BIOLOGICAL CHARACTERIZATION OF AN IMMUNOSUPPRESSANT FROM GROUP A STREPTOCOCCI*

By ARTIN H. MALAKIAN,‡ Ph.D., and JOHN H. SCHWAB, Ph.D.

(From the Department of Bacteriology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514)

(Received for publication 16 June 1971)

There is little doubt that the specific immune response is under direct genetic control at several levels (1). However, the extent to which environmental factors can modify regulation of the immune response is not well defined. The microbial flora of an animal certainly represents the most pervasive source of environmental influence; but the effect which bacteria may exert on the immune capacity, other than adjuvant properties, has received little attention. We propose that products of bacteria such as Group A streptococci, which are so common in the human environment, can have considerable qualitative and quantitative influence on the immune response.

In a preliminary report (2) we described a cytoplasmic component in extracts of mechanically disrupted Group A streptococci which suppresses the antibody response of mice against sheep red blood cells (SRBC).[1] This component exists in a very polydisperse complex with activity distributed over a broad size range which can be partially resolved by Sepharose chromatography. This paper describes the distribution of this material as it occurs in extracts of Group A streptococci processed in magnesium-free phosphate buffer and defines some of the parameters of its immunosuppressive effect on the host. The buffer composition is important insofar as it affects stability of ribosomes and aggregation. A subsequent paper[2] will demonstrate that under conditions of maximum ribosome stability the immunosuppressant factor is associated with ribosomes and can be derived from these structures as a low molecular weight protein.

Materials and Methods

Animals.—An outbred strain of Swiss-Webster mice from Carworth Farms, N. Y., and a closed colony bred for 20 yr at the University of North Carolina School of Public Health (Chapel Hill, N. C.) were used.

* This work was supported in part by grant AI-00949 from the U. S. Public Health Service.

‡ Present address: Department of Bacteriology, American University, Beirut, Lebanon.

[1] *Abbreviations used in this paper*: PBS, phosphate-buffered saline; PFC, plaque-forming cells; SF, Sepharose fractions; SRBC, sheep red blood cells.

[2] Schwab, J. H., H. R. Gaumer, A. H. Malakian, and R. R. Brown. 1971. An immunosuppressant from Group A streptococci. II. Identification of active component. Manuscript in preparation.

Bacterial Cultures and Fractions.—Group A, Type 3, strain D-58 streptococci were cultured for 18 hr at 37°C in Difco Todd-Hewitt broth (Difco Labs., Detroit, Mich.). Cells were collected by continuous flow centrifugation at 12,000 rpm in a Sorvall centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.), washed three times with cold saline, and resuspended in 0.05 M sodium phosphate buffer pH 7.2 plus 0.1 M NaCl to a concentration of 350 Klett units in a Klett-Summerson colorimeter (Klett Mfg. Co., New York) with a No. 54 green filter. This suspension was disrupted in a Braun shaker (Bronwill Scientific Inc., Rochester, N.Y.) for 3 min, using No. 12 glass beads and 1 drop of tributyl phosphate to prevent foaming. After settling of the glass beads, the supernate and washings of the glass beads were pooled and diluted with cold buffer to reduce viscosity. This suspension was then centrifuged at 12,000 *g* for 30 min to separate cell wall material (12p30) and the supernate centrifuged two successive times at 37,000 *g* for 45 min to remove remaining cell wall fragments and the large aggregates (37p45). The supernate (37s45) was the crude phosphate-buffered saline (PBS) extract, and some of this was further centrifuged at 100,000 *g* for 60 min to yield the 100s60 extract used in some experiments. Each of these sediments and supernates was dialyzed against water and lyophilized.

Measurement of Antibody-Forming Cells.—The hemolytic plaque technique of Jerne and Nordin (3) was used to detect direct plaque-forming cells (PFC) which we assume to be producing γM or 19S antibody. The indirect plaque technique developed by Šterzl and Řiha (4) was used to detect γG or 7S antibody-forming cells. The facilitating antibody was made by immunizing rabbits with mouse globulin eluted from a diethylaminoethyl (DEAE)-cellulose column with 0.005 M tris (hydroxymethyl) aminomethane (Tris) buffer pH 8.4. The optimum dilution of this antiserum was 1/150. At this dilution the antiserum inhibited 50% of the γM PFC. This factor was used to calculate the net γG PFC.

Mice were immunized intraperitoneally with 4×10^8 SRBC and hemolytic PFC measured in spleen cell suspension were collected 4 days later, unless otherwise specified. Duplicate measurements of PFC were done on spleen cell suspensions of each individual mouse.

Sepharose 2B Gel Filtration.—Ascending chromatography was done on Sepharose 2B (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) in a 95 × 2.5 cm column. 300 mg of 37s45 were applied to the column and fractions were eluted with 0.15 M NaCl at a rate of 10 ml/hr. Aseptic technique was carefully observed.

Analysis of Data.—Statistical calculations were done in the University of North Carolina Computer Center (Chapel Hill, N. C.) and probability of differences obtained by one-way analysis of variance.

RESULTS

Effect of Interval, Number of Injections, and Total Dose of Crude PBS Extract on Immunosuppressive Action.—

Four groups of mice were injected intraperitoneally with crude PBS extract (100s60) as follows: group I received one injection of 5.0 mg immediately before SRBC; group II was given six injections of 2.5 mg each, every other day and SRBC injected with the last dose; group III was given 13 injections every other day and SRBC with the last dose; group IV was the same as group III except the SRBC was given 7 days after the last (13th) injection. A control group given the same schedule of injections of saline was included with each experimental group. Direct PFC were determined 4 days after the injection of SRBC.

Table I shows that 1, 6, or 13 injections produced approximately the same degree of suppression of direct PFC when the SRBC were given with the last

injection. No suppression was obtained, however, after 13 injections if the interval between the last injection and SRBC was extended to 7 days.

In another experiment, groups of 10 mice were given a single injection of 5.0 mg of 100s60 at intervals of 1 or 2 days after SRBC or 1, 2, 7, or 14 days before SRBC. Highest suppression was obtained with an interval of 1 day (30% of control, $P = 0.007$) or 2 days (43% of control, $P = 0.01$) before SRBC. No significant suppression was observed at the other intervals. Comparable results

TABLE I

Effect of Interval, Number of Injections, and Total Dose of Crude PBS Extract on Primary Immune Response

Group*	Total 100s60 injected	No. mice	mg spleen/g‡	PFC/10^6 cells‡	% of Control
	mg				%
I 100s60 × 1	5	7	4.74 ± 0.87	128 ± 80	40
Control		7	5.43 ± 0.63	317 ± 153	
P§			0.12	0.01	
II 100s60 × 6	15	10	5.5 ± 1.16	125 ± 106	39
Control		10	5.5 ± 1.54	317 ± 192	
P			0.98	0.01	
III 100s60 × 13	32.5	5	7.52 ± 3.8	41 ± 42	44
Control		5	4.89 ± 1.1	183 ± 60	
P			0.18	0.01	
IV 100s60 × 13 (7 day)	32.5	5	5.7 ± 1.0	391 ± 176	72
Control		5	6.5 ± 1.1	546 ± 266	
P			0.28	0.31	

* For details, see text.

‡ Mean ±1 SD.

§ Probability calculated by one-way analysis of variance.

were obtained with a Sepharose fraction, although fewer intervals were studied (see below).

Filtration of Crude PBS Extracts of Group A Streptococci on Sepharose.— The immunosuppressive activity occurs in crude PBS extracts as a large complex distributed over a broad spectrum of aggregate sizes. Comparison of fractions derived by differential centrifugation shows that most of the activity is in the supernatant at 100,000 *g*, although some is sedimentable at this and lower forces. Activity soluble at 37,000 *g* is not retained by Sephadex G-200 (Pharmacia Fine Chemicals, Inc.) and is retained by an Amicon XM-100 membrane (Amicon Corp., Lexington, Mass.). This soluble crude extract was separated into several fractions by filtration on Sepharose 2B (Fig. 1). Each of

these Sepharose fractions (SF) is obviously heterogeneous and shows two to three precipitin lines in immunodiffusion. Assay for immunosuppression revealed that activity is associated with the entire range of molecular sizes represented in these fractions (Fig. 2). Nevertheless, a considerable increase in activity on a dry weight basis was achieved when compared with the initial crude extract (37s45). 40% of the material placed on the column was recovered. Fraction II (SF-II) contained the greatest total activity, considering that it had the bulk of recovered eluate (27%). Titration of SF-II showed a dose effect on the primary immune response to SRBC (Table II).

The effect of the interval of injection between SF-II and SRBC is shown in Table III. A high order of suppression is obtained with 1.0 mg SF-II injected

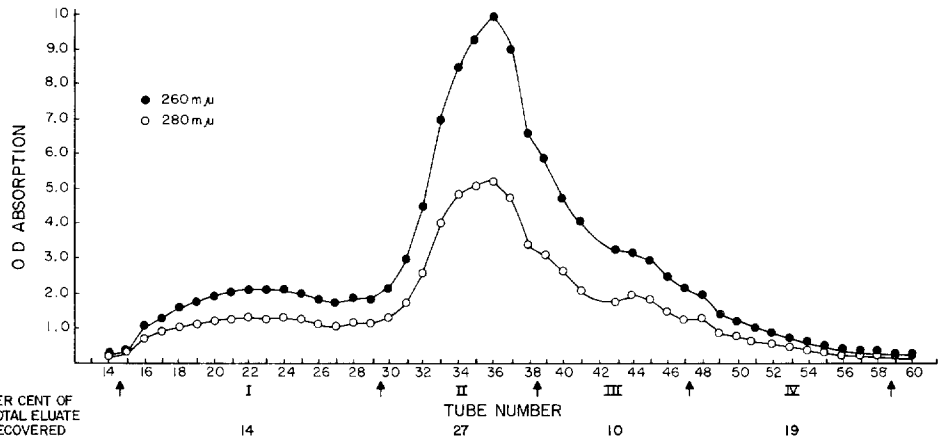

FIGURE 1. Ascending chromatography of PBS extract (37s45) on Sepharose 2B. 300 mg sample eluted with 0.15 M NaCl at flow rate of 10 ml/hr.

1 day before SRBC. Less effect, although still significant, is observed with an interval of 7 days before SRBC. When SF-II is injected 1 day after SRBC the response to SRBC is enhanced, although the difference is of low statistical significance.

Suppression by SF-II of Indirect and Direct PFC in the Primary and Secondary Immune Response.—To determine if SF-II delays rather than suppresses the primary immune response, PFC were measured in the spleens of mice injected with 1 mg of SF-II 24 hr before SRBC. Both direct and indirect PFC were determined 8 days after SRBC (Table IV). The direct PFC were very low at this time interval which limits reliability, but about 50% suppression was obtained, while the net indirect PFC were reduced 85%.

To determine the effect of SF-II on the secondary response, mice were injected with 1 mg of SF-II 24 hr before the second dose of SRBC, which was

injected 21 days after the primary immunization. No SF-II was given before the first injection of SRBC. Direct and indirect PFC were measured at both 2 and 4 days after the second injection of SRBC. As shown in Table V, the direct PFC per spleen were reduced significantly on day 4 ($P = 0.007$) and the net indirect PFC per spleen were reduced 79% on day 2 ($P = 0.004$) and 80% on day 4 ($P = 0.002$).

To determine the effect of SF-II on development of memory cells during

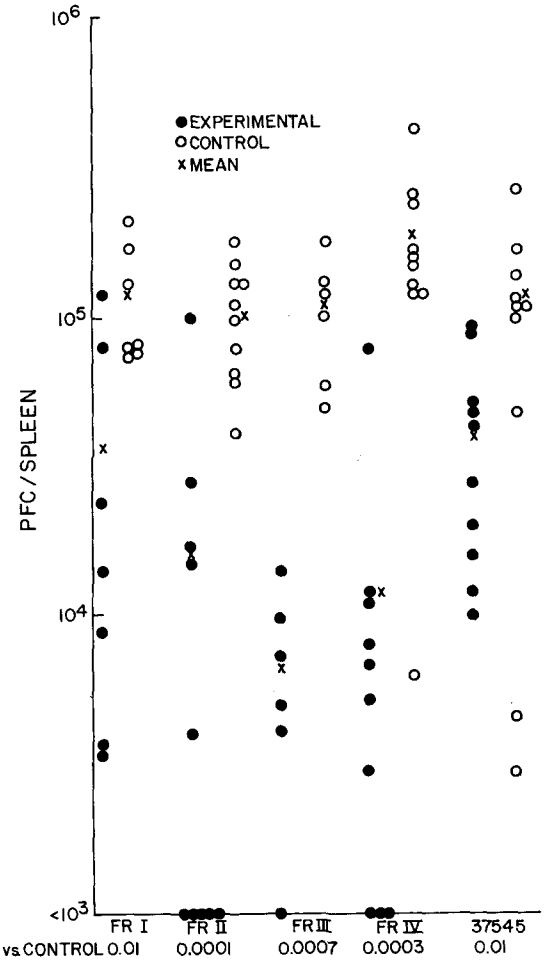

FIGURE 2. Suppression of primary immune response with fractions of PBS extract (37s45) obtained by Sepharose chromatography. Each group was injected with 5.0 mg (lyophilized weight) intraperitoneally. Each point represents one mouse. Because of the large number of mice, each group was assayed on a different day and a control group (injected with saline) was included with each assay.

TABLE II

Effect of Dose of SF-II on Primary Immune Response to SRBC

Group	Dose SF-II	No. mice	PFC/spleen × 10^4	% of Control	*P*
	μg			%	
I SF-II	200	7	6.0 ± 9.8	33	0.02
Saline	—	8	18.0 ± 8.9		
II SF-II	400	4	0.77 ± 0.48	6	0.001
Saline	—	5	13.6 ± 4.3		
III SF-II	1000	8	0.52 ± 0.3	2	0.0001
Saline	—	6	22.8 ± 9.4		

TABLE III

Effect of Interval between SF-II and SRBC

Group	Interval	No. mice	PFC/spleen × 10^4	% of Control	*P*
				%	
I SF-II	1 day after SRBC	7	28.2 ± 19.3	180	0.12
Control		7	15.6 ± 5.3		
II SF-II	1 day before SRBC*	8	0.52 ± 0.3	2	0.0001
Control		6	22.8 ± 9.4		
III SF-II	7 days before SRBC	8	7.3 ± 4.8	28	0.0004
Control		8	25.7 ± 9.4		

* Same as group III in Table II.

TABLE IV

Effect of SF-II on Direct and Indirect PFC in the Primary Response at 8 days after SRBC

Group	No. mice	Cells/ spleen × 10^8	PFC/spleen × 10^3		
			Direct	Indirect	Net indirect‡
Experimental*	8	2.7 ± 0.57	6.6 ± 8.2	10.4 ± 7.3	7.7 ± 4.8
Control	7	2.5 ± 0.24	13.3 ± 7.1	55.9 ± 15.7	50.9 ± 14.6
P		0.52	0.1	0.0001	0.0001

* Mice were injected i.p. with 1.0 mg SF in saline 24 hr before 4 × 10^8 SRBC i.p. Spleen cells were collected 8 days after SRBC. Controls received saline and SRBC.

‡ Corrected for 50% inhibition of γM by anti-IgG serum.

the primary immune response, 1 mg of SF-II was injected 1 day before the first injection of SRBC and a second injection of SRBC was given 3 wk later. SF-II was given only before the first SRBC. Direct and indirect PFC were measured 2 days after the second injection of SRBC. In spite of the high sup-

TABLE V

Effect of SF-II on Direct and Indirect PFC in the Secondary Response at 2 and 4 Days after SRBC

Group	Interval *days*	No. mice	Cells/ spleen × 10^8	PFC/spleen × 10^3		
				Direct	Indirect	Net indirect
Experimental*	2	6	2.95 ± 0.57	14.3 ± 12.8	24.2 ± 12.8	18.5 ± 7.6
Control	2	6	2.20 ± 0.32	17.7 ± 7.8	70.8 ± 31.6	63.6 ± 29.0
P			0.02	0.6	0.007	0.004
Experimental*	4	8	2.29 ± 2.9	4.3 ± 1.8	22.4 ± 19.6	19.7 ± 18.9
Control	4	8	1.85 ± 5.0	14.1 ± 8.5	101.3 ± 53.8	95.7 ± 52.3
P			0.05	0.007	0.001	0.002

* Two injections of 4 × 10^8 SRBC were given i.p. 21 days apart. 1 mg SF was injected i.p. 24 hr before the second dose of SRBC only. Spleen cells were collected for PFC measurement at 2 or 4 days after the last SRBC.

TABLE VI

Effect of SF-II on Direct and Indirect PFC in Secondary Immune Response when SF-II is Given Only before the First Dose of SRBC (Effect on Immunological Memory)

Group	No. mice	Cells/spleen × 10^8	PFC/spleen × 10^3		
			Direct	Indirect	Net indirect
Experimental	7	2.01 ± 0.27	14.5 ± 6.7	158 ± 68	152 ± 67
Control	6	2.05 ± 0.26	9.5 ± 5.0	122 ± 77	118 ± 75
P		0.80	0.16	0.62	0.58

TABLE VII

Effect of SF-II on Background PFC

Group	No. mice	mg spleen/g	mg thymus/g	Cells/spleen × 10^8	PFC/spleen
Experimental*	8	6.3 ± 2.7	2.7 ± 0.7	2.9 ± 0.57	554 ± 362
Control	8	4.9 ± 1.8	3.8 ± 0.9	2.3 ± 0.75	59 ± 51
P		0.23	0.15	0.100	0.002

* 1 mg SF-II injected i.p. 5 days before collection of spleen cells. Control group received saline. No SRBC given to either group.

pression of the primary response achieved with this dose of SF-II, and in contrast to the suppression of the secondary response when SF-II was given only before the second dose of SRBC, there was a slight increase in both direct and indirect PFC in the secondary response if SF-II was given only before the primary immunization (Table VI).

Effect of SF-II on the Background PFC in Hemolytic Plaque Test.—Most mammalian spleen cell suspensions produce a background of 19S PFC in direct hemolytic plaque tests without previous immunization with SRBC or other red blood cells. Spleens from unimmunized mice usually produce about 50–100 plaques when sheep erythrocytes are used in the test (5). To study the effect of SF-II on the background count, 1 mg of SF-II was injected into adult mice and the spleen cell suspensions were prepared 5 days later. Control mice

TABLE VIII

Effect of Intravenous Injection of SF-II on Primary Immune Response

Group	No. mice	mg spleen/g	mg thymus/g	Cells/ spleen $\times 10^8$	PFC/spleen $\times 10_\infty$
Experimental*	6	9.98 ± 1.84	2.88 ± 0.75	33.8 ± 25.1	24.7 ± 28.2
Control	5	5.98 ± 0.87	3.24 ± 1.05	2.8 ± 0.73	134.0 ± 37.9
P		0.00026	0.58	0.022	0.0006

* 1 mg SF-II was injected i.v. 24 hr before i.p. injection of 4×10^8 SRBC. PFC determined 4 days after SRBC injection.

were injected with saline. No SRBC were injected in either group. Table VII shows that SF-II produced a 10-fold increase of background counts.

Effect of Intravenous Injection of SF-II on the Primary Immune Response.—In all previous experiments both SF-II and sheep erythrocytes were injected intraperitoneally. To determine whether the effect of SF-II is altered by route of injection, 1 mg of SF-II was injected intravenously 24 hr before the intraperitoneal injection of red cells. The spleen cell suspensions were prepared 4 days after the injection of the antigen. As shown in Table VIII, intravenous injection of SF-II produced very significant suppression of the 19S PFC.

Effect of Enzyme Treatment of SF-II.—SF-II (1 mg/ml) in Tris buffer pH 7.2 was treated with pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N. J.; 20 μg/ml) for 16 hr at 37°C with a drop of chloroform added. A control sample was incubated without added ribonuclease. One-half of each sample was dialyzed against Tris buffer. There was a large decrease in absorbance at 260 mμ in both the test and control dialyzed samples, reflecting considerable endogenous ribonuclease. Each of the four samples was injected into mice 24 hr before SRBC in amounts corresponding to 400 μg of untreated

SF-II. Control mice received Tris buffer. There was approximately a 95% reduction of 19S PFC/spleen produced with all samples except the enzyme-treated but not dialyzed material, which contained nucleotide digest products known to enhance the immune response and hence would counteract the suppressant effect (Fig. 3). A sample of the ribonuclease-treated SF-II was subsequently treated with trypsin (20 μg/ml Tris buffer pH 8.2) for 9 hr at 37°C

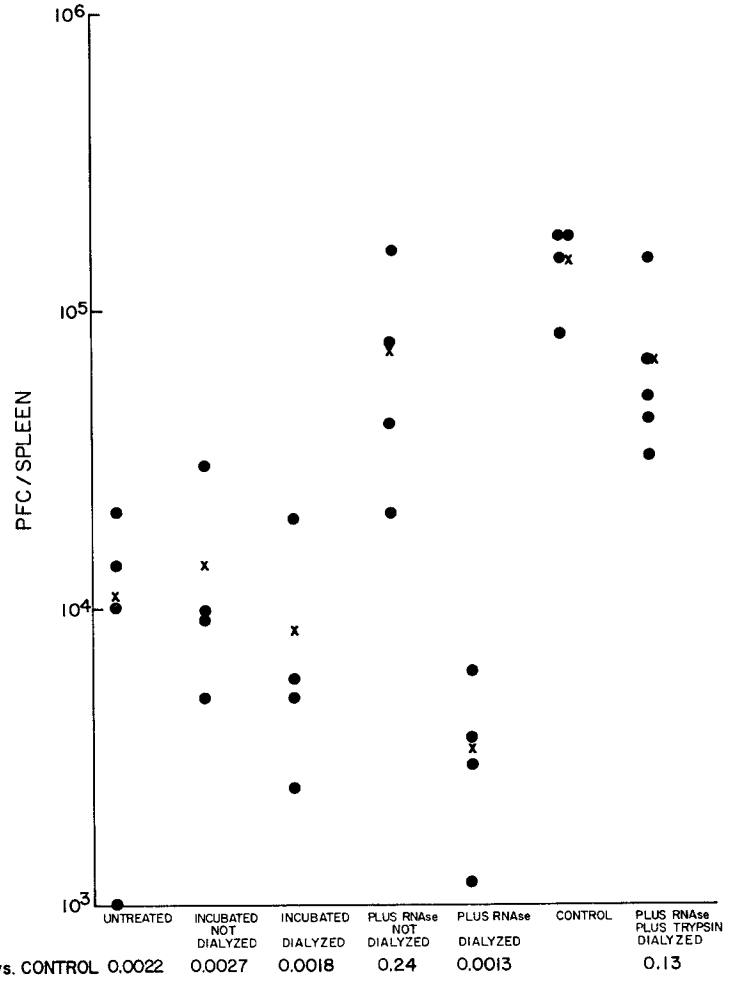

FIGURE 3. Effect of ribonuclease and trypsin on immunosuppressant activity of SF-II. Each point represents one mouse. SF-II was incubated with enzyme and dialyzed, as indicated. Each preparation was then injected into mice 24 hr before SRBC and PFC measured 4 days later.

and dialyzed against saline. This treatment destroyed most of the immunosuppressive activity (Fig. 3). Similar experiments demonstrated that pronase also destroyed the activity, and deoxyribonuclease had no effect.

Effect of SF-II on Immune Response to a Protein Antigen.—500 μg of SF-II were injected 1 day before 100 μg of heat-aggregated human γ-globulin. Serum was collected 8 days later and antibody measured by passive hemagglutination titration. The mean antibody titer of the SF-II–treated mice was four wells lower than the control group (Table IX).

TABLE IX

Effect of SF on Antibody Response to Aggregated Human γ-Globulin (HGG)

Group*	No. mice	Log_2 titer‡	Range
HGG + saline	5	7.5	2–16
HGG + SF	5	3.2	0–8

* 500 μg SF and 100 μg HGG in saline injected i.p.

‡ Mean passive hemagglutination titer of serum 8 days after injection of HGG.

DISCUSSION

Of all potential sources of immunoregulatory factors in our environment, bacteria are the most ubiquitous and have the most intimacy with animal tissues. The Group A streptococcus is probably the most common pathogenic bacterium for man, but there is no doubt that products of other microorganisms can also influence immunity, as indicated by recent reports in the literature (6–8).

The consequences to the host of this microbial source of immunosuppressive agents are difficult to ascertain. The importance of timing of suppressant factor injection relative to antigen, which can determine either suppression or enhancement of immunity also is observed with other agents such as 6-MP (9) or endotoxin (8). This complicates interpretation of the role of microbial immunosuppressants in host responses to infection, or possibly establishment of a neoplastic cell (10).

The immunosuppressive factor exists in PBS extracts of mechanically or osmotically disrupted Group A streptococcal cells as a polydisperse complex with activity distributed over a broad size range. After chromatography on Sepharose 2B, activity is distributed over the entire range of molecular sizes and the fractions are still very heterogeneous. This is nevertheless a very useful step since it provides material with about a 10-fold increase in potency over the crude extract and with considerably more consistency between batches. This is because of the separation from adjuvant factors such as ribonucleotides (11) or cell wall mucopeptides (12), which could counterbalance an immuno-

suppressive effect. Thus the Sepharose filtration provides a method for obtaining reasonable amounts of material and has enabled us to investigate various parameters of its activity. The protein nature of the factor is indicated by its susceptibility to trypsin and resistance to ribonuclease and deoxyribonuclease. Further characterization of the active component and identification of its source in the bacterial cell is described in a subsequent paper, which also demonstrates the resolution of adjuvant and suppressive fractions in the crude extract.[2]

A single injection of the crude PBS extract (37s60 or 100s60) 1 day before SRBC produced a significant suppression of antibody-forming cells (35% of control, $P = 0.01$). This was about the maximum degree of suppression which could be achieved with crude material, since higher doses or repeated injections did not produce a greater effect. In contrast, fraction SF-II obtained by Sepharose filtration produced 94% suppression with as little as 400 μg. We conclude that the maximum limit of suppression obtainable with crude material reflects a balance with the competing adjuvant factors. The fact that repeated injection does not *decrease* the degree of suppression obtainable with crude extract suggests that SF is not immunogenic in mice and therefore probably not functioning by antigenic competition.

Repeated injections of crude extract do not prolong the period of immunosuppression. After either single or multiple injections the immunosuppressive effect is lost when the interval before SRBC is extended to 7 days. On the other hand, after one injection of SF-II significant suppression is observed even with an interval of 7 days before SRBC.

The observation that the route of injection of SF is not important shows that it is not functioning by influencing antigen translocation from the peritoneum as reported to be the effect of phytohemagglutinin in mice (13). A feature which distinguishes this streptococcal factor from most other immunosuppressants is its very low toxicity. Even repeated large doses induce no clinically visible effect, although the histology of the bone marrow is modified.[3]

The background PFC in normal mouse spleen reflects immunocompetent cells stimulated by antigens in the environment cross-reactive with SRBC. The increase of this background level by SF reflects increased proliferation of these sensitized cells, and the observation that injection of SF 1 or 2 days after SRBC increases the number of PFC, is an analogous situation. The increase of background PFC does not indicate a cross-reactive antigen between SF and SRBC since no secondary response is noted when SRBC are injected at intervals up to 3 wk after SF, and addition of SF in vitro to spleen cell suspensions or the plating media in the PFC measurement does not reduce the development of hemolytic plaques.

The mechanism by which SF suppresses antibody formation is still conjectural. It cannot interfere with the late processes of induction or production

since injection 1 or 2 days after antigen is ineffective and may even be stimulatory. It does effect stimulation of immunocompetent cells to γM or γG antibody production in either a primary or secondary response, but does not alter induction of memory cells. Antibody formation against SRBC in mice requires both thymus and bone marrow cells. In this concept of two-cell interaction, the induction of memory is thought to be from thymus cells (14, 15). This suggests that the site of SF action is the prethymus or bone marrow cell, and current studies on lymphocyte populations of the mouse demonstrate that the streptococcal immunosuppressant is selectively blocking bone marrow stem cell development.[3]

SUMMARY

A component in extracts of Group A streptococci suppresses antibody formation in mice against heterologous erythrocyte and protein antigens. Large doses are not toxic and repeated injection does not change its effectiveness. It is most effective when injected 1 or 2 days before antigen and it is not suppressive when given after antigen. The active factor occurs as a large polydisperse complex and activity can be increased 10- to 25-fold by filtration through Sepharose 2B. Both direct (γM) and indirect (γG) antibody-forming cells are suppressed in primary and secondary responses. Injection before a primary response does not reduce memory cell development. It increases rather than depresses the "background" antibody-forming cells to sheep erythrocytes, and is equally effective if injected intraperitoneally or intravenously. Ribonuclease increases activity while deoxyribonuclease has no effect. Proteases destroy immunosuppressive action.

REFERENCES

1. McDevitt, H. O., and B. Benacerraf. 1969. Genetic control of specific immune responses. *Advan. Immunol.* **11**:31.
2. Malakian, A., and J. H. Schwab. 1968. Immunosuppressant from group A streptococci. *Science (Washington).* **159**:880.
3. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody producing cells. *Science (Washington).* **140**:405.
4. Šterzl, J., and I. Řiha. 1965. Detection of cell producing 7S antibodies by the plaque technique. *Nature (London).* **208**:858.
5. Hege, J. S., and L. J. Cole. 1967. Antibody plaque-forming cells in unsensitized mice. Specificity and response to neonatal thymectomy, X-irradiation and PHA. *J. Immunol.* **99**:61.
6. Renoux, G., M. Renoux, and R. Tinelli. 1970. Influence of Brucella endotoxins on the initiation of antibody-forming spleen cells in mice immunized with sheep red blood cells. *Infec. Immunity.* **2**:1.

[3] Gaumer, H. R., and J. H. Schwab. Unpublished results.

7. Spiller, L., K. Cochrum, and H. H. Fudenberg. 1968. Mycoplasma inhibition of PHA stimulation of lymphocytes. *Science (Washington)*. **161:**1148.
8. Franzl, R., and P. McMaster. 1968. The primary immune response in mice. I. The enhancement and suppression of hemolysin production by a bacterial endotoxin. *J. Exp. Med.* **127:**1087.
9. Schwartz, R. S. 1966. Specificity of immunosuppression by antimetabolites. *Fed. Proc.* **25:**165.
10. Allison, A. C. 1970. Tumour development following immunosuppression. *Proc. Roy. Soc. Med.* **63:**1077.
11. Merritt, K., and A. G. Johnson. 1965. Studies on the adjuvant action of bacterial endotoxins on antibody formation. VI. Enhancement of antibody formation by nucleic acids. *J. Immunol.* **94:**416.
12. Holton, J. B., and J. H. Schwab. 1966. Adjuvant properties of bacterial cell wall mucopeptides. *J. Immunol.* **96:**134.
13. Chan, P. C. Y. 1969. Immunosuppressive action of phytohemogglutinin. Effect on peritoneal macrophages. *Int. Arch. Allergy Appl. Immunol.* **36:**486.
14. Davies, A. J. S., E. Leuchars, V. Wallis, R. Marchant, and E. V. Elliot. 1966. The mitotic response of thymus derived cells to antigenic stimulus. *Transplantation.* **4:**438.
15. Jacobson, E. B., J. L'Oge-stehr, and L. Herzenberg. 1970. Immunological memory in mice. II. Cell interactions in the secondary immune response studied by means of immunoglobulin allotype markers. *J. Exp. Med.* **131:**1109.