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RED CELL-SENSITIZING ANTIGEN OF GROUP A STREPTOCOCCI

II. IMMUNOLOGICAL AND IMMUNOPATHOLOGICAL PROPERTIES

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ABSTRACT

The cell-sensitizing factor (SF) of group A streptococci is a teichoic acid which can sensitize mammalian cells to agglutination and lysis in the presence of anti-SF antibodies and complement. SF is highly immunogenic in the rabbit when bound naturally to some constituent of the streptococcus cell, but only feebly so when it is extracted from the cells by phenol. Both rabbit and human antibodies to SF, which are mainly associated with the macroglobulin fraction (IgM) of serum, are destroyed by treatment with 2-mercaptoethanol. While human anti-SF antibodies are readily destroyed by freezing and thawing and by heating to 58 C, the rabbit anti-SF antibodies are not destroyed at 64 C and are relatively resistant to repeated freezing and thawing. Complexes formed between SF and rabbit antibodies fix complement both in the absence and presence of red blood cells (RBC). Anti-SF antibodies interact with SF and prevent the latter from sensitizing RBC. Rabbits immunized with heat-killed streptococci and which developed anti-SF antibodies, developed severe arthritis when SF was injected into their knee joints. The arthritic lesions were characterized by a marked proliferation of the synovial membrane, a chronic inflammatory exudate and the accumulation of large numbers of lymphocytes in the form of "pseudolymphatic follicles." Nonimmunized animals failed to develop such lesions. It is suggested that sensitization of cells with SF during streptococcal infection may lead to passive immune cytolysis and may thus contribute to the pathogenicity of streptococci.

In previous reports (1–4) it has been shown that the cell-sensitizing factor (SF) of group A streptococci is a high molecular weight, heat stable material which contains glycerol, alanine, phosphate and glucose (teichoic acid). Unlike teichoic acids extracted from streptococci with trichloroacetic acid (5), the SF extracted with phenol (1, 4) is capable of sensitizing mammalian cells which, in the presence of anti-SF serum and complement, will cause lysis of the red cells (3). In a preliminary report (6) it was shown that rab-

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bits immunized with heat-killed streptococci developed severe arthritis when SF was injected into their knee joint. The present report further describes some of the immunological and immunopathological properties of SF.

MATERIALS AND METHODS

The extraction of the SF from streptococci, the immunization of rabbits with streptococci and the methods for the determination of passive hemagglutination titers and units have been described in detail in the previous paper (4).

Passive immune lysis was determined by the addition of 2 CH_{50} units of complement (normal guinea pig serum) (7) to the antibody dilution tubes in the presence of sensitized human red blood cells (RBC). The degree of lysis was determined by measuring the release of hemoglobin, as previously described (7).

Complement fixation by SF and anti-SF complexes. Complexes of various ratios of SF and anti-SF were prepared by incubating the reagents for 30 min at 37 C. Ten CH_{50} units of complement (C) were then added to the complexes and the amount of C not fixed to the complexes was determined with sheep RBC sensitized with rabbit anti-sheep hemolysin as previously described (7). The calculation of the number of C units fixed to the complex was done by subtracting the number of C units consumed by each reactant from the total number of C units fixed to the immune complex.

Ammonium sulfate fractionation. Rabbit or human sera with high titers of anti-SF antibodies were fractionated with 0.3 and 0.5 saturation of ammonium sulfate. The precipitated proteins were removed by high speed centrifugation, and the precipitate was dissolved in distilled water and dialyzed for 48 hr against saline. Anti-SF activity was assayed on the fractions.

Gel filtration and ion-exchange chromatography. Rabbit and human sera possessing anti-SF activities were filtered through columns of Sephadex G-200 (85 × 2.5 cm) (Pharmacia Chemicals, Uppsala, Sweden). The void volume of the column was determined with highly polymerized DNA (calf thymus, Calbiochem, USA). Aliquots eluted from the column were assayed for anti-SF activity as described.

Anti-SF sera dialyzed overnight against 0.01 M phosphate buffer, pH 7.4, were also chromatographed on DEAE-Sephadex columns (10 × 1.0 cm). Elution of the protein fractions was achieved by a stepwise increase in salt concentration (using 0.05 to 1.0 M NaCl buffered with 0.05 M phosphate, pH 7.4). The fractions eluted were assayed for anti-SF activity as described.

Treatment of serum with 2-mercaptoethanol. Equal volumes of anti-SF serum and 0.1 M 2-mercaptoethanol were incubated with constant mixing for 60 min at room temperature (8). The reaction was stopped by the addition of 0.1 M of iodoacetic acid. The reaction mixtures were dialyzed for 24 hr against saline.

The induction of arthritis. Four injections of 0.20 ml SF (derived from 20 ml streptococci, 2,000 Klett units) were given on four consecutive days into the right knee joint of rabbits which had previously been immunized with heat-killed type 4 streptococci. Saline was injected into the left knee joints. Only animals showing anti-SF titers of 50 and over were included in the study. Eight normal animals which were given an injection of SF into the knee joint served as controls. The severity of arthritis was evaluated by measuring the circumference of the knee joint and by the histological response (see below). The animals were killed 3 to 10 days following the last injection of SF. The synovial membrane was fixed in 4% formalin and paraffin sections were stained with hematoxylin and eosin.

The severity of the histological response was graded 0 to 3 as follows: 0, intact synovial tissue; 1, infiltration of the synovial membrane with small numbers of inflammatory cells; 2, marked hyperplasia of the synovium with villous formations accompanied by a chronic inflammatory exudate; 3, marked hyperplasia of the synovial membrane accompanied by severe inflammatory reactions and "pseudolymphatic follicles."

RESULTS

The immunogenicity of SF. The presence of high anti-SF antibody titers in rabbits immunized with streptococci and in sera of humans convalescing from streptococcal infections (unpublished results) suggested that SF is a good immunogen. To test whether SF isolated from the streptococci by phenol is similarly immunogenic, rabbits were immunized either with heat-killed streptococci or with SF. The amounts of SF used for immunization were roughly equivalent to twice the amount of SF which could be isolated by

TABLE 1. *Anti-SF antibodies in rabbits immunized with heat-killed streptococci or with SF*

Rabbit no.	*Immunizing agent*	*Passive hemagglutination titers (units). After weeks:* 1	2	3	4	5	6	7	8
1	Streptococci	—	100	400	1,600	—	—	—	—
2	"	50	400	800	800	800	—	—	—
3	"	50	800	800	3,200	3,200	—	—	—
4	"	50	1,600	1,600	—	—	—	—	—
5	"	100	5,000	—	6,400	6,400	6,400	—	—
6	"	50	50	100	100	200	200	200	—
7	"	50	100	100	400	400	400	800	800
8	"	50	200	400	400	800	400	—	—
9	"	—	100	200	800	800	800	—	—
10	"	50	200	3,200	1,600	1,600	—	—	—
11	"	—	200	800	1,600	1,600	—	—	—
12	SF	×	40	×	×	×	×	400	800
13	"	×	100	40	50	100	100	400	400
14	"	×	×	×	×	×	×	—	—
15	"	×	×	×	×	×	×	—	—
16	"	×	×	×	×	×	×	—	—
17	"	×	×	×	×	×	×	50	—
18	"	×	×	×	×	×	×	×	—
19	"	×	×	×	×	×	×	×	×
20	"	×	×	×	×	×	50	50	50

× = less than 25 units.

phenol from streptococcal suspensions used for immunization.

Table 1 shows that while rabbits immunlzed with streptococci developed high titers of anti-SF antibodies as soon as two weeks following the onset of immunization, most of the animals immunized with SF yielded only low titers of anti-SF antibodies up to eight weeks following immunization. Since streptococcal mucopeptide has recently been shown to enhance the formation of antibodies to bovine serum albumin (9), five rabbits were injected intracutaneously with SF-mucopeptide mixtures on two occasions, at an interval

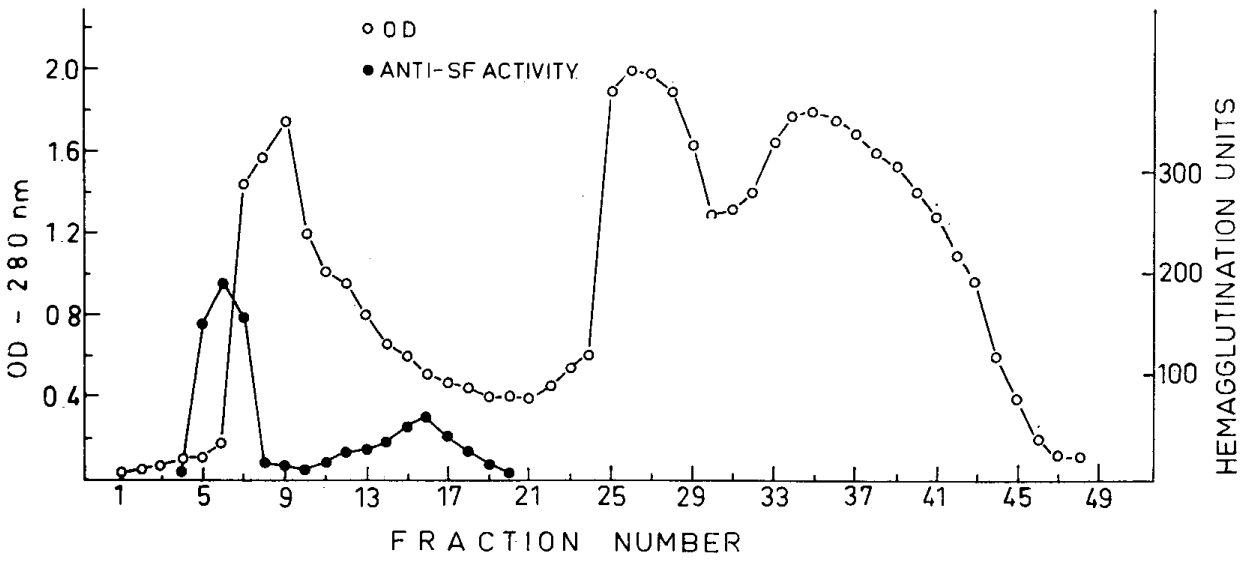

FIG. 1. Gel filtration of rabbit anti-SF serum on Sephadex G-200.

of one week. None of the rabbits developed any detectable amounts of anti-SF three weeks following the first injection. No attempts were made to employ Freund's adjuvant since this is considered to be an artificial method of immunization.

The nature of anti-SF antibodies. Gel filtration of rabbit and human anti-SF sera on Sephadex G-200 columns revealed that the immune sera were very rich in the macroglobulin fraction. All the anti-SF activity of the rabbit immune serum was eluted from the column in two peaks (Fig. 1). The first anti-SF peak, with a specific activity of 180 agglutination units/0.1 OD at 280 nm, appeared immediately following the void volume, which corresponded with the IgM fraction of normal rabbit serum. The second peak of anti-SF activity (20 agglutination units) was less sharp and appeared along the descending arm of the first protein peak. No anti-SF activity, however, appeared in the second protein peak which corresponded to the IgG fraction of rabbit serum. Gel filtration of human serum possessing anti-SF activity showed that all the antibody was concentrated in the first peak (IgM fraction). The results indicate that antibodies to SF were probably macroglobulins. To test this assumption further, the macroglobulin fraction possessing anti-SF activity, which was eluted from Sephadex G-200 columns, was treated with 2-mercaptoheptanol; 95% of the anti-SF activity was found to be lost after such treatment. In other experiments it was found that all anti-SF activity of rabbit immune serum was precipitated with 0.5 saturation of ammonium sulfate, but not with 0.3 saturation. It is also of interest that no anti-SF activity was found in any of several preparations of human fraction II (γ-globulin) obtained from commercial sources.

Ion-exchange chromatography of rabbit anti-SF serum on DEAE Sephadex columns revealed that all the antibody activity was

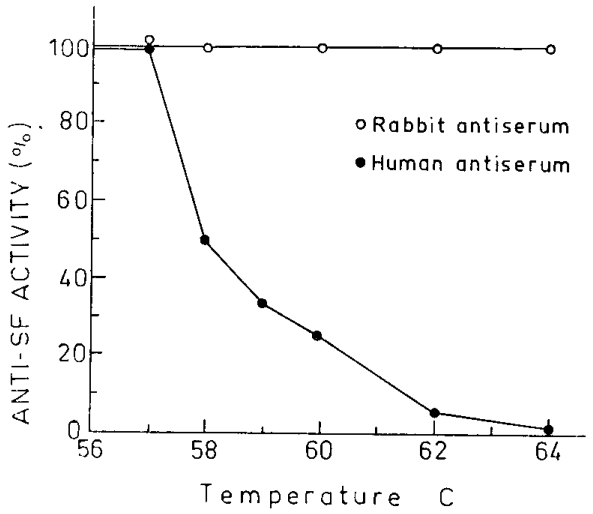

FIG. 2. Effect of temperature on the anti-SF activities of human and rabbit sera. Both antisera contained 256 hemagglutinating units/ml.

eluted from the columns with 0.5 M NaCl buffered with 0.05 M phosphate, pH 7.4. The results obtained from ammonium sulfate precipitation, gel filtration and ion-exchange chromatography experiments indicated, therefore, that anti-SF antibodies are of the macroglobulin type.

Repeated freezing and thawing of rabbit anti-SF serum (eight times in one day) did not result in a decrease in antibody activity. However, repeated freezing and thawing and long storage in the frozen state (–20 C) resulted in a significant loss of hemagglutination titers. On the other hand, freezing and thawing of human anti-SF serum (six times in one day) resulted in a 50 to 65% loss in hemagglutination titers.

Fig. 2 shows the effect of temperature on the activities of human and rabbit anti-SF sera. It appears that, unlike rabbit anti-SF, the human antibodies are readily destroyed by heating above 57 C.

Hemagglutination and C fixation by SF-anti-SF complexes. According to Jackson and Moskowitz (2) the polyglycerophosphate (PGP) moiety of SF was associated with its reactivity with antiserum (hemagglutination) while the alanine moiety was responsible for

TABLE 2. *Hemagglutination of RBC and C-fixation by SF-anti-SF complexes*

Reaction mixture	*Hemagglutination titers*	*Complement fixed (units)*
SF + anti-SF 1:500	400	2.7
SF + anti-SF 1:100	160	2.1
SF + anti-SF 1:50	80	1.3
SF + anti-SF 1:10	20	—
SF + normal rabbit serum 1:10	400	0

the binding of SF to RBC. The possibility that anti-serum to SF also contained antibodies against the alanine moiety was tested. SF was incubated for 30 min at 37 C with increasing amounts of anti-SF serum to allow complex formation. The immune complex mixtures were further incubated for 30 min at 37 C with RBC to achieve sensitization. The RBC were then washed twice to remove excess unbound reactants and used in hemagglutination reactions in the presence of anti-SF serum. Table 2 shows that immune complexes formed by increasing concentrations of anti-SF had a diminished capacity to sensitize RBC, as shown by the decrease in hemagglutination titers obtained. It was also found that SF-anti-SF complexes fixed complement. Some of the concentrations of the reactant used were, however, highly anti-complementary and could not be used to study C-fixation. The anti-C property of both the SF and anti-SF could not, however, be abolished by treatment with fluorocarbon previously shown to remove anti-complementary materials from certain antisera (10).

The induction of arthritis with SF. In a previous study (11), it was found that immune complexes derived from group A streptococcal extracellular antigens and rabbit antibodies caused severe arthritis in the rabbit knee joint. In other studies, it was found that streptococcal extracellular products (12), streptococcal sonicates (13), and streptococcal L-forms (14) induced severe chronic arthritis in the rabbit knee joint, while streptococcal cell walls, C-polysaccharide, mucopeptide and L-forms induced a severe myocarditis in the rabbit when injected intramyocardially (15). Since all the streptococcal antigens tested have been shown to be toxic to rabbit tissues, it was difficult to assess with certainty whether the toxicity induced by immune complexes prepared from streptococcal products and rabbit antibodies (11) was due solely to the inherent toxicity of the antigens or due to the phlogistic effect which might have developed by the introduction of immune complexes into the tissues.

In a preliminary experiment, it was found that the SF of streptococci was not toxic to

TABLE 3. *Arthritis in the rabbit right knee joint induced by SF*

No. of animals	*Time of sacrifice after injection of SF (days)*	*Anti-SF titer of sacrifice*	*Arthritis No. of animals showing lesions of various grades*[a]			
			0	*1*	*2*	*3*
Immunized with streptococci						
5	3	5,000	–	–	3	2
4	10	5,000	–	1	1	2
2	3	100	–	–	2	–
2	10	100	1	–	1	–
Nonimmunized						
4	5	0	3	1	–	–
4	10	0	4	–	–	–

[a] See Materials and Methods for explanation of grading.

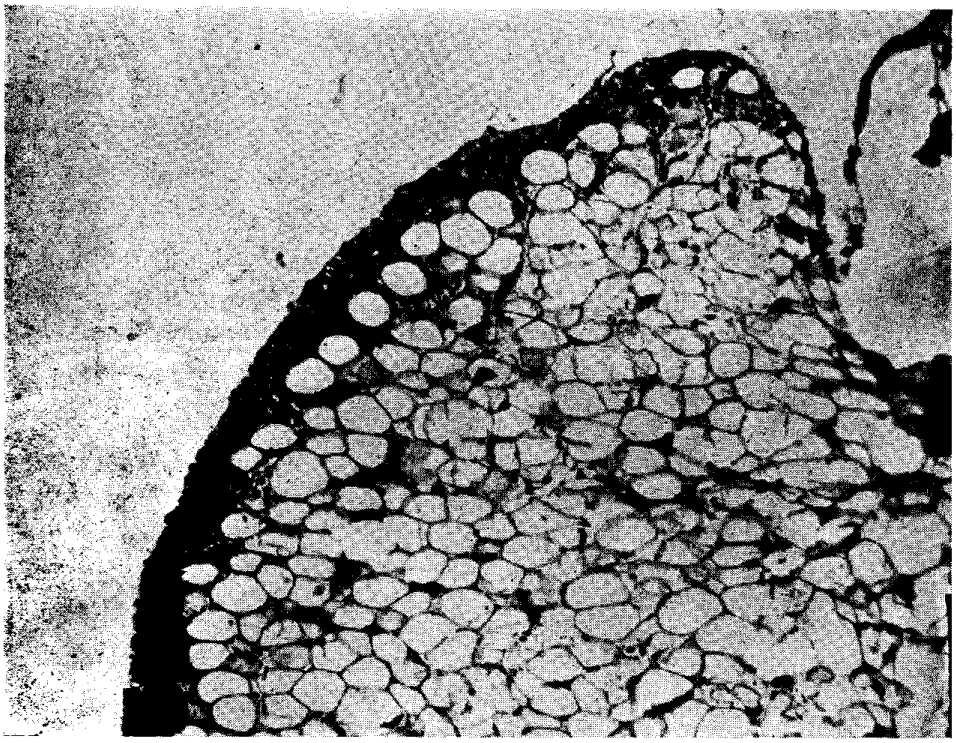

FIG. 3. Synovial membrane of a normal rabbit injected intra-articularly with SF. Note the mild superficial infiltration with mononuclear cells. Hematoxylin and eosin. × 105.

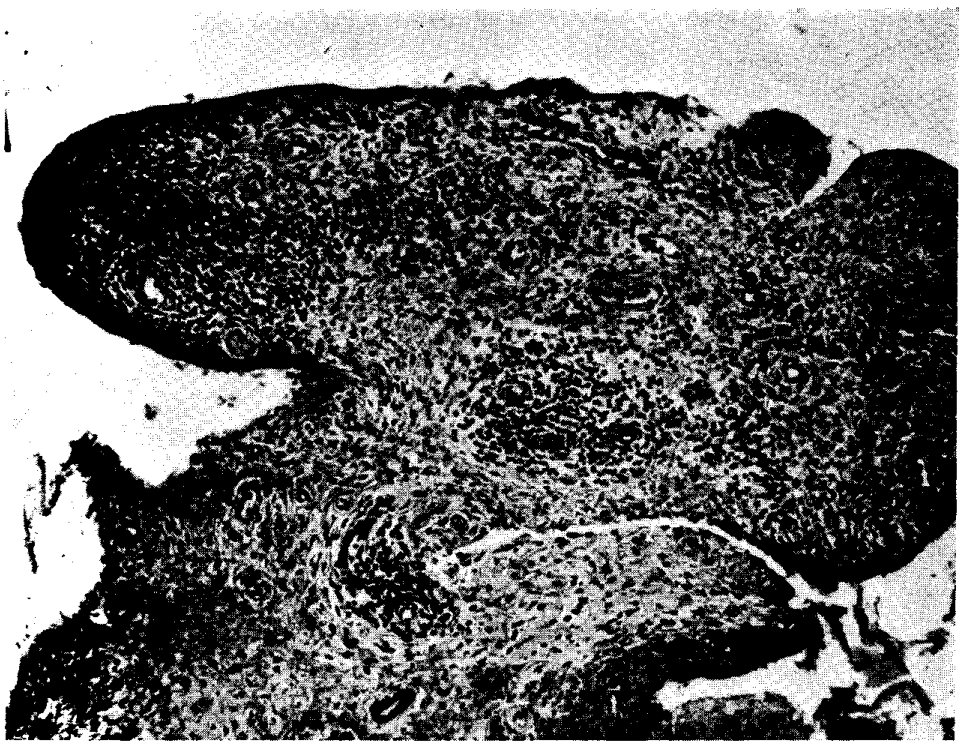

FIG. 4. Synovial membrane of a rabbit immunized with streptococci following the intra-articular injection of SF. The synovial membrane shows villous hyperplasia, and the adipose tissue is replaced by connective tissue heavily infiltrated with mononuclear cells. Hematoxylin and eosin. × 105.

the synovial membrane, the skin of rabbits, or to a variety of mammalian cells *in vitro* (3). Since SF has been found to sensitize mammalian cells to lysis in the presence of antibodies and complement (passive immune kill) (3, 16), it was of interest to study the capacity of the nontoxic SF to elicit passive immune arthritic lesions *in vivo*.

Table 3 shows that 11 of 13 rabbits previously immunized with streptococci and

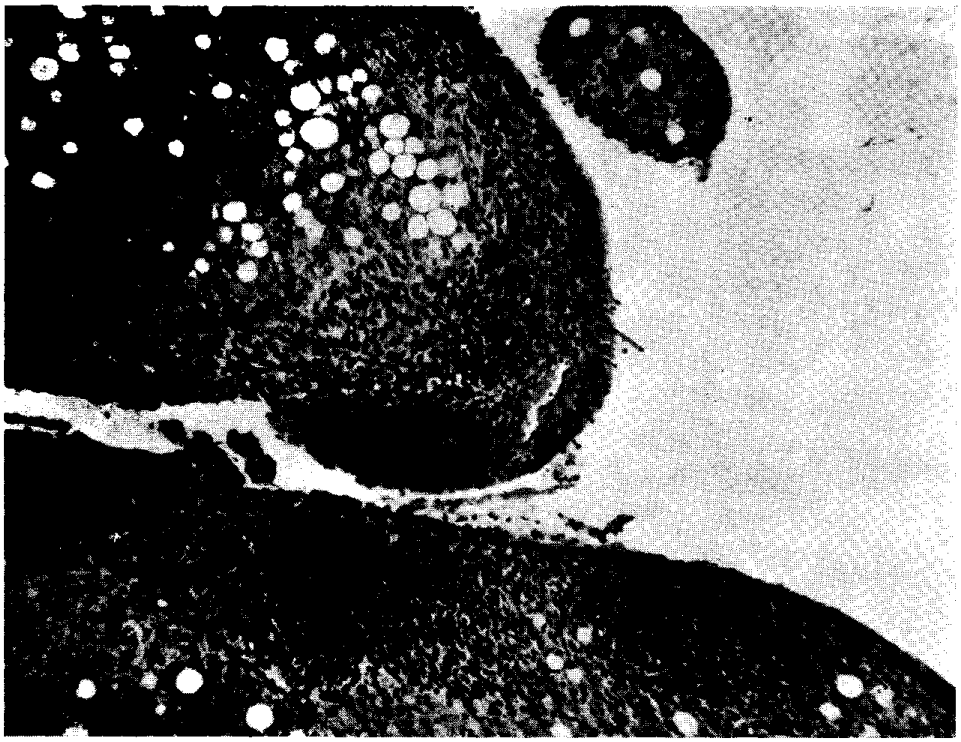

FIG. 5. Villous formations with fibrosis. Note the rounded dense accumulation of lymphatic cells tending to form "pseudolymphatic follicles." Hematoxylin and eosin. × 104.

which had anti-SF antibodies in their serum developed severe arthritis in the right knee joint when injected intra-articularly with SF. None of the animals which were given injections of saline into the left knee joint showed any lesions. On the other hand, only one out of eight nonimmunized rabbits developed very mild arthritis following the injection of SF (Fig. 3).

The first arthritic signs (swelling, tenderness and redness) appeared 24 hr following the injection of SF. In animals with low anti-SF titers, the arthritic lesions (10 days following the first injection of SF) were characterized by hyperplasia of the synovial membrane, villous formations and a severe inflammatory reaction which consisted of polymorphonuclears, lymphocytes and histiocytes (Fig. 4). Animals with high anti-SF titers also developed scar tissue and "pseudolymphatic follicles" in the hyperplastic villi (Fig. 5 and Table 3). The arthritic lesions were very similar to those induced in the rabbit knee joint by streptococcal extracellular products (12) or by immune complexes derived from streptococcal extracellular products and rabbit antibodies (11). They also bore a striking similarity to the joint lesions induced either by streptolysin S (17) or following the injection of streptococcal sonicates (13, 18).

The induction of cutaneous lesions with SF. All three animals immunized with streptococci developed erythema and nodular lesions in the skin (1 cm in diameter) 24 hr following a single i.c. injection of SF. Histologically, an acute inflammation with perivascular infiltrations of polymorphonuclear leukocytes was evident. Only very few monocytic cells were present in the lesions. The erythematous lesions subsided three to five days following the injection of SF. Nonimmunized animals developed only very slight erythema which disappeared within 24 hr and no histological changes were evident three days following the injection of SF.

DISCUSSION

The data presented demonstrate that SF is highly immunogenic when bound naturally to the streptococcus cell. On the other hand,

despite its high molecular weight, it is only poorly immunogenic when separated from the cells by phenol (Table 1). The low immunogenicity of SF may be due to several factors. It is possible that, like pneumococcal polysaccharide, SF is only poorly immunogenic in the rabbit. It was, however, also shown that no antibodies to SF could be raised in mice (19). Attempts have not, however, been made to demonstrate the immunogenicity of SF in other animal species. It is also possible that because of its high affinity to cell membranes, SF injected i.v. is immediately bound to RBC and WBC and thus does not reach the reticuloendothelial system (RES) in amounts sufficient to elicit an immune response. On the other hand, when naturally bound to some cell wall component of the streptococcus cell (carrier effect), it is readily engulfed by phagocytic cells, where it is released intracellularly together with mucopeptide in amounts sufficient to stimulate antibody formation. Although the streptococcal mucopeptide has recently been shown to function as an adjuvant (9), it failed to enhance the immunogenicity of SF.

Studies on the nature of the anti-SF antibodies revealed that unlike most of the anti-streptococcal antibodies (20) both human and rabbit anti-SF antibodies are associated with the macroglobulin fraction of serum.

Since rabbit anti-SF activity was present in two high molecular weight fractions (Fig. 1), it is possible that antibodies to SF in the rabbit are associated with both IgA and IgM. Recent studies (21) have indicated that both IgG and secretory IgA antibodies were induced in the colostrum of New Zealand red rabbits following the injection of group A streptococcal vaccine into the mammary gland, but no reference was made to the presence of antibody activity in the IgM fraction. On the other hand, the anti-SF activity of human and rabbit sera was always concentrated in the IgM fractions of the sera. Although both rabbit and anti-human SF sera were equally destroyed by 2-mercaptoethanol, the antibodies of the two animal species differed markedly in their heat stability. While human anti-SF antibodies were readily destroyed by heating above 58 C for 30 min, the rabbit antibodies withstood heating to 64 C (Fig. 2). The human anti-SF antibodies were also more labile to repeated freezing and thawing than the rabbit antisera. The nature of the difference between human and rabbit antibodies is still unknown. It is, therefore, suggested that only fresh human sera should be employed to titrate anti-SF activity (22).

As previously described (2, 4), SF is a teichoic acid containing PGP and alanine, where the PGP group constitutes the antigenic site and the alanine group is responsible for the binding of the SF molecule to the surface of the RBC (2). Since SF lost its capacity to sensitize RBC after it had interacted with anti-SF antibodies (Table 2), it is suggested that either antibodies with specificity for the alanine moiety have been raised, or that other nonimmune mechanisms, as yet unknown, are responsible for this effect.

As SF-anti-SF complexes fixed complement both in the absence and in the presence of RBC, it was postulated that animals with antibodies to SF could develop pathological lesions if SF sensitized the synovial tissue passively. It was indeed found that severe arthritis developed in the knee joints of immunized rabbits when SF was introduced intra-articularly. Similarly, acute inflammatory lesions were also induced by SF in the skin of immunized animals. On the other hand, nonimmunized animals failed to develop such lesions. The arthritic lesions which developed were characterized by proliferation of the synovial membrane, infiltrations with granulocytes and macrophages (Fig. 4) and by the appearance of "pseudolymphatic follicles," especially in animals with high anti-SF titers

(Fig. 5). The joint lesions were very similar to some of the lesions seen in rheumatoid arthritis in humans.

The mechanism by which SF induced arthritic lesions in immunized rabbits is not fully understood but it can be postulated that it involved passive immune sensitization *in vivo* (3, 16). It is likely that SF, which was introduced into the joints, was firmly bound to the synovial cells. Anti-SF antibodies which were present in the animals interacted with the sensitized cells and in the presence of serum complement caused passive immune cell lysis, as has been demonstrated in tissue cultures of rat heart (3). Some of the C constituents which functioned as chemotactic factors (23) attracted a large number of granulocytes which invaded the affected tissue and further enhanced the tissue damage through the release of lysosomal enzymes. It is also possible that circulating SF-anti-SF complexes which had been trapped in the synovial membrane triggered an acute inflammatory reaction of the Arthus type. Tissue damage could also have been induced by sensitized lymphocytes through delayed hypersensitivity mechanisms. Since the i.v. injection of SF in normal mice resulted in its localization in the heart, kidney, liver and spleen (19), it would be of great interest to study the outcome of such injection in animals with high serum titers of anti-SF.

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