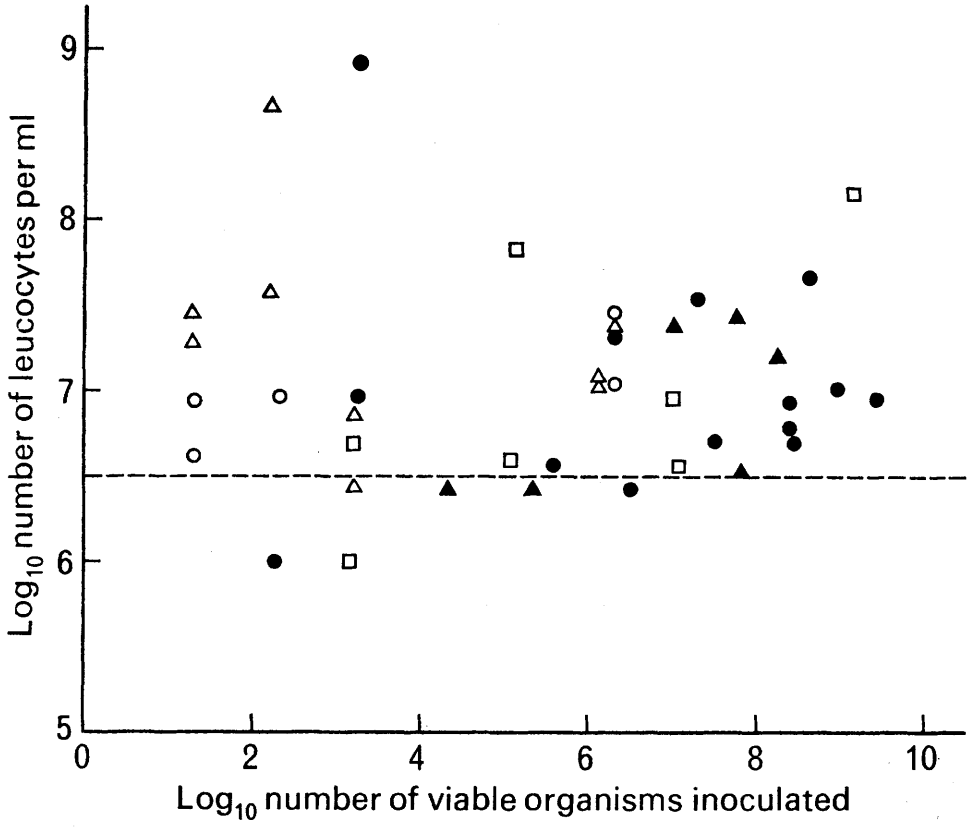

FIG. 3.—Leucocytes in guinea-pig chambers on the day after inoculation of various doses of gonococcal strains AS, BS, and AL. Persistent infections: ▲ = with strain AS; ● = with strain BS. Fleeting infections: △ = with strain AS; ○ = with strain BS; □ = with strain AL. Dotted line = median level of leucocytes in 35 uninoculated chambers.

RESULTS

Infection of chambers implanted in guinea-pigs

In preliminary experiments, stainless steel springs were used; but at the time of inoculation (30 days after implantation) they were found to be full of fibrous connective tissue, and only fleeting infections were obtained. The springs were superseded by the plastic chambers. By 30 days, these chambers had become encapsulated and filled with a dark red transudate without fibrous tissue. After the inoculation of the small-colony type strains AS or BS, persistent infections were obtained without betamethasone (table I). Strains BS (MID50 *c.* 10^3 organisms) and AS (MID50 *c.* 10^4 organisms) were similar in infectivity. Consistent infection was not achieved with the large-colony type strain AL despite the high inocula used. Infectivity could be increased by animal passage; the MID50 of strain BS passaged three times through guinea-pig chambers was about 5 organisms (table I).

TABLE III

Number of persistent infections produced by various strains of gonococci in rabbit chambers

Inoculum	Number of chambers persistently infected*/number tested						
	in the absence of betamethasone by strain			in the presence of betamethasone by strain			
	AS	BS	AS passaged†	AL	AS	BS	AS passaged†
0–50	...	...	...	...	1/2	...	...
50–500	...	...	0/2	0/2	0/2	0/2	4/4
500–5×10^3	...	...	0/2	0/4	4/6	0/3	2/2
5×10^3–5×10^4	...	...	0/2	0/2	1/3	0/5	2/2
5×10^4–5×10^5	...	...	0/2	1/6	1/5	1/4	2/2
5×10^5–5×10^6	...	0/1	0/2	0/2	4/5	0/1	2/2
5×10^6–5×10^7	0/4	0/1	...	1/4	3/4	...	...
5×10^7–5×10^8	0/2	0/2	...	0/1	0/4	...	...
5×10^8–5×10^9	...	0/2	...	0/4	...	...	...

... = Tests not done.

* Not less than 500 gonococci per ml of transudate for at least 4 days.

† Once in rabbits.

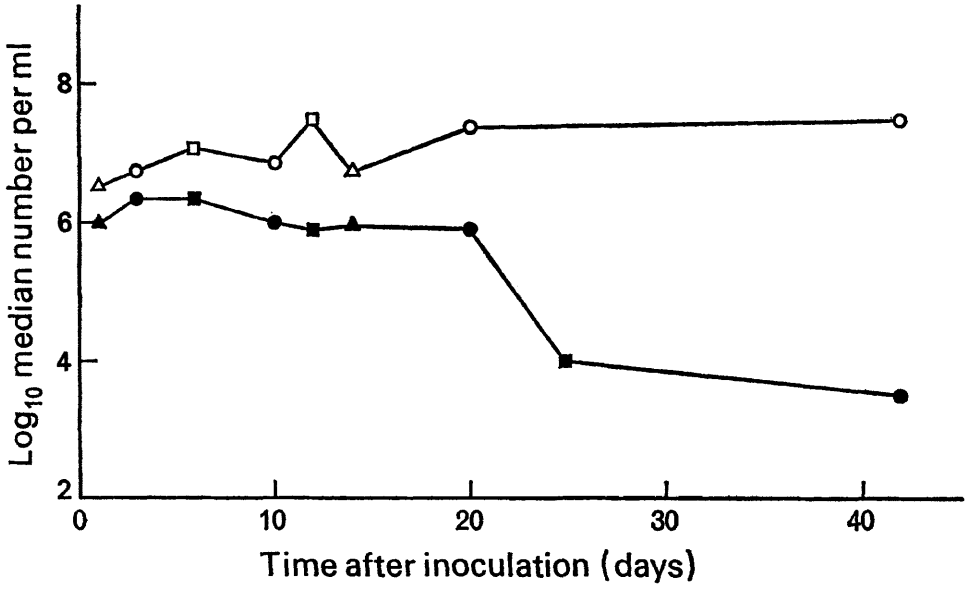

FIG. 4.—Concentration of gonococci (solid symbols) and leucocytes (open symbols) in rabbit chambers at the indicated time after inoculation. Each symbol represents the median concentration in 4 to 12 persistently infected chambers: ▲, △ = experiment 1; ●, ○ = experiment 2; ■, □ = experiment 3. Variously sized doses of gonococcal strains AS and BS were given. Median concentration of leucocytes in the chamber before inoculation = $1{\cdot}3\times10^6$ per ml.

Infections usually persisted several weeks, and of 23 chambers found to be infected on the day after inoculation with gonococci (day 1), all were infected on day 5 and in only two could live gonococci not be detected on day 21. The longest infections recorded were 146 and 118 days. Concentrations of organisms varied, but in the first 2–4 weeks after infection they were usually between 5×10^5 and 5×10^6 per ml (fig. 2). This level could be reached 24 h after infection but sometimes took several days. Because the concentration of gonococci in the exudates was around 1×10^6 per ml only occasional organisms could be seen in stained smears; these were mainly extracellular, and cell-associated organisms were extremely rare. In most cases the numbers of gonococci declined after 4–6 weeks. Observations on two animals suggested that infection did not spread from infected chambers to uninfected chambers in the same animal.

The cellular composition of the transudate in uninfected chambers was variable. Observations on 12 and 51 chambers respectively showed the erythrocytes varied from 8×10^6 to 6×10^8 (median 2×10^8) per ml and the leuco-

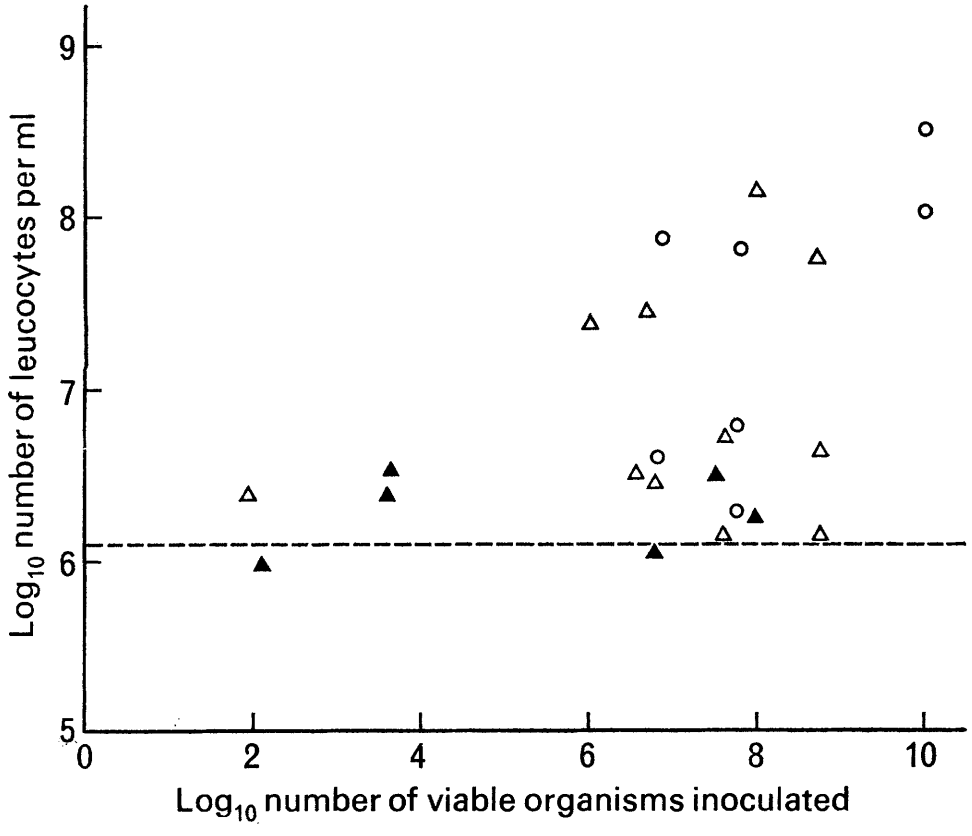

FIG. 5.—Leucocytes in rabbit chambers on the day after inoculation of various doses of gonococcal strain AS: persistent infection (▲), or fleeting or no infection (△), with multiple doses of betamethasone; fleeting or no infection (○) with a single or no dose of betamethasone. Dotted line = median concentration of leucocytes in 24 uninfected chambers.

cytes from 2×10^5 to 8×10^8 (median 3×10^6) per ml. The average contents of PMN and MNP are shown in table II, the remainder of the population being various lymphocyte-like cells.

After the inoculation of gonococci, leucocytes infiltrated into the chambers (fig. 2) raising the concentration to several times the control level. The level of infiltration was variable and did not appear to be related to the size of the inoculum (fig. 3) or the strain used. Eighty to 90% of these cells were PMN (table II). In successfully infected chambers, the leucocyte concentration continued to rise until, by day 10, a level of *c.* 2×10^8 cells per ml (fig. 2) was reached and this persisted for many days. The percentage of PMN in these chambers remained high (table II) whereas in unsuccessfully infected chambers the percentage of PMN fell with a concomitant rise in the percentage of MNP. Infected chambers contained many degenerate and disrupted cells.

Infection of rabbit chambers with gonococci

Plastic golf-balls and plastic centrifuge-tubes were used but centrifuge tubes were preferred because they could be autoclaved and were easier to implant successfully, although they tended to fill slowly with fibrous tissue. Both types of chamber became encapsulated and filled with pale yellow transudate.

Attempts to infect rabbits without using an anti-inflammation drug failed even with passaged organisms from betamethasone-treated rabbits; occasionally a few gonococci were cultured the day after infection, but none thereafter. A single dose of betamethasone similar to that recommended by Arko (1972) for dexamethasone, did not promote infection with higher inocula than those used by this author. With multiple doses of betamethasone, rabbits were infected with strain AS and more consistently, with this strain passaged once in rabbits (table III). Strains BS and AL produced persistent infections only on rare occasions. As in guinea pigs, infection usually lasted several weeks; of 32 chambers found to be infected 24 h after inoculation, 27 were still infected on day 5 and 24 on day 21. The longest recorded infection was 63 days. Concentrations of organisms in the tubes were similar to those in guinea-pigs (fig. 4) being initially 5×10^5–5×10^6 organisms per ml and tending to decline after 4–6 weeks of infection.

The cellular composition of the transudate in uninfected chambers was variable. Observations on 38 and 69 chambers respectively showed the erythrocytes varied from $<5\times10^5$ to 2×10^8 (median 5×10^6) per ml and the leucocytes from $<5\times10^4$ to 5×10^8 (median $1{\cdot}3\times10^6$) per ml. The proportions of PMN and MNP in the population were variable, and the mean values were 51% PMN and 44% MNP from observations on 24 chambers in six animals. The rest of the population consisted of lymphocyte-like cells. After the inoculation of gonococci, leucocytes infiltrated into the chambers (fig. 4) but persistent infections occurred only when the initial infiltration on the first days was low (less than 1×10^7 cells per ml; fig. 5) whereas guinea-pig chambers were successfully infected in the presence of higher concentrations of cells

N. GONORRHOEAE IN SUBCUTANEOUS PLASTIC CHAMBERS

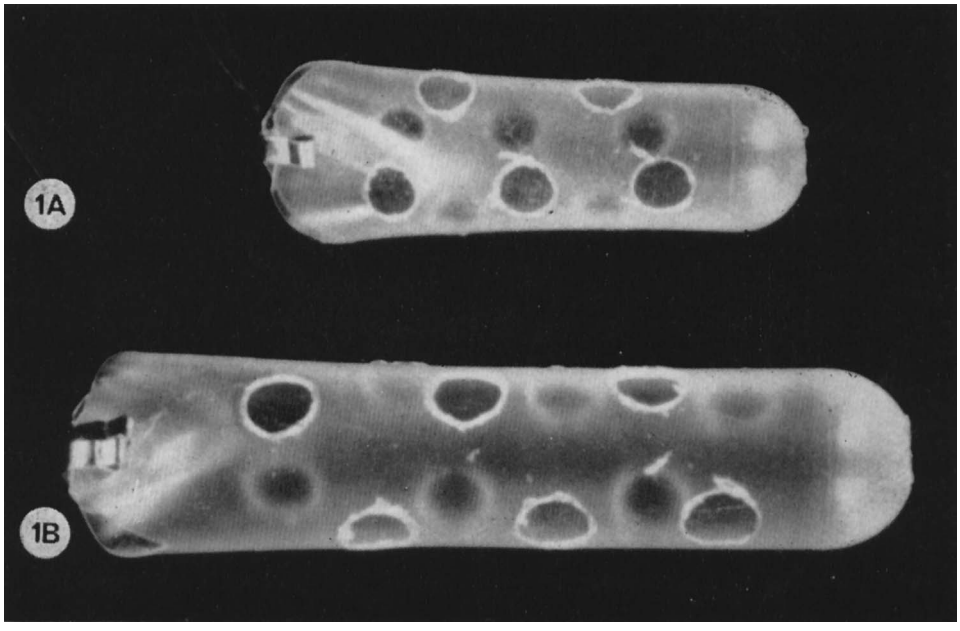

FIG. 1.—Chambers implanted into experimental animals: (*a*) guinea-pig tubes; (*b*) rabbit tubes. Natural size.

(fig. 3). In successfully infected chambers, the leucocyte concentration continued to rise until by day 10 a level of *c.* 2×10^7 cells per ml was reached, and this persisted for many days (fig. 4). This level was about one-tenth of the level in successfully infected guinea pig chambers (fig. 2) and was presumably due to the betamethasone treatment. On the first day after inoculation, whether or not the infection persisted, 91% of the infiltrating cells were PMN (from observation on 24 chambers in six animals) and in two persistently infected chambers the subsequent picture was similar to that in the persistently infected guinea-pig chambers, namely the high level of PMN was maintained. In the chambers not persistently infected, the level of PMN generally declined.

Discussion

Persistent infection of guinea-pig chambers has been achieved with small numbers of gonococci in the absence of anti-inflammation agents. The infections were induced in the face of a substantial inflammatory response which, as expected, was predominantly polymorphonuclear in the first instance. Somewhat surprising was the fact that the cellular content of infected chambers remained polymorphonuclear over many days; there was no significant replacement with mononuclear cells, although this occurred in chambers where infection was aborted. We have used the term infectivity rather than virulence in describing the ability of different colonial types to produce persistent infections in the chambers. Since these infections are established against an inflammatory response, the term virulence might have been used, but it was decided not to do so because of the manner of localising the infection.

The strains used resembled, in pilation and colony-form, those described by Kellogg *et al.* (1963 and 1968). In the guinea-pig chambers the infectivity of strain AS (resembling Kellogg type 1; MID50 *c.* 10^4) and strain BS (resembling Kellogg type 2; MID50 *c.* 10^3) were greater than that of strain AL (resembling Kellogg type 4; no persistent infection consistently established by large inocula) and were in good agreement with those mentioned recently by Arko (1974) for infection of guinea-pig "spring" chambers by Kellogg types 1 (MID50 *c.* 10^3) and 2 (MID50 *c.* 10^3). Furthermore, the infectivity of strains AS and BS was increased by animal-passage, so that infections could be established with only a few passaged organisms. The differences in infectivity of strains AS or BS and AL in guinea-pigc hambers paralleled the results of the experiments of Kellogg *et al.* (1968) in human volunteers, when large doses of types 1 and 2 produced infection but types 3 and 4 did not. Thus, the guinea-pig chambers provide a promising model for studying some aspects of the pathogenicity of the gonococcus, and the results obtained by using them might apply to man. Obviously, the mechanisms of early attachment to the urogenital mucosa cannot be examined in this model. It is, however, particularly appropriate for studying the next phase of gonococcal invasion, interaction with the humoral and cellular defence mechanisms, as well as any subsequent toxic effects of gonococci on host cells. Already the model has been used for immunological investigations (Arko, 1974).

Infection of rabbit chambers was less satisfactory. Betamethasone was required to promote persistent infection, which could be achieved only if leucocyte infiltration was low. Infection of the chambers could not be achieved without betamethasone even when animal-passaged organisms were used. Even when betamethasone was used, only strain AS produced persistent infections, and somewhat spasmodically until it was passaged. Strains BS and AL did not infect on most occasions. Thus, the growth of gonococci in rabbit chambers did not seem to be a good animal model for studying gonococcal pathogenicity.

SUMMARY

Perforated plastic chambers implanted subcutaneously in guinea-pigs and rabbits became encapsulated and filled with sterile transudate. When these chambers in guinea-pigs were inoculated with various strains of *Neisseria gonorrhoeae*, persistent infections were achieved without the use of anti-inflammation agents and in the presence of a substantial predominantly polymorphonuclear inflammatory response. Two strains with small colonies similar to types 1 and 2, and one strain with large colonies similar to type 4 of Kellogg *et al.* (1963 and 1968), showed differences in infectivity comparable with those that might be expected in man, and passage through guinea-pig chambers increased this infectivity. Rabbit chambers could not be infected without the use of an anti-inflammation drug (betamethasone), and differences in infectivity between strains were not as clear cut. The growth of *N. gonorrhoeae* in chambers in the guinea-pig provides a convenient model system for studying some aspects of the pathogenicity of this organism.

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