HUMORAL AND CELLULAR ASPECTS OF THE IMMUNE RESPONSE TO THE SOMATIC ANTIGEN OF SALMONELLA ENTERITIDIS

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In 1927 White (1) established that the somatic polysaccharides on the surface of Gram-negative bacteria confer on these organisms their characteristic serologic specificities. This was followed in the early 1930's by the pioneer work of Boivin (2) who extracted and isolated these enterobacterial "glucidolipids" and revealed that the same complex carried both the antigenic and toxic attributes of Enterobacteriaceae. The tempo of investigations on these endotoxins increased considerably thereafter, but most studies were concerned with the diverse physiologic derangements and pharmacologic effects evoked in mammals by these materials. The biologic effects of these agents were so dramatic as to largely overshadow their antigenic properties. Nonetheless, a few studies were made which still account for much of the present information on the immunogenic properties of these endotoxic polysaccharide complexes (3, 4).

A number of contemporary investigations established additional capabilities for these bacterial polysaccharides, notably their capacity non-specifically to increase resistance to infection (5), to function as powerful adjuvants (6), and to exert proliferative effects on the reticuloendothelial system and lymphoid elements (7). In retrospect these findings were portents that the somatic polysaccharide complexes might possess unique immunological attributes. Recently several methods have been developed which seemed especially useful for further assessment of the immune response to these antigens, *viz.*, the highly sensitive bactericidal assay for antibacterial "O" antibodies (8), the detection of both 7S and 19S forms of these antibodies (9, 10), and the adaptation of these antigens to the procedure for *in vitro* enumeration of antibody-forming cells (11). This communication records the results of an investigation in which these methods were employed to ascertain the characteristics of the immune response to the somatic antigens of Gram-negative bacteria.

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Materials and Methods

Rabbits.—Animals of both sexes were obtained from closed colonies maintained by the National Institutes of Health Animal Production Unit. The rabbits were kept in air-conditioned rooms at 24°C, housed 1 to a cage, and fed pelletted rabbit chow and greens *ad lib.* For the humoral antibody study the Flemish Giant breed, 4 to 7 months old, was employed; New Zealand Albinos, 4 to 5 months of age, were used for all of the cell population studies.

Antigens.—Two antigenic preparations derived from *Salmonella enteritidis* were kindly made available for this investigation by our colleague, Dr. E. Ribi. They were conventional aqueous-ether extracts of viable bacilli precipitated by 68 per cent ethanol and not subjected to further refinement (12). Preparation 1 was used throughout for the humoral antibody portion of this work while preparation 2 was used for the cellular studies. Chemical analyses and bioassay data on these products are given in Table I.

Immunization and Bleedings.—After a preinjection bleeding, rabbits were given a single intravenous injection of antigen preparation into the marginal ear vein, and at appropriate intervals, they were bled from the same vein. Serum was separated by centrifugation and stored without preservatives at −20°C.

Serum Fractionation.—The two molecular species of immunoglobulins (7S and 19S) were separated by ultracentrifugation in 10 to 37 per cent sucrose density gradients (13). Representative fractions were assayed for antibody activity by the bactericidal and passive hemagglutination procedures.

Serum Antibody Determinations.—

Bactericidal assay: The bactericidal technique was selected as the principal assay for circulating antibody inasmuch as its precision and sensitivity made it possible to distinguish between serum titers which differed from one another by only 10 per cent and also to encompass a wider range of titers than with other assay systems (8). To dilutions of serum samples, precolostral calf serum[1] free of salmonella antibody was added as a complement source, and the mixture was incubated with 100 viable cells of *Salmonella typhosa* 0901 for 1 hour at 37°C. The reaction mixtures were plated, incubated overnight, and the number of surviving organisms determined for each serum concentration. From this dose-response data antibody activity was expressed as the SD_{50}; *i.e.*, the reciprocal of the serum dilution interpolated as that which would kill 50 per cent of the bacterial inoculum.

Enumeration in Vitro of Antibody-Forming Cells.—

The technique employed was essentially that described by Jerne *et al.* (11) with some modifications.

Coating of erythrocytes with somatic polysaccharide: For application of this technique to the study of the immune response to these polysaccharides, the *S. enteritidis* endotoxin was incubated with 0.02 N NaOH at 37°C for 18 hours (14); this modified antigen was taken up readily by sheep red cells.[2] Erythrocytes were exposed to a series of concentrations of the modified antigen and these cells were then used in hemolytic titrations with rabbit antiserum to *S. enteritidis*. On the basis of these data, the concentration yielding coated cells of optimum reactivity was 5μg/ml 1 per cent sheep erythrocytes; this was the amount routinely

[1] Colorado Serum Company, Denver, Colorado.

[2] An extensive series of experiments were performed with homologous, coated erythrocytes since it was desired to use such cells in order to cancel out the ubiquitous normal anti-sheep hemolysins. It was found that rabbit red blood cells, coated with the *S. enteritidis* polysaccharide could participate in immune hemolysis with guinea pig complement but as compared to sheep erythrocytes they performed erratically and were refractory to lysis (15). This approach was therefore abandoned and sheep erythrocytes coated with *S. enteritidis* polysaccharides were used routinely.

employed thereafter. Cells were coated for each experiment, and their acquired antigenic specificity was checked by tests with antiserum to *S. enteritidis*.

Complement source: During early phases of the work, fresh guinea pig serum was used as the complement source. However, lyophilized[3] guinea pig serum was adopted subsequently as a more stable and potent product. Titrations of these reconstituted sera showed that a dilution of 1/10 consistently provided an excess of complement in that maximum numbers of plaques were developed.

Preparation and standardization of blood leucocyte suspensions: Blood obtained from immunized rabbits by cardiac puncture was drawn into heparin at a final concentration of 0.05 mg/ml. To one volume of blood was added two volumes of 3 per cent dextran (molecular weight 188,000) in saline and the specimen was gently mixed for 10 minutes at room temperature. The blood-dextran mixture was transferred to 40 ml conical centrifuge tubes and allowed to stand 30 minutes at 4°C. The supernatant plasma, which generally contained a ratio of one leucocyte to four red cells, was then removed and centrifuged at 150 *G* for 10 minutes at 4°C. The packed leucocytes were resuspended in Eagle's medium 2 (without added glutamine, serum, or antibiotics). Differential and total chamber counts of these suspensions were made to determine the percentage of mononuclear cells.

TABLE I

Chemical Composition and Biological Activity of S. Enteritidis Somatic Polysaccharide Preparations

Antigen preparation No.	Nitrogen	Phosphorus	Carbohydrate	Hexosamine	Fatty acids	Lethality for mice (LD_{50})	Non-specific resistance to infection (ED_{50})	Sarcoma 37 tumor damage (TD_{50})	Pyrogenicity (FI_{40})
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*μg*	*μg*	*μg*	*μg*
1	4.1	1.1	50	1.8	13	310	0.26	0.31	0.10
2	3.2	0.83	51	2.6	8.3	410	0.50	0.22	0.07

Preparation and standardization of cell suspensions from lymphoid tissues: Spleen and thymus were removed into Eagle's medium, and macerated by gently scraping against a No. 40 mesh stainless steel screen (14 gauge). The suspension was centrifuged to a maximum of 150 *G* for several seconds to remove gross tissue debris; the supernatant was centrifuged at 150 *G* for 10 minutes at 4°C, the cells resuspended, and the total cell count determined as for peripheral leucocytes. Other organs were processed as follows: the appendix was opened, washed free of intestinal contents, and the mucosa lightly scraped (with a scalpel directly into Eagle's medium. The cellular contents of individual lymph nodes were expressed by gentle pressure with forceps. Bone marrow was expelled from the tibia with an applicator stick into a 12 ml conical centrifuge tube containing 5 cc Eagle's medium. The cells were shaken vigorously for 1 minute and allowed to stand for 10 minutes at 4°C. Aspiration of the fatty layer was followed by filtration of the supernatant fluid through glass wool. Enumeration of cells was made by chamber count. In the event of high antibody titers in serum, cells from the aforementioned sources were washed by centrifugation at low speed (40 *G*) to reduce the amount of associated antibody.

Between the time cells were harvested and used in experiments, suspensions were kept at 4°C; at which temperature their plaque-forming capability was maintained without serious loss for several hours. It was therefore feasible to replate such suspensions in the event that

[3] Probio, Inc., Nyack, New York.

the initial experiment showed the range of cell concentrations was inappropriate for accurate enumeration of plaque-forming cells (PFC). Since in replicate plating experiments, agreement was within ±5 per cent, dependence was placed on the correlation between the number of plaques formed by progressive increments of cells plated, rather than on replicate plating at a single dilution. Furthermore, this assured the inclusion of the appropriate concentration of cells. For uniformity of reporting and to permit ready comparison, data are given in terms of PFC per 10^6 lymphoid cells (except for peripheral blood cells where the values refer to 10^6 leucocytes).

In most experiments which involved a number of cell suspensions, it was not practical to determine the per cent viability of each suspension. However, observations over many months showed that cell viability, as assessed by exclusion of nigrosine, ranged from 92 to 99 per cent. All data reported are uncorrected for viability. In view of this and the known limitations of the method for enumeration of PFC, the values reported are regarded as minimum figures for these particular conditions.

RESULTS

Humoral Response.—

Effect of antigen dose: Previous studies in this laboratory on *S. typhosa*–somatic polysaccharide had provided information on the agglutinin titers developed by rabbits in response to a single intravenous injection of various amounts of these antigens (4). Accordingly, to encompass the range of immune responses to a single antigenic stimulus, the quantities of the *S. enteritidis* antigen selected for test ranged from 0.002 μg to 20 μg.[4] Groups of 3 rabbits were given a single intravenous injection of endotoxin and at intervals the animals were bled according to a predetermined schedule over the first 3 weeks; thereafter the levels of antibody served as the guide for obtaining further blood samples. Equal volumes of sera from the rabbits in each group were pooled and these specimens were subjected to assay for specific bactericidal activity. In addition, selected pools were also fractionated by sucrose density gradient ultracentrifugation (13). Individual fractions containing 19S and 7S globulins were then assayed for bactericidal activity. The overall findings with regard to the antibody produced in response to this 10,000-fold dosage range of somatic polysaccharide are shown in Fig. 1.

Sera obtained 48 hours after stimulation with antigen consistently showed no bactericidal activity greater than preinjection levels. However, by 60 hours additional bactericidal antibody invariably was present in considerable amounts, even in response to the lowest dose of antigen (0.002 μg). Except for

[4] In addition to their immunogenic properties these complexes also display characteristic toxicity; these physiological effects are especially severe in rabbits. The particular antigen preparation 1 used for these studies on humoral antibody was less toxic than preparation 2. At 20 μg it evoked discernible toxic reactions in rabbits; nonetheless, the animals tolerated this dose and produced a good immune response. However, attempts to increase this dose to levels of 200 μg, or even 60 μg, resulted in fatal shock in all the animals employed. Consequently, with this preparation it was not possible to include such a high range of antigen concentration.

the very largest dose employed (20 μg), which produced a peak response at 6 to 8 days, maximum levels of antibody were attained by day 5. It can be seen that these peak titers were correlated with the quantity of antigen administered, although in view of the 10-fold increments of antigen injected the differences in titers are relatively modest. The peak titers of individual rabbits within a group varied considerably as well, and indeed some produced higher titers than did others given a larger dose; analogous findings on antibody-

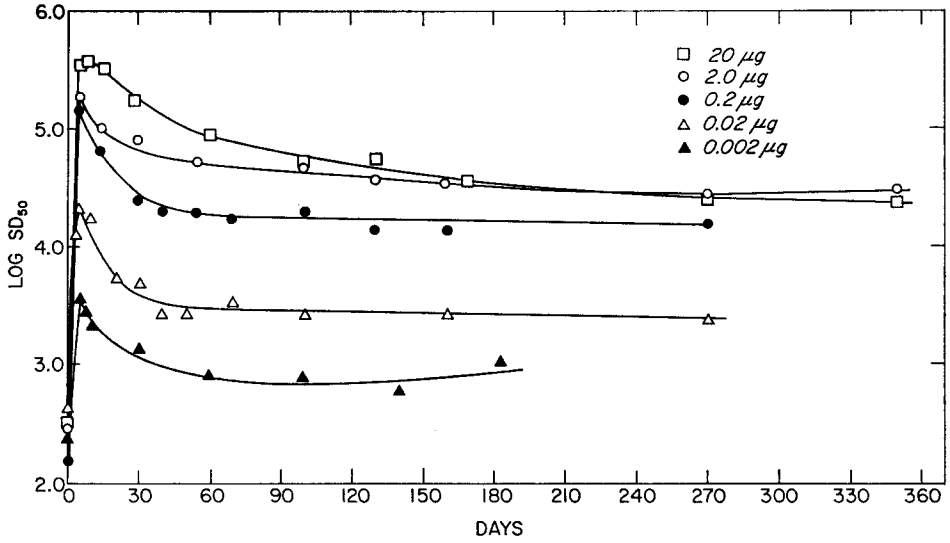

FIG. 1. Antibody response to *Salmonella enteritidis* somatic polysaccharide. Bactericidal antibody titers (SD_{50}) of pooled rabbit sera immediately before and at various intervals after a single intravenous injection of antigen.

producing cells are recorded in another section of this report. The antibody levels for the 20 μg dose remained near maximum for several days. In other groups the titer decayed sharply after peak by the 6th or 7th day, and continued to fall over a period of some 3 weeks, with approximately a 50 per cent decrease in titer over 6 to 10 days. These curves are generally similar in contour, despite the many variables referable to both the host and the bioassay. During the 2nd and subsequent months the decline was very gradual; indeed, only minor decreases in titer were seen between 2 and 12 months. The levels at which the titers stabilized appeared to be proportional to the dose of antigen employed.

Effect of a second injection of antigen: Since, in all doses studied, the titers had not returned to preinjection levels 6 months later, the recall experiments could not be carried out as had been planned originally. Nonetheless, a few

experiments were made at 190 days in rabbits that had received 0.002 to 20 μg and whose antibody titers had declined 90 per cent in this interval. The antibody response to a second, equal dose at this time is shown in Table II. The salient features of this restimulation were as follows: (*a*) no significant rise in antibody above preinjection levels was discernible for at least 48 hours; (*b*) a considerable increase in antibody was seen during the next 24 hours; (*c*) peak titer occurred at 5 to 7 days; (*d*) for the higher doses of antigen the peak titer was in the same general range as that following the first injection; and (*e*) at the lower doses (0.002 to 0.02 μg) there was evident a sharp increase in maximum titer. One animal which initially had made a minimal response

TABLE II

Humoral Response to a Second Injection of Somatic Polysaccharide*

Animal	Antigen dose‡	Bactericidal antibody titer			
		First injection		Second injection	
		Maximum	Day	Maximum	Day
	μg				
1	20	400,000	6	400,000	6
2	2	215,000	5	215,000	5
3	0.2	119,000	5	385,000	5
4	0.02	31,000	4–5	102,000	6
5	0.006	4,550	5	83,330	6
6	0.002	1,860	5	90,000	6

* Second injection given 187 to 192 days following primary stimulation.

‡ The dose of antigen was the same for both injections.

to 2.0 μg (½ the average peak titer for the group) did appear to synthesize more antibody upon restimulation.

Type of antibody produced: We have previously reported (10) that while the 7S antibody can be produced in rabbits stimulated with these polysaccharides, this form of antibody was not a prominent feature of the response which is dominated throughout by the production of macroglobulin antibody. Indeed, to obtain any significant amount of the low molecular weight antibody, it was necessary to subject rabbits to massive multiple doses (9) or unusual schedules of daily injections (16) of antigen. In the present work, involving a single dose of antigen, it was anticipated that the larger doses of 2 to 20 μg would be the only amounts effective in this respect. Sucrose density gradient ultracentrifugation of several sera showed that peak response was overwhelmingly 19S and that this was the only detectable form of antibody present in the period of exponential decline. The contour of the antibody curves for the different amounts of antigen (Fig. 1) showed that by day 30 the exponential decline

had leveled off. This finding indicated that an immunoglobulin with a much longer half-life might now be present. Accordingly, antibody assays were made on the serum pools at this time interval as well as those obtained 5 months later. The findings on three representative sera are shown in Fig. 2. In no case was 7S antibody present in amounts which exceeded 1 per cent of the total.

Cellular Response.—The ease with which sheep erythrocytes can be coated with somatic polysaccharides and the efficiency of the coated cells in immune hemolysis were indications for the applicability of the technique of localized hemolysis in gel to investigation of the immune response to these polysaccharides at the cellular level. Pilot experiments confirmed the assumption that polysaccharide-coated sheep erythrocytes were indeed appropriate indicator cells for following the cellular aspects of the immune response to somatic antigens. Since it was necessary to sacrifice the experimental animals for enumeration of plaque-forming cells (PFC) we decided to use a single dosage of antigen. The antigen preparation, No. 2, employed for the cellular studies was toxic for rabbits; since 5 μg was the largest amount that was tolerated and gave a strong immune response it was employed in this investigation.

PFC in spleen: It is known, from studies with heterologous erythrocytes, that when injected intravenously, the immune response, presumably to their surface polysaccharides, is focused largely in the spleen (11). Accordingly, most of the experiments to establish the temporal relationship between immunization and response were concerned with the enumeration of PFC in spleen. When aliquots of 20 to 40 $\times$ 10^6 spleen cells of normal, unstimulated NIH New Zealand albino rabbits were plated in parallel on antigen-coated and on normal sheep rbc few PFC were detected. The values for 12 untreated animals are given in Table III and show that with possibly one exception there are only minor differences in the numbers of PFC revealed by the two kinds of indicator cells. The values for PFC reported here are in each instance corrected for the low "background" activity against normal sheep rbc. When the numbers of PFC on the antigen-coated erythrocytes exceeded by more than 10 (per 20 $\times$ 10^6 spleen cells) those obtained on normal rbc this was considered to be a positive immune response. A total of 113 rabbits were sacrificed at various intervals after immunization to determine the number of splenic PFC; the data provided by these experiments are shown in Table IV. Even at the interval of 14 to 18 hours after 5 μg of antigen, a large proportion of the animals tested (16 of 30) had already developed very appreciable numbers of specific PFC in spleen. The values for those 16 reactors ranged from 5 to 85 PFC per 20 x 10^6 cells; estimates of the total PFC in spleen ranged from 121 to 672 with an arithmetic mean of 326. These findings have prompted a more detailed study of the early inductive phase of this immune response; the work is in progress and results will be reported separately. During the first 24 to 36 hours after antigen a significant proportion of the animals fail to develop an immune response detectable

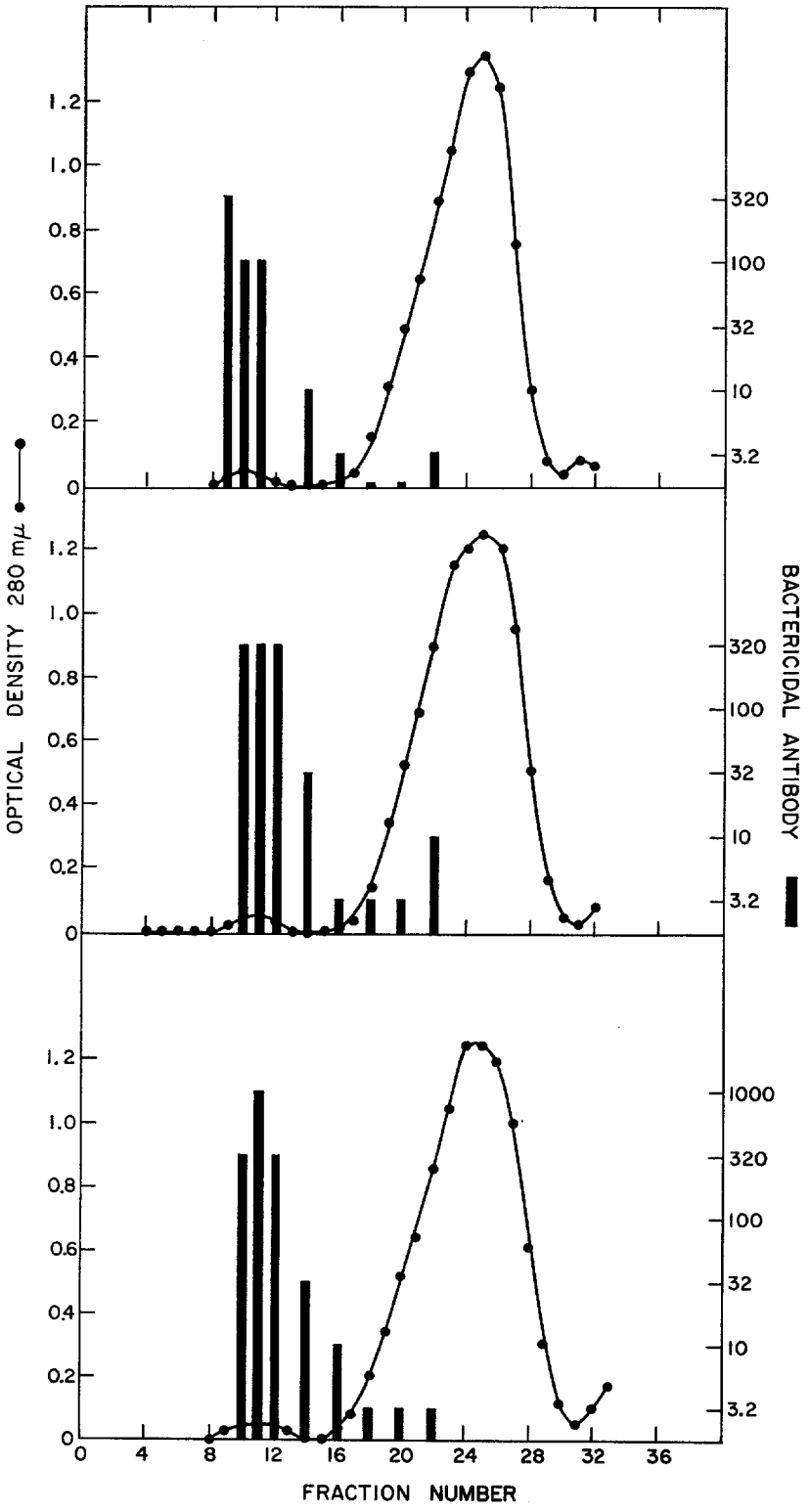

FIG. 2. Sucrose density gradient ultracentrifugation of pooled rabbit antisera obtained 160 days after a single intravenous injection of 0.2 μg (top); 2 μg (middle); and 20 μg (bottom). Sedimentation is from right to left. Bactericidal antibody titers expressed as the reciprocal of the fraction dilution killing at least 70 per cent of the bacterial inoculum.

TABLE III

Tests for Splenic Plaque-Forming Cells in Normal NIH Rabbits

Animal No.	No. of PFC in 20 × 10^6 splenocytes	
	Plated on uncoated sheep RBC	Plated on polysaccharide-coated sheep RBC
1	27	29
2	16	21
3	6	10
4	15	10
5	15	13
6	25	33
7	16	21
8	6	10
9	8	10
10	15	18
11	15	37
12	25	25

TABLE IV

Population Dynamics of Antibody-Producing Spleen Cells in Rabbits Receiving 5 μg of S. Enteritidis Polysaccharide

Time Postimmunization	No. of animals tested	No. of PFC per 10^6 splenocytes	
		Values for individual rabbits	Arithmetic means
days			
14–18 hrs.	30	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0.3, 0.4, 0.5, 0.5, 0.5, 0.7, 0.7, 1.9, 2.0, 2.1, 2.2, 3.1, 3.3, 3.3, 3.7, 4.3	1.8
1	8	0, 0, 0, 0, 1.5, 2.4, 4, 9	2
2	6	2, 18, 22, 25, 300, 500	144
3	10	6, 43, 66, 90, 100, 122, 140, 170, 1300, 6400	844
4	7	150, 255, 700, 720, 1750, 2224, 3480	1326
5	21	250, 500, 935, 1100, 1458, 1360, 1370, 1500, 1515, 1700, 1736, 2000, 2000, 2500, 2750, 4192, 4195, 4300, 4400, 7360, 8576	2625
6	6	43, 347, 450, 550, 1408, 1976	796
7	6	36, 129, 170, 192, 900, 1287	452
8–10	4	$\frac{8}{30}$ $\frac{8}{190}$ $\frac{9}{18}$ $\frac{10^*}{75}$	78
12–18	4	$\frac{12}{32}$ $\frac{13}{95}$ $\frac{16}{25}$ $\frac{18}{25}$	44
22–31	4	$\frac{22}{2}$ $\frac{24}{30}$ $\frac{25}{25}$ $\frac{31}{15}$	18
40–51	4	$\frac{40}{24}$ $\frac{47}{20}$ $\frac{49}{6}$ $\frac{51}{0}$	13
90–167	3	$\frac{90}{8}$ $\frac{120}{4}$ $\frac{167}{2}$	5

* The underscored number indicates the number of days postimmunization.

by this methodology. However by 48 hours all animals tested were positive; thereafter the number of PFC rose rapidly, consistently reaching peak at or near day 5, at which time the average number of PFC was 2625 per 10^6 splenocytes or a commitment of about 0.25 per cent of the cells in spleen to the synthesis of specific antibody. The rate of decline in the population of antibody-forming cells was very rapid and by days 12 to 18 had been reduced to an average of 44 per 10^6 spleen cells; this was only about 2 per cent of the average value at day 5. Thereafter, the number diminished gradually; specific PFC were stil

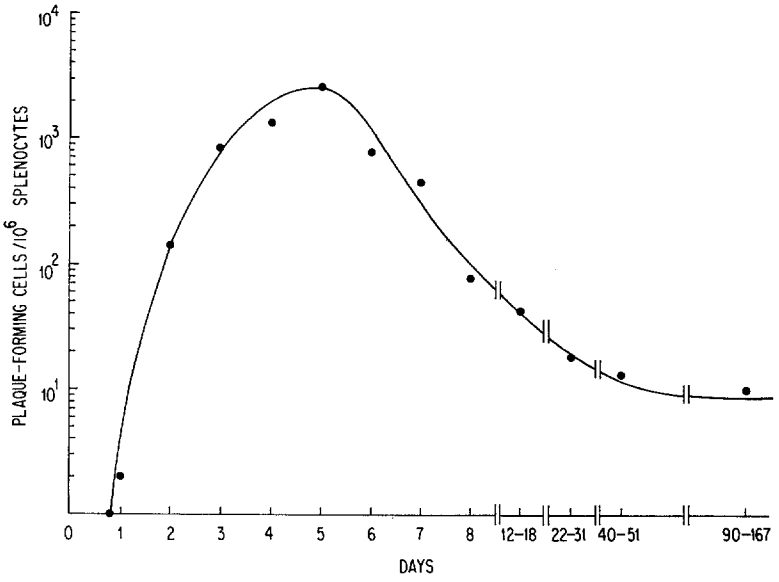

FIG. 3. Population changes of antibody-forming cells in spleen after injection of 5 μg of antigen.

present even at 167 days, the longest period tested. It was not practical to determine whether, and when, the number of PFC would once again recede to preimmunization values. The general pattern of the cellular response, including the appearance of PFC, the attainment of maximum levels, and their decline and persistence is illustrated in Fig. 3.

Effects of a second injection of antigen: A total of 22 rabbits were restimulated with 5 μg of *S. enteritidis* polysaccharide 3 months after the first injection of antigen. The changes in splenic cell population associated with this second immune response are shown graphically in Fig. 4. The values at 90 days for rabbits given only a single stimulus (note designation in Fig. 4) were the only guide as to the expected range at this time. At 18 hours after the second in-

jection, 2 rabbits yielded values of 6 and 17 PFC per 10^6 splenocytes; shortly thereafter the development of plaque-forming cells was markedly accelerated, 40 PFC per 10^6 spleen cells (approximately a 7-fold increase over the primary response) were present at 24 hours. By 48 hours this number had risen to 1112 (a 9-fold increase over the initial response at this time). Average peak response

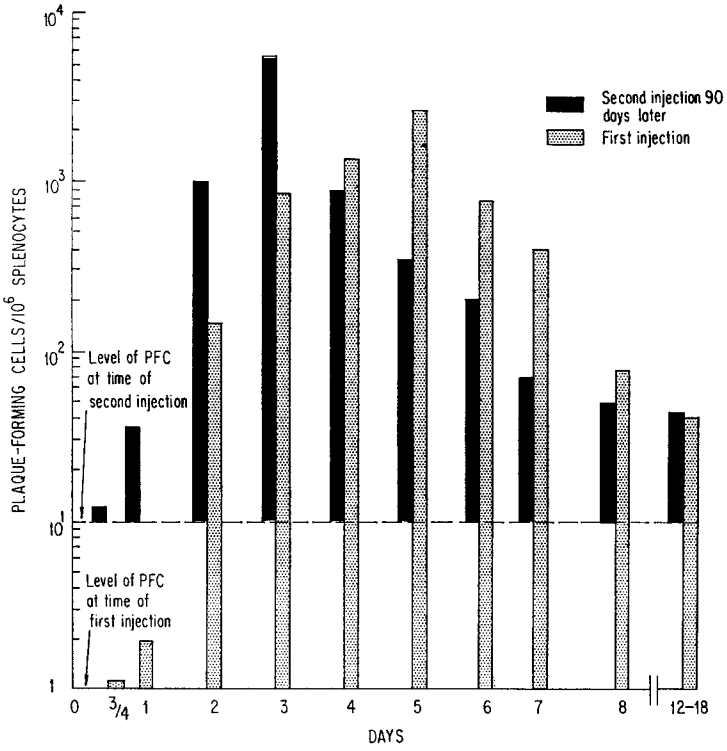

FIG. 4. Effect of a second injection of 5 μg of antigen on the development of antibody-forming cells.

was at 3 days in contrast to 5 days for animals receiving their first injection. This peak averaged 5000 PFC, approximately twice the value obtained with the initial stimulus. Circulating antibody was also measured in each of these animals; the peak humoral response occurred at day 5 rather than day 6 or 7 as had been the case following the initial injection, however no increase in average titer was detectable.

Effect of dose level of antigen: Subsequently experiments were carried out to determine the number of splenic PFC developed in response to an extended range of antigen dosage. Rabbits were given a single intravenous injection of

5, 1, 0.2, 0.05, or 0.01 μg of somatic polysaccharide and spleen cell suspensions tested 5 days later, when the number of PFC as well as the serum titer was known to be at or near maximum (at least for the 5 μg dose). A summary of the data obtained in these experiments is given in Table V and show the range in which the dose of antigen, as would be expected, is a major factor in determining the magnitude of the ensuing cellular response. Administration of 1 μg evoked a response generally similar to that obtained with 5 μg, and frequently even greater. The response to 0.2 μg was, however, markedly reduced and this did not change greatly as the dose of antigen was further decreased. All rabbits given 0.01 μg responded well. Three rabbits injected with 0.002 μg

TABLE V

Effect of Quantity of Somatic Polysaccharide on Number of PFC Developed in Spleen

Quantity of antigen injected intravenously	No. of PFC per 10^6 splenocytes*	
	Values for individual rabbits	Arithmetic means
μg		
5	(Given in Table IV)	2625
1	21, 24, 140, 150, 6800, 9000, 10,000, 10,000	4017
0.2	8, 25, 214, 400	162
0.05	17, 25, 62, 740, 1365	444
0.01	5, 8, 235, 257, 593	220

* 5 days after immunization.

responded with averages of 2.75, 2.2, and 1.4 PFC/10^6 cells. It is possible that the time at which PFC reach their maximum may differ considerably for the smaller doses of antigen. These findings on the effect of quantity of antigen are consistent with our earlier data in which response was measured in terms of O agglutinin titers (4) as well as those in the preceding section of this report based on bactericidal titers.

PFC in Various Lymphoid Tissues.—

Peripheral leucocytes: It has been reported recently that human peripheral blood lymphocytes, on exposure *in vitro* to agents such as phytohemagglutinin and various specific antigens can be transformed into blast cells synthesizing γ-globulin (17). Moreover, Hulliger and Sorkin had shown that buffy coat leucocytes from rabbits hyperimmunized with human serum were capable of producing specific antibody (18). Accordingly, at various intervals after administration of a single intravenous injection of 5 μg of somatic polysaccharide, leucocytes were separated from heparinized blood and tested for elaboration of specific antibody. From the first pilot experiments it was apparent that

PFC were indeed present in peripheral blood (19) and buffy coat cells were thereafter examined along with spleen cells. The results of tests on a total of 72 rabbits given either a single injection of antigen or a recall stimulus 3 months after the first injection are grouped in Table VI. It will be seen that within 48 hours after antigen, specific PFC have made their appearance. The data indicate that the highest proportion of reactors and the largest numbers of PFC

TABLE VI

Antibody-Forming Cells in the Peripheral Blood of Rabbits Immunized with S. Enteritidis Somatic Polysaccharide

No. of injections of 5 μg of antigen	Interval between injection of antigen and test for PFC.	No. of PFC per 10^6 blood leucocytes	
		Values for individual rabbits	Arithmetic means
	days		
1	1	0, 0	0
	2	0, 0, 0, 15, 50	13
	3	5, 9, 10, 10, 15, 18, 80, 130	35
	4	0, 25, 70, 375, 382	171
	5	0, 0, 0, 0, 6, 20, 123, 128, 150, 161	59
	6	0, 0, 0, 0, 12, 90, 118	32
	7	0, 0, 0, 0, 0, 0	0
	8–49	0, 0, 0, 0, 0, 0, 0, 0, 0	0
	days after second injection		
2 (90 days apart)	1	0	0
	2	13, 17, 31	20
	3	0, 168, 190, 374	183
	4	10, 65	37
	5	0	0
	6–11	0, 0, 0, 0, 0, 0	0

developed, occurred earlier than that in spleen. The blood PFC declined very rapidly and were no longer detectable after day 6. The data on the effect of a second injection of antigen 90 days later indicate an accelerated appearance, decline, and disappearance of specific PFC from peripheral blood (20). No correlation was observed between the percentage of lymphocytes in the leucocyte suspensions and the number of plaques obtained. The values given in Table VI are for total leucocyte suspensions without reference to the cell types present. Since the animals employed were sacrificed for parallel studies on other lymphoid organs, study of serial blood samples from individual animals was not made.

Thymus: Specific plaque-forming cells were found in the thymuses of many rabbits 5 days after intravenous injection of somatic polysaccharide (21). How-

ever, as is shown by the data in Table VII, further tests failed to disclose PFC in the thymuses of rabbits at intervals ranging from 16 hours up to 4 days or from 6 through 40 days after injection of antigen. In contrast, the majority of the animals tested at day 5 had specific PFC in this organ. At this time the thymuses were generally shrunken and strikingly acellular. A reduction in cellularity was evident within 1 day after injection; this depletion progressed to a maximum at day 5 when some thymuses were reduced to as little as 15 per cent of the normal weight. Organ cellularity, as measured by total chamber count of thymic cell suspensions was diminished to as little as 1 per cent of the number present in thymuses of normal controls. Parallel histologic examination disclosed no evidence of an inflammatory reaction or of

TABLE VII

Antibody-Forming Cells in Thymus after Immunization with Somatic Polysaccharide

Interval between 5 μg antigen and assay for PFC	No. of PFC in entire thymus*	Rabbits positive / Total rabbits
16 hours to 4 days	40 (4 days)	1/18
5 days	99, 270, 476, 500, 610 630, 700, 768, 1500 1596, 1770, 3154, 9744	13/17
6 to 40 days		0/14

* Increments of 20 to 40 × 10^6 cells were plated.

cell destruction; the change reflected primarily a loss of cortical cells. After day 5 a progressive return of cellularity began; by day 22 to 30 the gross appearance, number of cells, and the histologic picture were essentially that of a normal thymus.

Other lymphoid organs: Tests for the presence of PFC were also made on appendix (10 rabbits), mesenteric lymph nodes (15 rabbits), and on tibia bone marrow (26 rabbits). At various intervals after injection of 5 μg of antigen, increments of 20 to 40 million cells from these sites were tested as described previously. In the appendix, high background activity against normal sheep erythrocytes (2 to 10 PFC/10^6 cells) was encountered and the values for polysaccharide-coated rbc were no greater. In mesenteric nodes background activity was negligible without any increase in numbers of PFC specific for *S. enteritidis*. In bone marrow, specific PFC were found at 3 days in all 4 rabbits tested (average 6 PFC/10^6 cells); at day 5, PFC were present in 10 of 13 animals with an average of 38 PFC/10^6 cells. There has been uncertainty (22) whether cells in bone marrow actively contribute to the systemic immune response. These preliminary observations are relevant to the general issue and suggest that further studies based on localized hemolysis would be useful.

DISCUSSION

The data obtained in this study of the immune response to the somatic polysaccharide of *S. enteritidis* show that the surface antigens of Gram-negative bacteria are strongly immunogenic for the rabbit although there still remains uncertainty as to the extent to which their associated toxic attributes may contribute to their antigenicity. A single intravenous injection of microgram amounts of these polysaccharide complexes elicits a rapidly developing cellular response centered largely in the spleen. Circulating 19S antibody and the plaque-forming cells (PFC) in spleen elaborating this macroglobulin reach maximum levels at days 5 to 6, recede rapidly, but then persist for many months. Generally the number of PFC and circulating antibody levels are proportional to the dose of antigen employed.

Up to now erythrocytes have been employed exclusively both as antigen and the *in vitro* indicator of antibody synthesis by lymphoid cells. In the present work other possibilities inherent in the method have been realized by utilizing the capacity of a typical enterobacterial somatic polysaccharide to coat sheep erythrocytes (14) and to participate in complement-dependent immune reactions (4). Since these bacterial polysaccharides evoke an almost pure macroglobulin response and as 19S antibody is known to be the most efficient immunoglobulin in lytic reactions (23) it followed that the immune response to these antigens could be characterized at the cellular level by this technique with an efficiency comparable to that of the red cell itself.

The characteristics of the immune response to sheep erythrocytes have recently been explored by means of the *in vitro* technique for enumeration of antibody-forming cells (11, 24–26). There is a striking similarity in the overall pattern of cellular response of mice to erythrocytes with that reported here for a typical somatic polysaccharide; this was not expected in view of differences in methodology, the species of animals involved and the use of cellular *versus* "soluble" antigens. However, there are also major differences in the results of these investigations. Our finding that an increase in specific PFC is discernible much earlier than had been seen previously is particularly noteworthy. In their studies of the immune response of the mouse to sheep erythrocytes, both Sterzl (24) and Jerne (11) had unequivocal evidence for plaque-forming cells being present in spleen within 48 hours after stimulation. The data for intervals prior to this time were considered by these workers to be of questionable significance as the number of PFC in a considerable proportion of the unimmunized controls were in the same general range. In the system employed in the present work it was consistently found that there was very little background activity for the *S. enteritidis* specificity *viz.* in the spleens of normal rabbits the number of PFC with coated erythrocytes seldom exceeded those seen with uncoated red cells. In the work reported here each experiment in-

cluded spleen cells plated with normal sheep erythrocytes as well as with polysaccharide-coated red cells; occasionally rbc coated with *Shigella* somatic polysaccharide were included as additional controls. Consequently, when PFC were detected with coated cells in numbers greater than those observed with normal erythrocytes this was construed as positive evidence for a specific cellular response.

We have obtained unequivocal evidence for the appearance of specific PFC as early as 14 to 18 hours after a single intravenous injection of bacterial somatic antigen. In view of their capacity to evoke proimmunity (5) and their activity as powerful adjuvants (6) it seems reasonable to assume that this early induction of antibody synthesis is a property characteristic of the somatic polysaccharides. However, it remains to be determined whether this is the only type of antigen capable of evoking such an accelerated immune response. Alternatively, this may be a minimum, but unexpectedly early recall response. It may also be that other attributes of this system serve to favor detection of small numbers of antibody-producing cells.

From studies on the inhibition of the antibody response by the nucleic acid inhibitors thioguanine and 5-fluoro-2 deoxyuridine, Frisch and Davies (27) and Merritt and Johnson (28) have obtained evidence that events of a critical nature probably take place at or about 18 hours after injection of antigen. This interpretation was based on the fact that during this initial period these workers obtained *no* inhibition of subsequent antibody synthesis. Thus, the first division of antibody-forming cells or their precursors may take place at or near this time. The appearance of PFC in the interval of 14 to 18 hours in some animals is consistent with their findings.

The detection in many rabbits of specific PFC by 14 to 18 hours after initial stimulation with antigen preceded any discernible increase in circulating antibody by more than 30 hours. This delay need not be due entirely to a lack of sensitivity of the bactericidal assay. A significant increase in antibody concentration in the circulation surely requires some interval between the initial elaboration of antibody and a perceptible rise in titer. Muschel and Treffers (29) reported that 6 to 7 $\times$ 10^9 molecules of antibody were required for killing 50 per cent of the bacterial inoculum they employed (SD_{50}). Their calculations were, however, based on a molecular weight of 158,000 for antibody, whereas 900,000 is now known to be the more appropriate value (30) for the bactericidal antibody involved in these tests. Consequently, only 1 to 2 $\times$ 10^9 molecules of macroglobulin antibody are likely to be required for an SD_{50}. To effect a doubling of the serum antibody concentration (2 SD_{50}) by a normal rabbit weighing 3 kilos[5] synthesis of an average of 6 $\times$ 10^{11} molecules would be required. Jerne (11) has estimated that one PFC can produce 5 $\times$ 10^3 molecules

[5] Based on dilution of intravenously injected radioisotopes, blood volume is estimated to be 400 ml.

of antibody per second. Employing his calculations and assuming a total of 3×10^8 lymphoid cells per spleen and 37 PFC/10^6 cells at 48 hours after antigen injection it is estimated that at this time the spleen could produce 3.33×10^9 molecules of antibody per minute. In that case, 3 hours should be sufficient for this organ *alone* to effect a measurable increase in serum bactericidal antibody.

Complex formation between the initial antibody and residual antigen is one generally accepted reason for some of the delay between synthesis and appearance of detectable antibody (31). Also, some time probably elapses between elaboration from the cell and its transfer into the circulation. There may also be an inhibition mechanism which prevents elaboration of antibody during the initial period. For example, it is known that the somatic polysaccharides (endotoxins) exert profound effects on the adrenals and cause the release of considerable amounts of corticosteroids. These in turn, are known to be capable of interfering with antibody formation. Accordingly, it is possible that while cells with antibody-forming potential make their appearance within 14 to 18 hours after antigen, the active elaboration of antibody *in vivo* is effectively suppressed by the high concentration of adrenal hormones. It may be that the removal of these cells from the host environment and their maintenance in tissue-culture media permits the active production of antibody to proceed unchecked. In all probability the extended lag period is due to a combination of these and still other factors, all or a part of which contribute to a delay in the appearance of antibody in the circulation after both primary and secondary injections.

The immune response of the rabbit to these somatic polysaccharides of Gram-negative bacteria is characterized by predominance of 19S macroglobulin antibody (32). It is possible to elicit the production of some γ2-globulin as well, but this necessitates intensive stimulation with massive amounts of antigen. In the present work, our previous findings in this regard have been extended in several ways. For one thing, small but detectable amounts of 7S antibody were discernible in response to a single injection of 0.2 μg antigen. For another, the 7S antibody was seen as early as 30 days postinjection, and indeed it was still present at 170 days; throughout this period it constituted only a minute proportion of the total antibody produced. Furthermore, we have found (16) that repetitive stimulation with this antigen, comprising 9 daily injections of 0.25 μg, produced more 7S antibody than an equal amount of antigen given as a single injection. These findings stand in marked contrast to the experience of many investigators with other antigens since the appearance of 7S antibody was not associated with any effect on either the continuance of 19S antibody synthesis or on the subsequent production of 7S antibody itself.

[6] This value is the geometric mean, which was employed as a more conservative figure than the arithmetic mean of 144 given in Table IV.

There appeared to be some disparity between the levels of antibody maintained and the number of PFC which persisted in the spleen as the principal organ of antibody production. Rabbits that had received antigen in amounts ranging from 0.02 to 20 μg displayed an initial sharp decline from peak titers followed by a plateau which persisted for more than a year. As might be expected, the height of the plateau was related to the amount of antigen administered and represented in each instance a substantial increase above background levels. It was anticipated that the numbers of PFC would reasonably account for the continuing high titers especially in view of the known short half-life of macroglobulin antibody (33). However, after 30 days significant numbers of PFC were found only in spleen; they now constituted less than 1 per cent of the average peak response, while average bactericidal antibody titers in these same animals were 3 to 10 per cent of the maximum values. The relative lytic inefficiency of 7S antibody (23) could be excluded as a significant factor inasmuch as this form of antibody was shown to constitute only a minute proportion of the immune response. It is possible that a substantial number of cells synthesizing bactericidal antibody are present which are non-functional in localized hemolysis for either of two reasons; the amount of antibody liberated is insufficient to form visible plaques, or the immunoglobulin now has qualitatively different properties. There is the further possibility that at this stage PFC are not readily dissociated from the organ structure and are therefore not discerned. Finally, there may have occurred a redistribution of PFC into sites other than spleen (34). Alternatively, it is conceivable that the relatively small number of cells discernible in spleen are indeed responsible for the continuing titers. In that event, either these cells are producing antibody at a markedly accelerated rate or the antibody they synthesize is of a functional capacity considerably in excess of that produced at the time of peak response. A further possibility is that small amounts of residual antigen persist and continue to stimulate additional lymphoid cells at various sites, *e.g.*, bone marrow, liver, lungs, etc., which although present in very small numbers, taken together might account for these substantially elevated levels of antibody.

Because of the rapidity with which antibody is produced following injection of somatic polysaccharides the impression prevails that this represents a secondary response to this kind of antigen. This widely held view probably stems from an awareness of the lifelong intimate contact of mammals with Gram-negative bacteria and the ubiquity in mammals of natural antibodies reactive with these somatic antigens. It is inevitable that comparisons are made repeatedly with the response to protein antigens where extensive experience has shown that the presence of circulating antibody is associated with an accelerated response to a subsequent injection. Since these polysaccharides possess physico-chemical properties quite different from those of proteins, it does not follow that they necessarily possess similar immunogenic attributes. Indeed,

their striking effects on cells of the RES and their potent adjuvant action attest to the probability that they represent a category of antigens with unique capabilities.

A recall injection of the higher doses of somatic antigen did not produce a humoral response which differed appreciably from the pattern following the first injection. However, the cellular response differed markedly, taking the form of a more rapidly occurring increase in PFC (Fig. 4). A highly significant number of PFC appeared within 24 hours which culminated in a maximum population at 3 days instead of the 5 days required for the first injection: thus, 3 months after the initial stimulus the same dose of antigen now evoked the appearance of twice as many PFC in 3 days instead of the 5 days required initially. Acceleration of response by 2 days would be less meaningful in a protein system with an initial peak response requiring 10 to 16 days, but in the present system, the reduction represents a compression or acceleration of 40 per cent, a change of considerable magnitude. This, together with the demonstration of an enhanced production of antibody in rabbits given a recall injection of minimal amounts of antigen (Table II), indicates that a kind of secondary reaction does indeed occur in this system. These findings are therefore interpreted as indicating that the immune response to this category of somatic polysaccharides involves both primary and secondary characteristics which are distinctive, probably differing in many ways from the familiar pattern associated with protein antigens. Investigations are now in progress to determine the conditions for elicitation of a "secondary" response by somatic antigen, as well as the type of antibody produced and the extent to which the humoral aspects of this recall are reflected in the appearance of increased numbers of PFC.

SUMMARY

A study was made of the cellular and humoral aspects of the immune response of the rabbit to the somatic polysaccharide of *Salmonella enteritidis*. The response to a single intravenous injection was characterized by the appearance of elevated titers of bactericidal antibody between 2 and 3 days later. The maximum titer was dose-dependent and occurred between 5 and 7 days, thereafter declining rapidly during the first month. The significant stabilized levels which then persisted for at least 1 year were also dose-dependent. Most of the antibody produced (>99 per cent) was associated with the macroglobulin fraction of serum.

Plaque-forming cells (PFC) elaborating antibody specific for this somatic antigen were detected and enumerated by the technique of localized hemolysis in gel employing polysaccharide-coated sheep erythrocytes. Significant numbers of PFC were encountered in the spleen as early as 14 to 18 hours after a single intravenous injection of antigen; after 36 hours the number of PFC rose rapidly and culminated in a maximum population at 5 days, followed by a rapid

decline and plateau similar to that for circulating antibody. The spleen was the principal organ involved in the systemic response, but other lymphoid tissues including bone marrow, peripheral blood leucocytes, and thymus contributed significantly.

After an interval of 3 months the effect on humoral antibody titers of a second injection of antigen was dependent on the amount of polysaccharide administered; markedly greater titers were now obtained with 0.02 to 0.002 μg, whereas 0.2 to 20 μg resulted in a duplication of the initial humoral response. The cellular response to a second dose of 5 μg was accelerated; larger numbers of PFC appeared more rapidly, attained a maximum population by day 3, and exceeded the primary response by a factor of two. This acceleration in the attainment of maximum numbers of PFC and the increased bactericidal antibody titers following a second injection of limiting amounts of antigen suggest that these somatic polysaccharides may in fact evoke a "secondary" type of response in the rabbit.

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