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Characterization of the Antibody Response to Type III Pneumococcal Polysaccharide at the Cellular Level

I. DOSE-RESPONSE STUDIES AND THE EFFECT OF PRIOR IMMUNIZATION ON THE MAGNITUDE OF THE ANTIBODY RESPONSE

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Summary. The cytokinetics of the antibody to Type III pneumococcal polysaccharide (SSS-III) were characterized by an immuno-plaque procedure using erythrocytes sensitized with SSS-III. Prior immunization, irrespective of the doses employed, did not result in the development of immunological memory; instead, low-dose paralysis was produced in mice previously immunized with all doses of SSS-III.

Dose-response studies revealed that within a given dose range, there was a direct relationship between the immunizing dose and the magnitude of the antibody response obtained. The dose-response curve for SSS-III showed a single optimal dose for immunization; doses only slightly in excess of the optimal dose produced a significant reduction in the magnitude of the antibody response. The implications of these findings, with respect to the development of paralysis to SSS-III, are discussed.

INTRODUCTION

Pneumococcal polysaccharides (SSS) are naturally occurring linear polymers composed of hundreds of identical saccharide sub-units (How, Brimacombe and Stacey, 1964). Because of their structural simplicity and uniformity, these antigens are ideally suited for use in basic studies on the antibody response.

In the present work, the influence of immunizing dose and prior immunization on the magnitude and the cytokinetics of the antibody response to Type III pneumococcal polysaccharide (SSS-III) were evaluated. An adaptation of the technique of localized haemolysis-in-gel was used for the detection of specific antibody-producing cells (Baker, Stashak and Prescott, 1969). For sub-optimal and optimal doses of SSS-III, there was a direct relationship between the magnitude of the cellular and humoral antibody response produced and the dose of antigen used for immunization. Prior immunization, irrespective of the doses employed, did not lead to the development of immunological memory. Instead, transient low-dose paralysis was demonstrable upon re-immunization with an optimal dose of antigen. A tentative model for the induction of low-dose paralysis to SSS-III is proposed.

MATERIALS AND METHODS

Antigen

Purified nitrogen-free Type III pneumococcal polysaccharide was prepared by a calcium phosphate modification of the procedure of Felton, Kauffmann and Stahl (1935). It is the same preparation used in earlier work on the antibody response to SSS-III (Baker, Stashak and Prescott, 1969; Baker and Stashak, 1969), and by Felton, Kauffman, Prescott and Ottinger (1955) in their original studies describing the phenomenon of immunological paralysis.

Animals

Female BALB/c mice (8–12 weeks of age), obtained from the Rodent and Rabbit Production Section of the National Institutes of Health, were used.

Immunization procedure

Various amounts of SSS-III were administered intraperitoneally in a volume of 0·5 ml saline. Mice immunized with sheep erythrocytes (SRBC) received a single intraperitoneal injection of a known number of washed erythrocytes in 0·2 ml saline.

Detection of serum antibody and antibody-forming cells

Serum antibody and splenic antibody-forming cells specific for SSS-III were detected by the specific haemolysis of SSS-III-coated sheep erythrocytes and by a slide version of the technique of localized haemolysis-in-gel, respectively (Baker *et al.*, 1969); SRBC were coated with SSS-III by a chromium chloride coupling procedure (Baker *et al.*, 1969). In studies on the antibody response to SRBC, native SRBC were used in the above procedures. Unless otherwise stated, serum antibody levels were expressed as the reciprocal of the $\log_2$ of the highest dilution of antiserum producing complete haemolysis of a standard suspension of coated (or uncoated) erythrocytes; the cellular antibody response was expressed as the number of specific plaque-forming cells (PFC) produced per spleen.

Although immunization with SSS-III did not lead to the development of antibody or PFC capable of lysing native SRBC, uncoated SRBC were used as controls in all assays for SSS-III-specific PFC to permit an adjustment to be made for the small number of SRBC-specific background PFC present. Generally, about 200–400 such background PFC were present in the spleens of non-immunized mice. SSS-III-specific PFC were not present in the spleens of non-immunized mice (Baker and Stashak, 1969).

RESULTS

THE INFLUENCE OF IMMUNIZING DOSE ON THE DEVELOPMENT OF DIRECT AND INDIRECT SSS-III-SPECIFIC PFC

In previous studies, class-specific anti-γ-globulin sera were used to characterize the types of cells involved in the antibody response to SSS-III (Baker and Stashak, 1969). While only PFC synthesizing antibody of the IgM class could be detected two types of IgM-producing PFC were found. These were referred to as direct PFC, i.e. PFC which could be detected without the use of class-specific anti-γ-globulin sera, and as indirect PFC which could be detected only with the aid of antisera specific for murine IgM. Since

both types of PFC were present in the spleens of all mice immunized with SSS-III, we sought to determine whether the dose of antigen used to immunize would selectively favour the development of either type of PFC.

Maximal numbers of both types of PFC were detected, 5 days after immunization. Also, about equal numbers of direct and indirect PFC were present at 5, 7, 10 and 14 days after immunization (Baker and Stashak, 1969). Consequently, we elected to assess the effect of immunizing dose on the development of both types of PFC by comparing the numbers of direct and indirect PFC produced, 5 days after immunization with various doses of SSS-III.

The data derived from such studies (Table 1) show that within a given dose range (0·001–0·5 μg) the number of direct and indirect PFC produced was dose-dependent.

TABLE 1

MAXIMAL NUMBERS OF DIRECT AND INDIRECT SPLENIC PFC PRODUCED IN MICE IMMUNIZED WITH VARIOUS AMOUNTS OF SSS-III*

Immunizing dose, μg/mouse	Direct PFC/ spleen	Indirect PFC/ spleen
0·001	100	—
0·005	380	960
0·01	1980	780
0·05	5260	6100
0·10	8760	5700
0·25	11,960	10,600
0·50	16,260	23,600
1·0	9860	10,000
5·0	3600	7260
10	580	1200
25	40	0
50	0	0
100	0	—
200	0	—

* Pooled spleen cell suspensions obtained from six to eight mice were used for all determinations.

Since the greatest number of PFC were produced in response to 0·5 μg of SSS-III, that dose was considered to be the optimal dose for immunization. Immunization with doses greater than the optimal dose resulted in a reduction in the magnitude of the cellular antibody response.

The data of Table 1, when presented in the form of a bivariate distribution (Fig. 1), yield a curve with a slope very close to unity (slope $\pm$ SE of the slope = $0{\cdot}99 \pm 0{\cdot}28$). This suggests that, within the range of immunizing doses employed, about equal numbers of both types of PFC were produced in response to SSS-III. Therefore, the results of the remaining studies to be described were based on the number of direct PFC produced. It seemed reasonable to assume that in so doing, the data obtained would be representative of the macroglobulin antibody response to SSS-III in general.

EFFECT OF PRIOR IMMUNIZATION ON THE MAGNITUDE AND THE CYTOKINETICS OF THE ANTIBODY RESPONSE

Groups of mice were immunized with 0·001 μg, 0·005 μg, or 0·5 μg of SSS-III; these were doses which regularly elicited a marginal, a very weak, or a maximal antibody

response, respectively (Table 1). Then, at 2, 14, or 25 days after primary immunization (priming), the mice were re-immunized with an optimal dose (0·5 μg) of SSS-III, and the magnitude of the resultant antibody response was assessed 5 days later. The responses obtained were compared to the response elicited by an optimal dose in mice that had not been immunized previously (control group).

The data presented in Table 2 clearly show that all of the doses used for priming reduced the magnitude of the antibody response to an optimal dose of antigen. In comparison to the control group, 90, 96 and 98 per cent fewer specific PFC were elicited in response to an optimal dose of SSS-III given 2 days after priming with 0·001 μg, 0·005 μg

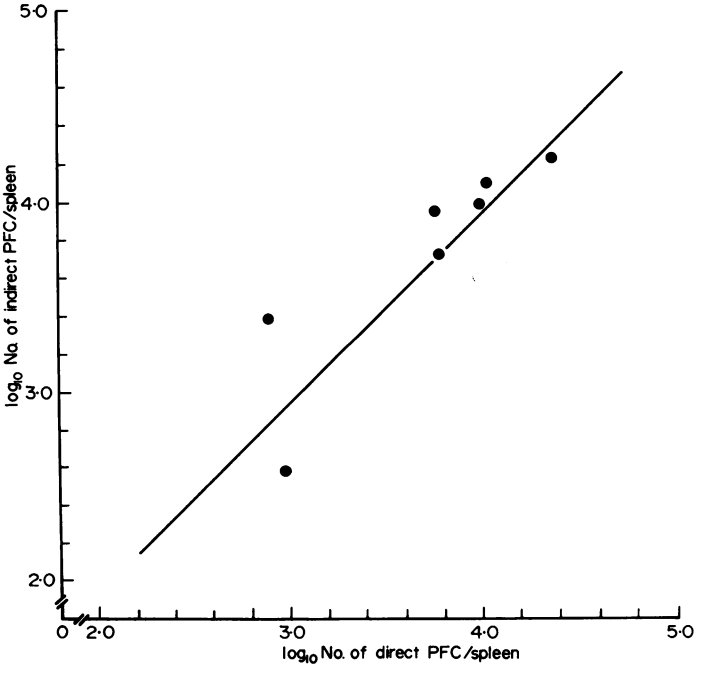

FIG. 1. Bivariate distribution of the numbers of direct and indirect PFC obtained in mice immunized with different amounts of SSS-III.

or 0·5 μg of SSS-III, respectively. With the same priming doses, about 65, 88 and 98 per cent fewer PFC were produced in response to an optimal dose given 25 days after priming. Corresponding reductions in the mean serum antibody levels were also noted.

Different results were obtained when the time interval between priming and re-immunization was extended. The kinetics of the primary antibody response to an optimal dose (0·5 μg) of SSS-III, and the kinetics of the response obtained in mice given a second injection of SSS-III (0·5 μg), 6 months after primary immunization, are given in Fig. 2. Primary immunization with this dose was shown to induce the greatest degree of unresponsiveness encountered in mice re-immunized 2–25 days after priming. With the possible exception that the antibody response elicited following re-immunization may have been more sustained, no differences were apparent in the kinetics of the primary and secondary responses obtained. PFC and serum antibody levels increased at about the same rate and to the same extent. The serum antibody level, which in all likelihood is a

measure of the total antibody response evoked, was directly related to the number of splenic PFC detected. This suggests that the response elicited in the spleens of mice immunized with SSS-III very closely approximates the total cellular antibody response produced. We were unable to detect SSS-III-specific PFC in the blood, in the mesenteric

TABLE 2

EFFECT OF PRIOR IMMUNIZATION WITH VARIOUS AMOUNTS OF SSS-III ON THE MAGNITUDE OF THE ANTIBODY RESPONSE OBTAINED FOLLOWING RE-IMMUNIZATION WITH AN OPTIMAL DOSE (0·5 μg) OF SSS-III*

Interval between injections of SSS-III	Dose used for prior immunization					
	0·001 μg		0·005 μg		0·5 μg	
	PFC/spleen	Titre†	PFC/spleen	Titre	PFC/spleen	Titre
2 days	600	4	600	4	240	3
	4800	5	320	4	240	0
	1000	4	280	4	200	0
	100	3	800	4	300	3
	300	3	720	4	220	4
	(1360)	(3·8)	(544)	(4·0)	(240)	(2·0)
14 days	Not done	Not done	4400	4	2600	6
			1800	4	1400	6
			6900	6	1500	3
			1400	0	2200	6
			5500	4	1700	6
			3000	3	1300	0
			2600	5	1100	0
			3700	6	1300	0
					3200	0
			(3662)	(4·0)	(1811)	(3·0)
25 days	4200	5	1080	4	160	3
	5970	5	1760	1	440	3
	3400	5	1520	5	440	3
	4600	5	1200	5	280	3
	4000	5	1680	5	400	4
	(4434)	(5·0)	(1448)	(4·0)	(344)	(3·2)
Controls‡	10,380	8440		8 7		
	8920	9600		7 7		
	19,400	16,400		8 7		
	7000	8800		7 6		
	19,000	18,800		9 7		
	17,200	10,900		7 7		
	(12,900)			(7·2)		

* The values listed represent determinations made using individual mice, 5 days after re-immunization. Mean values are in parentheses.

† $\log_2$ of the highest dilution of serum giving complete haemolysis of antigen sensitized erythrocytes.

‡ Responses obtained in twelve mice 5 days after primary immunization with 0·5 μg SSS-III.

lymph nodes or in the bone marrow of mice immunized with an optimal dose of SSS-III.

In the same study, about equal numbers of direct and indirect PFC were detected at corresponding time intervals after re-immunization. As was the case for the primary response to SSS-III (Baker and Stashak, 1969), IgG producing PFC were not detected following re-immunization. Under such test conditions, the antibody response elicited upon re-immunization was in all respects nearly identical to that observed for a primary response.

MAGNITUDE OF THE ANTIBODY RESPONSE OBTAINED IN MICE PREVIOUSLY IMMUNIZED AND RE-IMMUNIZED WITH VERY LOW DOSES OF SSS-III

Because of the small numbers of PFC produced, the very low serum antibody levels attained, and the variation encountered in the response to marginally and weakly immunogenic doses of SSS-III, it was difficult to evaluate the effects of priming on the magnitude of the antibody response elicited in mice re-immunized with these doses. Large numbers of mice are required for such studies in order to reveal differences which may be statistically significant. However, the results of preliminary studies showed that, in general, priming with sub-optimal doses of SSS-III did not alter the capacity of mice to respond to sub-optimal doses administered 2–4 weeks after priming.

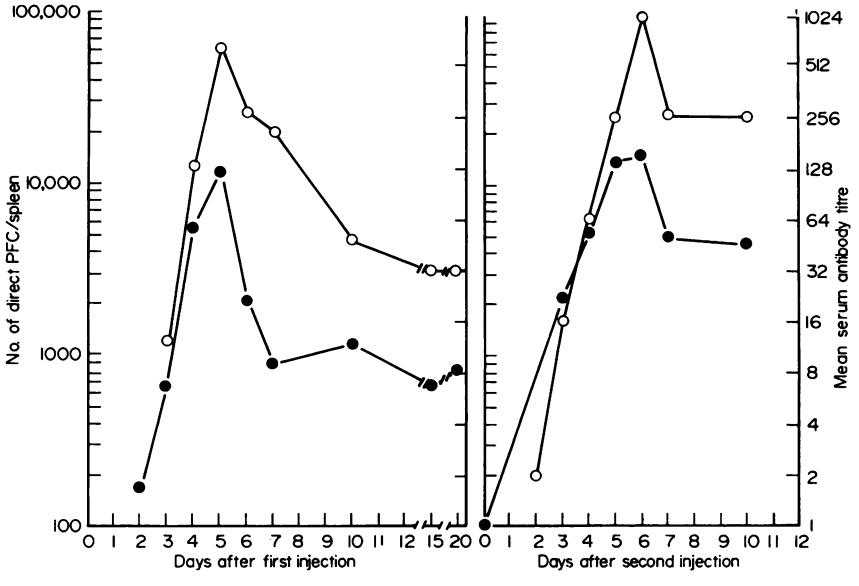

FIG. 2. Kinetics of the cellular and humoral antibody response obtained in groups of mice given one or two injections of 0·5 μg SSS-III. The second injection of SSS-III was given 6 months after primary immunization with the same dose. The values shown are mean values derived from individual determinations using six to eight mice. ●, PFC/spleen; ○, serum antibody titre.

DOSE-RESPONSE STUDIES CONDUCTED WITH SSS-III AND SRBC

The results of a comparative study on dose-response relationships obtained with SSS-III and SRBC are summarized in Figs 3 and 4. Pooled spleen cell suspensions, obtained from six to eight similarly treated mice, were used to determine the maximal number of specific PFC produced in response to each immunizing dose of antigen. The antibody titres shown are the means of titrations performed on individual serum samples from the same six to eight mice.

The dose-response curves obtained illustrate that striking differences exist in the manner in which immunizing doses affect the magnitude of the antibody response produced by both antigens. In the case of mice immunized with 0·01–0·5 μg of SSS-III (Fig. 3), the magnitude of the cellular and humoral antibody response obtained is a linear function of the immunizing dose; a ten-fold increase in the immunizing dose resulted in a corres-

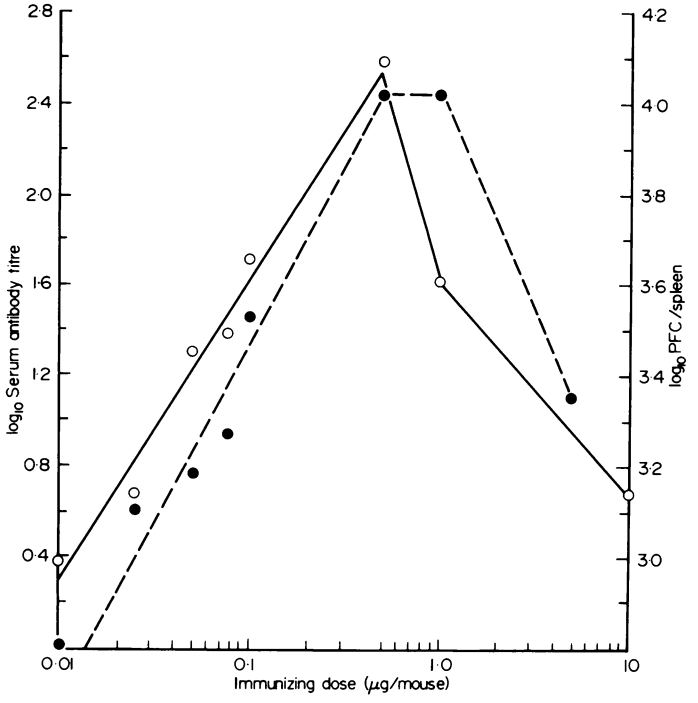

FIG. 3. Maximal serum antibody levels and maximal numbers of direct PFC obtained for mice immunized with various amounts of SSS-III. ●, PFC/spleen; ○, serum antibody titre.

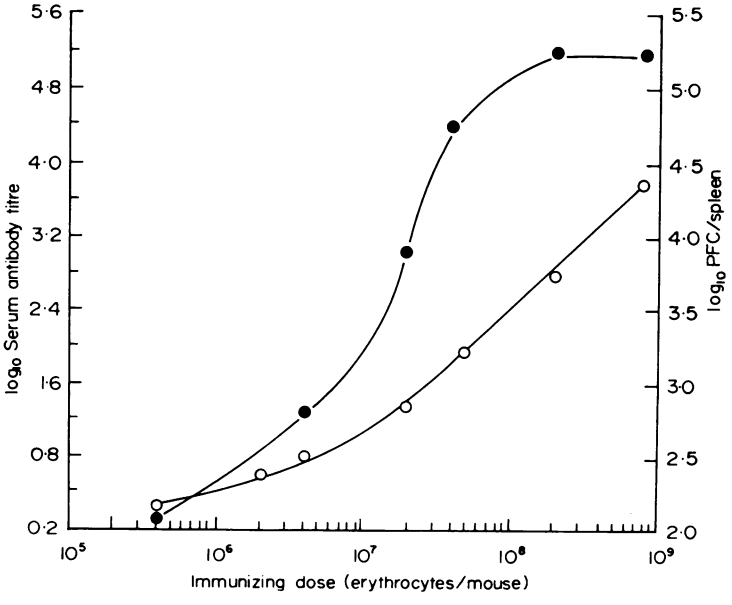

FIG. 4. Maximal serum antibody levels and maximal numbers of direct PFC obtained for mice immunized with various numbers of sheep erythrocytes. ●, PFC/spleen; ○, serum antibody titre.

ponding ten-fold increase in both the number of specific PFC and the serum antibody level. As noted for the primary antibody response (Fig. 2), there was good agreement between the number of splenic PFC produced and the serum antibody level obtained. Similar relationships were not observed in dose response studies with SRBC (Fig. 4); there was no direct relationship between immunizing dose and the magnitude of the antibody response, and the serum antibody levels attained were not related to the number of splenic PFC produced.

A unique feature of the dose-response curve for SSS-III is its prominent sharp peak and single optimal immunizing dose; immunization with doses only two to three times greater than the optimal dose resulted in a significantly reduced antibody response. No SSS-III-specific PFC or serum antibody could be detected in mice receiving 50 μg or more of the antigen (Table 1). In contrast, the dose-response curve for SRBC exhibited a 'plateau effect' in which immunization with doses of SRBC ten times or more greater than the lowest optimal dose (2×10^8 SRBC) produced a maximal antibody response.

DISCUSSION

In this work and in previous studies (Baker and Stashak, 1969), only PFC synthesizing antibody of the IgM class were detected following primary immunization and re-immunization with SSS-III; however, two types of IgM-producing PFC (direct and indirect) were found. Equal numbers of both types of PFC were produced in response to all doses of SSS-III, and the numbers obtained were dose-dependent (Fig. 1; Table 1). These observations show that the antibody response to SSS-III in mice is exclusively an IgM response. The role of direct and indirect PFC in the development of immunity and paralysis to SSS-III has not been established. In contrast, PFC producing antibody of the IgM, IgG and IgA class can be detected in mice immunized with formalin-treated Type III pneumococci (unpublished observations).

Short-term IgM memory has been demonstrated following primary immunization with low or sub-optimal doses of bacteriophage ΦX 174 (Uhr and Finkelstein, 1963), poliovirus (Svehag and Mandel, 1964), or SRBC (Sercarz and Byers, 1967). In such studies, the dose of antigen used for priming stimulated the formation only of IgM antibody and IgM memory was expressed upon re-immunization shortly after the peak of the resultant primary response. Also, Nossal, Austin and Ada (1965) demonstrated IgM memory to flagellin and flagella under circumstances in which primary immunization did not give rise to detectable serum antibody.

In the present work, an attempt was made to demonstrate short-term IgM memory to SSS-III under a variety of test conditions. None of these, including those shown to be ideal for producing the effect with other antigens, led to the development of immunological memory; instead, all treatments produced a state of unresponsiveness which persisted—depending upon the dose of SSS-III employed—for several weeks (Table 2). Because of the small amounts of antigen used to produce the effect, such unresponsiveness has been termed low-dose paralysis, in contrast to the longer-lasting high-dose paralysis regularly induced with large doses (100–500 μg) of SSS (Felton *et al.*, 1955). Both types of paralysis differ from the low-zone and high-zone paralysis produced with bovine serum albumin (Mitchison, 1968); only a single injection of low or high doses of SSS-III was required to induce paralysis, and paralysis could be induced even with an optimally immunogenic dose of SSS-III.

The low-dose paralysis obtained with SSS-III also differs from the tolerance produced in rats with multiple injections of small numbers of SRBC (Rowley and Fitch, 1965); paralysis to SSS-III was of longer duration and could be induced in the absence of detectable serum antibody and PFC. Marginally and weakly immunogenic doses of SSS-III gave rise to no, or at best exceedingly small, amounts of serum antibody and PFC; yet such doses were just as effective as an optimal dose in inducing paralysis. Also, the same degree of unresponsiveness to SSS-III was evident in mice previously immunized with an optimal dose, regardless of whether re-immunization took place well before or well after the appearance of circulating antibody or PFC (Table 2; Fig. 2). In view of these considerations, it seems unlikely that circulating antibody plays an important role in the development of low-dose paralysis to SSS-III.

The dose-response curve for SSS-III illustrates that within a given dose range (0·01–0·5 μg), there is a linear relationship between the immunizing dose and the magnitude of the serum antibody and PFC response obtained (Fig. 3); such a relationship was not observed in dose–response studies with SRBC (Fig. 4). The differences noted might be due to the fact that while SSS-III is a linear polymer of uniform composition (How *et al.*, 1964), SRBC represent a collection of numerous determinant groups. By increasing the numbers of SRBC used to immunize, one also increases the opportunity for different minor determinant groups to become involved in the antibody response. The most significant features of the dose-response curve for SSS-III are its prominent sharp peak and its single optimal dose for immunization. Doses only two to three times greater than the optimal dose of SSS-III produced a response which was significantly less than maximal; but, doses of SRBC ten times or more greater than the lowest optimal dose still gave a maximal response. Thus, the shape of the dose-response curve for SSS-III is unique in that it does not show the 'plateau effect' observed for SRBC and for other antigens (Nossal *et al.*, 1965; Dixon, Jacot-Guillarmod and McConahey, 1966). Such a pattern illustrates the exquisite sensitivity of lymphoid cells to supra-optimal amounts of SSS; this could account for the relative ease with which paralysis can be induced with these antigens. To our knowledge, the only other instances in which a similar dose–response relationship has been reported were obtained in adoptive transfer studies in which large amounts of protein antigens inhibited directly the antibody response produced by limited numbers of primed lymphoid cells (Mäkela and Mitchison, 1965; Celada, 1967).

The results obtained in dose response studies with SSS-III also imply that immunity and paralysis are processes which occur concomitantly following primary immunization. Whether paralysis or immunity predominates depends upon the dose of antigen used to immunize. With an optimal dose, a balance is obtained between both processes in which maximal immunity is achieved; however, sub-optimal or supra-optimal doses of SSS-III can shift the balance in favour of the expression of more immunity with less paralysis, or more paralysis with less immunity, respectively. The relationship between concomitant immunization and immunological paralysis has been reviewed by Dresser and Mitchison (1968). It would appear from the present work that SSS-III provides the experimental model system *par excellence* for studying the interaction of both processes since immunological memory does not occur.

Immuno-cytoadherence studies have shown that the antibody response to SSS—and to related capsular polysaccharide antigens—is characterized by the early and rapid appearance of large numbers of cells with cell-associated antibody present at their surface (Baker, Bernstein, Pasanen and Landy, 1966; Baker and Landy, 1967; Howard, Elson,

Christie and Kinsky, 1969). In the past, we have referred to such cells as antigen-reactive cells (ARC), simply because of their demonstrable specific reactivity for antigen.* It has been shown that such reactivity is mediated by newly synthesized antibody (Baker *et al.*, 1966; Howard *et al.*, 1969). While ARC have been detected as early as 5–12 hours after immunization, the time at which ARC can first be detected, the rate at which ARC increase in number, and the maximal number of ARC produced are dose-dependent. With an optimal immunizing dose of SSS, maximal numbers of ARC (about 40,000 ARC/spleen) can be detected, 2 days after immunization (Baker and Landy, 1967).

At about the time maximal numbers of ARC can be detected (2 days after immunization with an optimal dose of SSS), serum antibody and specific PFC first begin to appear (Baker and Stashak, 1969); this is followed by a progressive increase in serum antibody and PFC until maximal levels of serum antibody and maximal numbers of PFC (about 40,000 direct and indirect PFC/spleen) are present, 5 days after immunization (Table 1; Fig. 2). The excellent agreement noted between the maximal number of ARC and PFC produced, and the fact that PFC and serum antibody are not detected until maximal numbers of ARC have been produced suggest that ARC may be converted to PFC. We have evidence showing that there is a direct relationship between an increase in the relative rate of antibody synthesis by PFC and the rate of increase in splenic PFC in mice immunized with SSS-III (Baker, Stashak, Amsbaugh and Prescott, 1971). Thus, the conversion of ARC to PFC could be brought about by a progressive increase in the rate of antibody synthesis which occurs during the immune response to SSS-III. This implies that the increase in splenic PFC is largely the result of a differentiative—rather than a proliferative—process, and that the terms ARC and PFC may represent functional definitions for antibody-producing cells which differ mainly in their relative rates of antibody synthesis. In this context, Bussard and Lurie (1967) reported that the development of PFC by peritoneal exudate cells stimulated by antigen *in vitro* is largely the result of a differentiative process. Another model, which embodies both differentiation and the proliferation of PFC, has also been proposed to account for the increase in splenic PFC in mice immunized with SRBC (Dutton and Mishell, 1967). At present we are attempting to assess the role of both processes in the antibody response to SSS-III.

Since appreciable numbers of ARC can be detected, not only during the initial stages of the antibody response to SSS, but also for several weeks or months after primary immunization (Baker and Landy, 1966; Howard *et al.*, 1969), one might anticipate that previously immunized mice—in contrast to non-immunized mice—would respond differently upon re-immunization. This is indeed the case. In the present work, the antibody response to an optimal dose of SSS-III in previously immunized mice was significantly reduced in magnitude (Table 2). Others have shown that previously immunized mice are more susceptible to the induction of paralysis than are non-immunized mice (Siskind and Howard, 1966), and that a transient phase of weak immunity preceeds the development of paralysis to tolerogenic doses of SSS (Matangkasombut and Seastone, 1968; Howard and Siskind, 1969). These findings suggest that immunity may play an important role in the induction of paralysis to SSS.

There are at least two simple mechanisms which could account for the development of the low-dose paralysis described in the present work. The first involves the capture of

*In this report the term ARC is used to refer to cells which not only specifically react with antigen, but also form antibody (Baker *et al.*, 1966). The term has also been used by Mitchell and Miller (1968) to describe a particular cell of thymic origin which does not form antibody.

supra-optimal amounts of antigen by previously stimulated lymphoid cells or ARC. As a result, paralysis would be induced in a substantial portion of the population of cells capable of responding to SSS-III. The dose-response curve for SSS-III (Fig. 3) illustrates the sensitivity of lymphoid cells to supra-optimal amounts of the antigen and supports such a view. However, due to the more efficient capture of antigen by previously stimulated cells, one would also expect that the antibody response to sub-optimal amounts of SSS-III in primed mice would be greater in magnitude than that produced in unprimed mice. The results of preliminary experiments have not shown such an effect. Alternatively, previously stimulated lymphoid cells may, for a given period of time, be resistant to restimulation by antigen; thus, prior immunization could lead to the terminal differentiation of precursor cells to ARC but not to PFC. Such a process would likewise reduce the number of precursor cells available for ultimate conversion to PFC upon re-immunization. The capacity to respond to an optimal dose of SSS-III would be restored with time as new precursors become available. At present, sufficient information is not available to enable one to choose between these or other mechanisms for the induction of paralysis to SSS.

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