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Streptococcal Growth and Toxin Production In Vivo

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Streptococcal growth in vivo was studied with inoculated micropore filter chambers which were implanted into the peritoneal cavities of mice. Eight of nine group A strains and one group B strain grew in vivo, achieving concentrations of 10^7 to 10^9 CFU/ml in the chambers. Experiments with the Richards strain showed that the number of viable organisms remained high at 5 and 8 weeks after infection. The use of specific inhibitors and appropriate toxin-negative strains demonstrated that both cytolytic toxins produced by group A streptococci, streptolysin S and streptolysin O, were present in the culture fluids harvested from the chambers. This finding represents the first evidence that streptolysin S is produced in vivo. The host response to streptococci growing in vivo was examined by following the increase in serum antistreptolysin O levels. The response was first detected 2 weeks after chamber implantation and appeared to be maximal after 5 weeks. In addition, the production of antibody to streptococcal cell surface antigens was demonstrated indirectly with fluorescein-labeled anti-mouse immunoglobulin G.

Although the properties of pathogenic bacteria are often studied after in vitro growth, there is substantial evidence that organisms grown in vivo may differ from those grown in vitro (11, 12). The growth environment of a microorganism in vivo is complex and undoubtedly changes during the course of infection; such an environment cannot be duplicated in vitro. As a result, factors that contribute to the virulence of an organism may be produced under in vivo but not in vitro growth conditions. On the other hand, substances produced during in vitro growth may not be produced when the same organism grows vivo and then to be recovered so that the properties and products of the organisms can be analyzed. These include semipermeable collodion sacs (10), dialysis tubing (5), chambers sealed with dialysis tubing or micropore filters (6), plastic balls (2), plastic centrifuge tubes (14), etc.

The growth of streptococci in vivo has been studied previously by placing the organisms in celloidin capsules (16) or in diffusion chambers made from Plexiglas (7) or nylon sheets (13). In none of these studies, however, were the products or properties of the streptococci grown in vivo examined. More recently, streptococcal growth in tissue cages implanted in rabbits has been studied (8, 15). Knoll et al. (8) found that erythrogenic toxins types A and C could be detected in the tissue-cage fluids primarily during the first week of infection with group A streptococci. Two encapsulated strains of group B streptococci were reported by Wagner et al. (15) to grow in the tissue cages, and the thickness of the capsule differed from that found when the strains were cultivated in vitro. Unencapsulated strains did not grow in tissue cages.

Chambers consisting of plastic syringe barrels sealed with membrane filters (pore size, 0.22 μm) were devised by Day et al. (3) to study staphylococcal growth in vivo and have recently been used by Finn et al. (3a) to examine the properties of *Escherichia coli* grown in vivo. The present report describes streptococcal growth and toxin production in similar chambers implanted intraperitoneally in mice. The results demonstrate that both group A streptococcal cytolytic toxins are produced in vivo.

MATERIALS AND METHODS

Bacteria. The strains of *Streptococcus pyogenes* and *Streptococcus agalactiae* used in this study are listed in Table 1. The strains were maintained on sheep blood agar plates at 4°C. For inoculation into the chambers, the organisms were grown overnight in tryptic soy broth plus 2% yeast extract and then diluted in medium 199 to a concentration of approximately 10^5 CFU/ml.

Animals. BALB/c mice, 8 to 12 weeks old, were used in all experiments.

Preparation, implantation, and recovery of chambers. Cylinders were cut from polypropylene syringe barrels, washed, and sterilized as previously described

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TABLE 1. Streptococcal strains

Streptococcal strain	M type	Cytolytic toxin production		Source of streptococcal strain
		Streptolysin O	Streptolysin S	
Group A				
Richards	3	+	+	H. Slade
Lang	11	−	+	R. Lancefield
Blackmore	11	−	+	R. Lancefield
DS-1821-75	NT[a]	+	−	R. Facklam
RT⁻	3	+	−	This laboratory[b]
530	NT	ND	+	Northwestern Memorial Hospital
TLK	NT	ND	+	Northwestern Memorial Hospital
Williams	NT	ND	+	Northwestern Memorial Hospital
Group B				
090R[c]				R. Lancefield

[a] NT, Not typed; ND, not determined.
[b] A streptolysin S-negative mutant obtained by treating strain Richards with nitrosoguanidine.
[c] Produced hemolysin.

(3). Sterile micropore filter disks (pore size, 0.22 μm; Sartorius or Millipore Corp.) were aseptically attached by softening the ends of the cylinders in the pilot flame of a Bunsen burner and then pressing the cylinders against the disks. Before the second disk was sealed onto a cylinder, 0.1 ml of diluted bacterial suspension (approximately 10^4 CFU) was added to the chamber. The completed chambers were placed in medium 199. Mice were anesthetized with halothane, and two to four chambers were inserted into the peritoneal cavity.

To recover the chambers, the mice were killed by cervical dislocation. After the peritoneal cavity was aseptically exposed, the tissue adhering to the chambers was gently removed, and the chambers were placed in a sterile petri dish. The contents of the chamber were removed with a sterile syringe, and a portion was diluted in tryptic soy broth plus 2% yeast extract for viable count determinations on blood agar plates. The remaining suspension was centrifuged in an Eppendorf microfuge for 5 min, and both the cell pellet and the supernatant were retained.

Hemolysin assays. The contents of the infected chambers were recovered and incubated with 5 μl of 20 mM dithiothreitol for 10 min. After centrifugation for 5 min in an Eppendorf microfuge to remove the streptococcal cells, cytolytic toxin activity was assayed by making serial dilutions of the chamber supernatants in phosphate-buffered saline placed in the wells of microtiter plates. An equal volume (25 μl) of washed rabbit erythrocytes (2% suspension) was added to each well, and the plates were incubated at 37°C for 30 min. The extent of lysis was estimated with a scale ranging from negative (−) to complete (+++) lysis. Wells showing (++) lysis were designated as the endpoint, and the reciprocal of that dilution was defined as the number of hemolytic units in a given sample. In some assays, cholesterol (0.005 M) was added to the first well of a plate to inhibit the activity of streptolysin O. An equal volume of trypan blue (50 μg/ml) was added to each well of a series to inhibit streptolysin S.

Antistreptolysin O. To quantitate the antistreptolysin O produced, mice containing implanted chambers were killed at various times by cervical dislocation. The heart was exposed and cut, and the blood that accumulated in the thoracic cavity was collected. After clotting, the blood was centrifuged, and the serum was stored at −20°C. The antistreptolysin O titer was determined by making serial dilutions of the sera in microtiter wells that contained sufficient streptolysin O to produce (++) hemolysis. The greatest dilution producing complete inhibition of hemolysis was taken as the endpoint. All sera in a given experiment were assayed at the same time, and sera from untreated mice in the same litter were used as controls.

Fluorescent-antibody studies. The production of antibodies to streptococcal cell surface antigens was examined indirectly with fluorescein-labeled rabbit anti-mouse immunoglobulin G (IgG) (Miles Laboratories, Inc.). Streptococcal cells from chambers implanted for various periods of time were washed in phosphate-buffered saline and resuspended in the antibody preparation for 20 min at 37°C. The cells were then washed three times in phosphate-buffered saline and suspended in mounting buffer (30% phosphate-buffered saline, 70% glycerol [vol/vol]). A small drop was placed on acid-cleaned slides, a cover slip was sealed in place, and the preparations were viewed with a fluorescent microscope.

Other procedures. The amount of protein present in the chamber supernatants was determined with a protein assay kit (Bio-Rad Laboratories). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out as described by Laemmli (9).

RESULTS

Growth of streptococci in vivo. The growth of nine group A streptococcal strains and one group B streptococcal strain was studied in intraperitoneal chambers for periods ranging from 6 h to 7 days. Figure 1 shows typical results for three group A strains. The Richards strain began to grow within 6 h after implantation and reached a concentration of greater than 10^8 CFU/ml in 24 h. In addition to the Richards strain, four other laboratory strains (i.e., streptococcal strains that had been maintained on complex media for several years) showed similar levels of growth (>10^8 CFU/ml) in the chambers. Strain TLK, a recent clinical isolate that had been passaged very few times in the laboratory, also grew rapidly, attaining a concentration of >10^7 CFU/ml at 24 h. Two additional recent isolates showed a slower pattern of growth but also reached concentrations of 10^7 CFU/ml after 4 days of growth. Strain 530, however, grew for only 24 h and then was lost; three separate

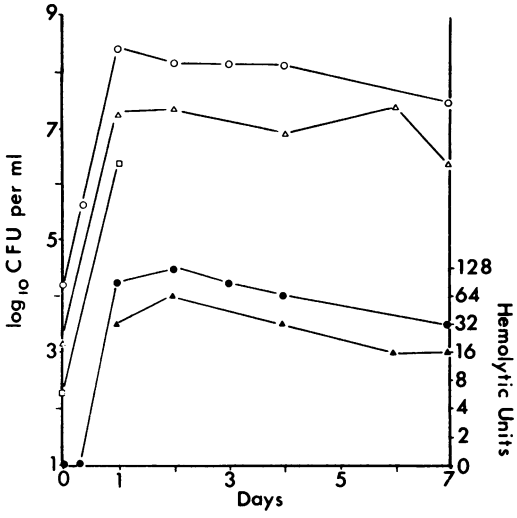

FIG. 1. Growth and hemolysin production in vivo of three group A streptococcal strains. Duplicate chambers containing 10^2 to 10^3 CFU/ml of each strain were implanted intraperitoneally in mice at day 0. At various times, the chambers were recovered and the contents were assayed for viable counts (open symbols) and hemolysin (closed symbols). Symbols: ○ and ●, strain Richards; △ and ▲, strain TLK; □, strain 530.

attempts to cultivate this strain in vivo produced the same result.

Examination of the cells grown in vivo at various times by light microscopy revealed that, after 24 h, the streptococcal cells were present primarily as diplococci and single cells, with a few short (four-cell) chains. The same general picture was seen after 48 h, although the numbers of four-cell chains had increased, and a few longer chains were found. By 72 h, most of the cells were in short chains and the number of longer chains (10 to 20 cells per chain) had increased. After 5 days of in vivo growth, most of the cells were present as long chains; many of the chains contained greater than 20 cells.

Production of cytolytic toxins in vivo. To determine whether the streptococcal cytolytic toxins, streptolysin O and streptolysin S, were produced under in vivo growth conditions, the chamber contents were recovered, centrifuged, and tested for lytic activity in microtiter plate wells containing rabbit erythrocytes. Significant hemolytic activity was present in the chamber fluid of the Richards and TLK strains at 24 h, and this activity persisted for at least 7 days (Fig. 1). To determine which cytolytic toxin was responsible for hemolysis, the hemolytic titrations were also carried out in the presence of cholesterol or trypan blue or both. Cholesterol and trypan blue are inhibitors of streptolysin O and streptolysin S, respectively. The results indicated that, although both toxins were present in the chamber fluid, most of the hemolytic activity was due to streptolysin S; i.e., trypan blue greatly decreased the amount of hemolysis, whereas cholesterol had only a slight effect.

To show more clearly that both cytolytic toxins were produced in vivo, strains Blackmore and Lang, which produce only streptolysin S, and a mutant of the Richards strain, RT^-, which produces only streptolysin O, were studied. All three strains showed significant growth and toxin production in vivo (Fig. 2). The lytic activity present in the chambers containing the Blackmore and Lang strains was completely inhibited by trypan blue, and cholesterol had no effect. Conversely, cholesterol completely inhibited the hemolysis produced by RT^- chamber supernatants, but trypan blue was without effect. In later experiments, lecithin, which is also an inhibitor of streptolysin S, was used to confirm the presence of that toxin in the chamber fluid. Preliminary experiments with group B streptococcal strain 090R indicated that the group B hemolysin was also produced in vivo after 24 h of growth.

The proteins present in the chamber fluids were examined by recovering duplicate control, uninoculated chambers, and chambers inoculated with the Richards strain at 24, 48, and 72 h. After centrifugation, protein determinations were made on the chamber fluids, and the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The super-

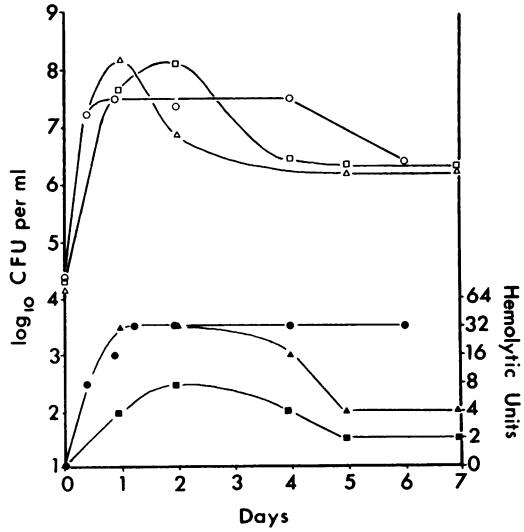

FIG. 2. Cytolytic toxin production in vivo. Strains Blackmore (○, ●) and Lang (△, ▲), which produce only streptolysin S, and strain RT^- (□, ■), which produces only streptolysin O, were grown in intraperitoneally implanted chambers in mice for up to 7 days. The viable counts (open symbols) and hemolysin titers (closed symbols) were determined at various times.

natants from the Richards strain-inoculated chambers that had remained in mice for 1, 2, or 5 weeks were also examined in this way. The protein concentrations ranged from 4.0 to 6.3 mg/ml, but there was virtually no difference between control and inoculated chambers. Examination of the sodium dodecyl sulfate-polyacrylamide gels showed a large number of protein bands present in both control and inoculated chamber fluids (data not shown). However, the gels were complicated and distorted by the presence of an extraordinarily large and diffuse band in the region corresponding to the heavy chain of immunoglobulin. Nevertheless, it was possible to identify two, and possibly three, proteins that were present in the control chambers but were diminished or absent in the inoculated chambers.

Immune response to streptococci grown in vivo. The immune response of the host to the streptococcal cell surface and to extracellular antigens was examined in mice over a 5-week period. Implanted chambers containing the Richards strain were recovered at various times, and the viable count of streptococci in the chambers and the titer of antistreptolysin O in the serum were determined. The results of two such experiments are shown in Fig. 3. The number of viable bacteria in the chambers remained high ($>10^7$ CFU/ml) over the 5-week period. The antistreptolysin O activity of the serum was negligible at 0 and 1 week, but it began to increase at 2 weeks and was maximal at 5 weeks.

The immune response to streptococcal cell surface antigens was assessed indirectly with fluorescent antibody. Streptococci were recovered from chambers at 24 and 48 h, and 1, 2, 3, and 5 weeks and stained with fluorescein-labeled rabbit antimouse IgG antibody as described above. No fluorescence of the cells was detected with streptococci grown for up to 2 weeks in the chambers or with control cells grown in vitro. After 3 weeks of growth in vivo, however, fluorescent staining of the streptococci was readily observed, and by 5 weeks, the streptococcal chains were intensely stained with the fluorescent antibody.

DISCUSSION

The results demonstrate that the chamber system described by Day et al. (3) is a useful procedure for studying the growth of streptococci in vivo. With one exception, all group A streptococcal strains tested grew in vivo, achieving concentrations of 10^7 to 10^8 CFU/ml. Although it appeared that strains maintained in the laboratory grew more rapidly and to higher concentrations than did recent isolates of group A streptococci, more strains will have to be tested before a definitive statement can be made. The number of viable streptococci in the chambers remained high for at least 1 week, at which point most experiments were terminated. Long-term experiments with the Richards strain revealed that even after 5 and 8 weeks, there were greater than 10^7 CFU/ml in the chambers. In comparison, in vitro cultures of the Richards strain are completely nonviable within 4 to 5 days (data not shown). Day et al. (3) found that the viable count of staphylococci remained high ($>10^7$ CFU/ml) after 9.5 months in vivo and suggested that the in vivo chamber resembles a chemostat with a continuous influx of nutrients and an efflux of metabolic products.

The presence of hemolytic activity in the chamber fluids of the Richards and other strains that was inhibited by cholesterol but not by trypan blue indicated that streptolysin O was produced in vivo. This was confirmed by the finding that RT$^-$, a streptolysin S-negative mutant of the Richards strain, produced hemolytic activity with the same characteristics and by the increase in antistreptolysin O activity of serum obtained from the mice during in vivo streptococcal growth. The levels of streptolysin O within the chambers were always quite low, perhaps due to the rapid diffusion of molecules from the chambers (3).

The most interesting observation was that streptolysin S was produced during in vivo growth of the streptococci. This cytolytic toxin is not immunogenic (4), and heretofore there has been no evidence that streptolysin S is synthesized in vivo (1, 4). Most of the hemolytic

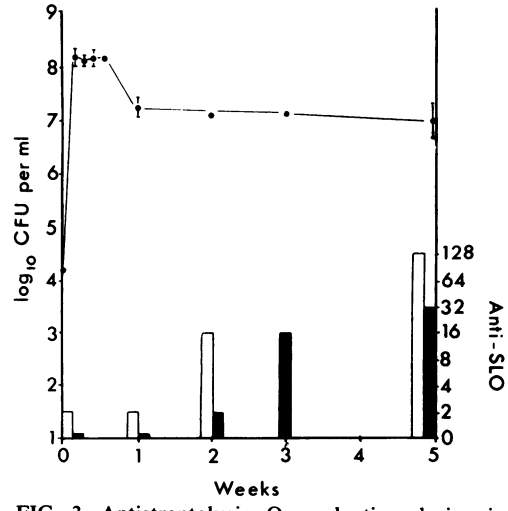

FIG. 3. Antistreptolysin O production during in vivo streptococcal growth. Strain Richards was cultured in vivo for up to 5 weeks, and the viable counts were determined at various times (●). The bar graph shows two experiments in which the antistreptolysin O (Anti-SLO) titer was determined at weekly intervals.

activity present in the chamber fluids appeared to be streptolysin S, i.e., it was inhibited by trypan blue and lecithin but not by cholesterol. Hemolytic activity possessing the characteristics of streptolysin S was found in chambers inoculated with Blackmore or Lang strains, two group A strains that are known to produce streptolysin S but not streptolysin O. Streptolysin S is a small peptide toxin that is found as a cell-bound hemolysin or in a soluble form associated with certain carrier molecules (1, 4). In the experiments described in Fig. 1 and 2, the chamber fluids were centrifuged to remove the streptococci; when the fluids were tested before centrifugation, the hemolytic titer usually increased by one well in the microtiter plates. Thus, a significant proportion of the streptolysin S found in the chambers is present in a soluble form, perhaps associated with one or more of the numerous host proteins that accumulate in the chambers. When examined over a longer period of time, the hemolytic activity produced by the Richards strain, most of which was streptolysin S, was found to remain relatively high (within one dilution well of the maximum) for 2 to 3 weeks; however, by 5 weeks, little or no hemolysin could be detected.

The chamber implant system also allows one to study the host response to bacterial growth in vivo. The increasing antistreptolysin O activity of the mouse serum (Fig. 3) indicated that antibody to this toxin is produced during streptococcal growth in the chambers. The response could be detected 2 weeks after implantation and reached a maximum at 5 weeks. In one experiment (data not shown), the antistreptolysin O activity of the serum was still high (within one dilution well of the maximum) after 8 weeks. An immune response to streptococcal cell surface antigens was also demonstrated. The presence of immunoglobulins on the streptococci was first detected after 3 weeks of growth with fluorescein-labeled antimouse IgG, and the reaction was even more intense after 5 weeks. With similar chambers (3a), it was shown by slide agglutination tests that immunoglobulins were tightly bound to all *E. coli* grown in vivo. As the chambers are impermeable to cells of the immune system, the stimulation of antibody production presumably occurs as a result of bacterial degradation and diffusion of cellular debris from the chambers.

The results of this study provide further evidence that the chamber implant system is a useful procedure for studying the properties and products of microorganisms grown in vivo. In addition, it allows one to examine the host response under conditions in which the microorganisms survive in the host for days or weeks.

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