THE ANAMNESTIC ANTIBODY RESPONSE TO TYPE III SPECIFIC PNEUMOCOCCAL POLYSACCHARIDE*

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Type specific pneumococcal polysaccharides are considered to be very weak immunogens and, at the same time, quite specific antigenic determinants. They are weak immunogens in the sense that they elicit very meager primary antibody responses in several species and that they initiate with great ease a state of specific unresponsiveness (1–5). They appear to be excellent antigenic determinants as demonstrated by the production of large amounts of polysaccharide specific antibody by rabbits immunized with the whole pneumococcal organisms. Indeed, Pincus et al. (6) have described the production of 11 mg anti-type III specific pneumococcal polysaccharide (S III) antibody per ml of serum in rabbits immunized with type III pneumococci (Pn III). It is of interest that this antibody had a limited degree of thermodynamic heterogeneity. The pneumococcus organism is therefore required, as a vehicle or carrier, to induce a sustained immune response to the specific polysaccharide.

Weakly immunogenic S III can nevertheless stimulate a substantial anamnestic antibody response in rabbits previously immunized with killed type III pneumococci[1] (7). Certain aspects of this secondary response to S III are characteristic:

(*a*) It may be obtained only after a relatively long interval (6 months or more) is allowed to elapse between the primary and secondary immunizations (7).

(*b*) The response is not sustained and the serum anti-S III antibody level declines rapidly after reaching peak concentration, approximately 7 days after boosting (7).

(*c*) Even at times when a secondary response can be obtained with S III, spleen cell cultures from immunized animals are not stimulated to increased DNA synthesis by S III (7) although such responses are observed when protein or hapten-protein antigens are added to spleen cell cultures derived from rabbits immunized to these antigens (8).

These observations indicate that S III may stimulate specific anti-S III cells (either precommitted primary or memory cells) to differentiate and to produce specific

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[1] MacLeod, C. M. Personal communication.

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antibody but is not capable, or is only marginally capable, of causing sustained proliferation of such cells. The long interval required for successful secondary immunization might be ascribed to the time necessary for the accumulation of a sufficient number of S III specific memory cells so that their direct differentiation would result in production of measurable amounts of anti-S III antibody. Alternatively, it might be due to an increase in the affinity for S III of the sensitive cell population and to a requirement of relatively efficient binding of the polysaccharide in order that differentiation be stimulated, as was shown to be the case with secondary responses to the 2,4-dinitrophenyl hapten on heterologous carriers (7). To choose between these possibilities, we have measured the antigen binding characteristics of the anti-S III antibodies produced at various times after immunization, and before and after secondary responses. We have detected only small changes in relative affinity with time which have not been consistent. Similar observations have been made by Pappenheimer et al. in their study of the immune response of rabbits to Pn VIII. In addition, these authors have noted a limited degree of heterogeneity of anti-S VIII rabbit antibodies (9).

If the concept that S III stimulates the direct differentiation of antigen sensitive cells to antibody producing cells without their significant replication is correct, one can predict that repeated secondary challenges with S III should deplete the pool of antigen sensitive cells and result in progressively smaller anti-S III antibody responses. In the present study, this prediction has been tested and found to be correct.

Materials and Methods

Female New Zealand white rabbits weighing 2–2.5 kg were immunized intravenously over a 2 wk period with four 0.1 mg nitrogen doses of a killed Pn III vaccine prepared as described previously (7). They were bled several times in the ensuing months and were secondarily immunized 8 or 9 months later with 0.5 mg of S III intravenously. S III (Lot 310) was the generous gift of Professor Michael Heidelberger. The rabbits were bled at 4, 7, 11, and 21 days after secondary immunization and then received a tertiary intravenous immunization at day 21 with 0.5 mg of S III. The same bleeding and immunization schedule was then repeated except that after the quaternary (and final) immunization, animals were bled only on days 7 and 21.

Serum concentrations of anti-S III antibody were determined by quantitative precipitin analyses according to the method of Heidelberger and Kendall (10).

1–2 ml samples of sera were studied by gel filtration through Sephadex G-200 equilibrated in a solution of Tris-HCl buffer, pH 8.0, 0.1 M and NaCl, 0.5 M. Samples were collected and pooled as illustrated in Fig. 1. The pools were concentrated either by pressure dialysis or by 50% saturated ammonium sulfate precipitation and subsequent dialysis. Quantitative precipitin analyses were performed on the concentrates. Electrophoresis in acrylamide gel was performed in 8.4 M urea as described by Reisfeld and Small (11) on washed anti-S III immune precipitates, prepared at equivalence in the presence of 0.01 M ethylenediaminetetraacetate and containing approximately 400 μg of antibody and 40 μg of S III. These precipitates were dissolved in 7 M guanidine, reduced with 0.1 M dithiothreitol and then alkylated with 0.22 M iodoacetate. Similar electrophoresis were performed on 400–500 μg of purified rabbit anti-DNP antibodies, purified rabbit IgG, S III, and purified rabbit IgG to which 50 μg of S III had been added.

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Binding studies were carried out utilizing ^{131}I labeled *p*-OH-benzyl-S III (S III*) prepared as previously described (12). Under the conditions of the assay, 1 nanogram (ng) of S III* yielded approximately 1100 cpm. For equilibrium binding experiments, antisera were diluted 1:100 with PBS[2] (0.15 M NaCl and .01 M phosphate, pH 7.6) and further diluted with 1:100 normal rabbit serum (NRS) in PBS to a concentration of 200 ng/ml of anti-S III. 0.5 ml (100 ng) of anti-S III was mixed with either 1, 2, 5, 10, or 20 ng of S III* in 0.1 ml of the same diluent, incubated at 4°C overnight and then counted in a well type crystal scintillation counter. 0.6 ml of cold saturated ammonium sulfate was added and, after 1 hr, the mixture

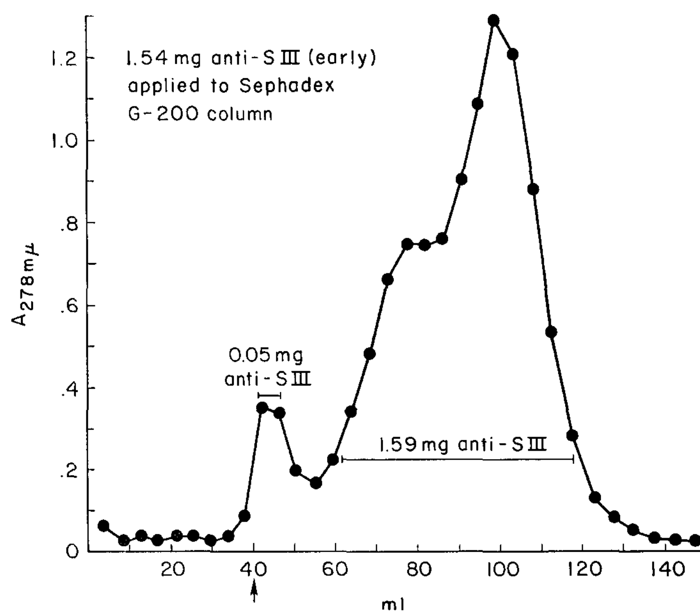

FIG. 1. Gel filtration on Sephadex G-200 of anti-S III antiserum obtained from rabbit 83 shortly after immunization with Pn III. The arrow represents the void volume of the column.

was centrifuged for 30 min at 2500 rpm, 4°C, and a measured aliquot of supernatant was counted.

Studies of dissociation kinetics were performed by preparing 20 ml of solution containing 200 ng of anti-S III and 1 ng of S-III*/ml. After overnight incubation at 4°C, 5 ml was removed. To the remaining 15 ml, 50 μl containing 150 μg of unlabeled S-III was added and 0.5 ml samples were removed after 1, 2, 3, 5, 10, 30, 120, and 300 min. These samples were immediately mixed with 0.5 ml of cold saturated $(NH_4)_2SO_4$. After centrifugation, supernatant counts were determined. 0.5 ml samples of the initially removed 5 ml were mixed with either $(NH_4)_2SO_4$ or PBS and supernatant counts measured to determine the amount of S III bound at equilibrium (time 0) and the total counts in the system. The results are expressed as the per cent of bound counts at time 0 which dissociated at any given time.

[2] Phosphate buffered saline.

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RESULTS

A. Properties of Early and Late Anti-S III Antibodies.—

Several comparative studies of the anti-S III antibody produced shortly after primary immunization and later in the immune response were performed in an attempt to gain further insight into the cell population dynamics which allow successful secondary responses to the polysaccharide when a relatively long interval has elapsed after the primary immunization. These included (*a*) the estimation of the molecular class of the antibody; (*b*) a study of the banding

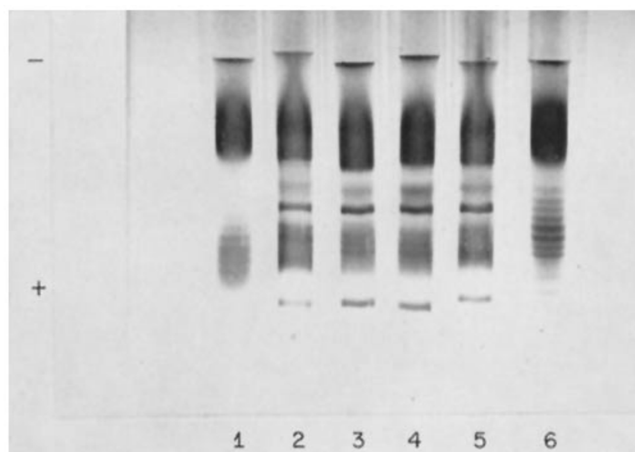

FIG. 2. Acrylamide gel electrophoresis of reduced and alkylated immunoglobulins performed as described in methods. Samples 2–5 are specific precipitates of S III and rabbit antisera. They were prepared from individual rabbits bled at following times: 2. Rabbit 87, shortly after primary immunization with Pn III. 3. Rabbit 87, 7 days after secondary immunization with S III. 4. Rabbit 84, shortly after primary immunization with Pn III. 5. Rabbit 84, 7 days after secondary immunization with S III. Sample 1 is rabbit IgG and Sample 6 is specifically purified rabbit anti-DNP antibody obtained 365 days after immunization with DNP-BGG.

patterns of its dissociated light chains in acrylamide gel electrophoresis; (*c*) the measurements of equilibrium binding characteristics of the antibodies and of dissociation kinetics of S III*–anti-S III complexes.

Gel filtration on Sephadex G-200 was performed with sera obtained from rabbit 83 shortly after primary immunization with Pn III and after secondary response to S III. The bulk of the precipitating anti-S III antibody in both sera emerged from the column significantly after the void volume and thus was not of the IgM class. Fig. 1 illustrates one such chromatogram.

Acrylamide gel electrophoresis of reduced and alkylated washed specific precipitates of anti-S III and S III revealed an antibody light chain banding pattern which was virtually identical in preparations from different rabbits

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and from the same rabbit at different times in the immune response (just after primary response to the whole organisms or just after secondary response to S III). This pattern (illustrated in Fig. 2) consists of a prominent fast band, a rather broad but fainter slur of intermediate mobility and an intense slower band, followed by two bands of lesser intensity which migrated even more slowly. These light chain patterns were quite different from those obtained on electrophoresis of either reduced alkylated pure IgG in the presence or absence of S III or of reduced, alkylated, purified, early or late anti-DNP antibodies.

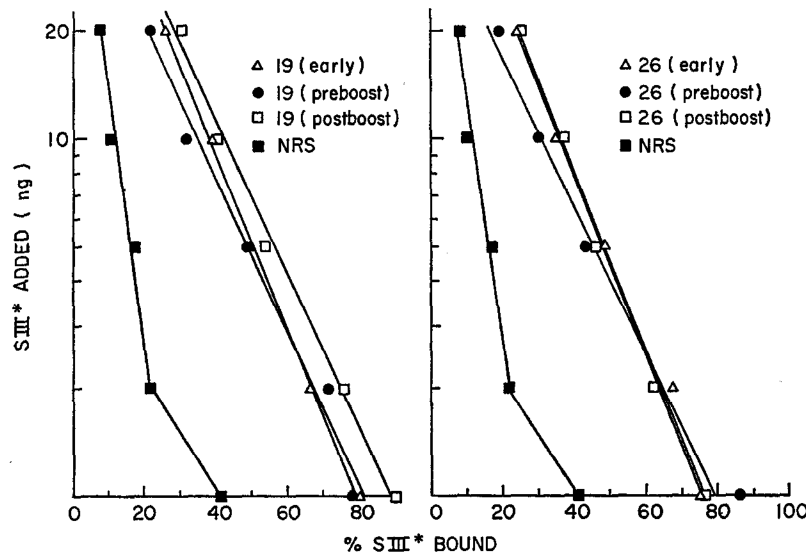

FIG. 3. Equilibrium binding of varying amounts of S-III* by 100 nanograms of anti-S III. These studies were performed on antisera obtained from rabbits 19 and 26 shortly after primary immunization with Pn III, 9 months later (immediately prior to secondary immunization with S III), and 7 days after secondary immunization with S III.

Indeed no obvious counterparts of the two most intense bands were noted in these other preparations. The anti-S III light chain pattern thus appeared to be that of an electrophoretically heterogeneous population with the predominance of some light chain subpopulations which appeared characteristic of anti-S III antibody.

Binding studies were carried out with anti-S III and S III*, a polyvalent molecule with respect to antigenic determinants. 50% saturated ammonium sulfate was shown to precipitate S III* which was bound to antibody while that which was free remained in solution.

A series of equilibrium binding experiments are illustrated in Fig. 3. These are studies of antisera produced by two individual rabbits shortly after primary

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immunization with Pn III, just prior to, and 7 days after, secondary immunization with S III. Very little difference is noted between these antisera with respect to the fraction of antigen bound for any given amount of antigen added (from 1 to 20 ng) to 100 ng of anti-S III. This behavior was noted with several additional anti-S III antibodies, and probably reflects a similarity in binding affinity of these rabbit antibodies for S III. Although the concentration of effective antigenic sites is not known and therefore affinities could not be directly determined, one can show that it is quite unlikely that all antibody combining sites are saturated under the experimental conditions at which binding was studied. Indeed, if all the antibody sites were bound at a ratio of antibody to S III of 100:1, and considering a molecular weight of antibody of 150,000 and a valence of 2, the effective molecular weight of an antigenic site would have to be 750. Although this number is not unreasonably low for an individual site, it would seem highly unlikely that *every* such site on a polyvalent S III molecule could be occupied by antibody in view of problems of steric hindrance. Furthermore, a considerable number of antigenic sites present in the native S III are probably ineffective because of the presence of the *p*-OH-benzyl groups which had been added to the polysaccharide in order to prepare the radioactive compound (see Methods). Finally, when precipitin analyses are made at antibody concentrations 10,000 or more times greater than those used in these studies, a 45 to 1 antibody to antigen ratio is required to precipitate all the antibody (10). Thus, it seems very probable that these binding studies reflect the affinity of anti-S III for S III. The conclusion can be made that all the antibody populations studied are quite similar in regards to their binding constants for S III. Although small differences are, indeed, noted, no constant change in affinity of anti-S III antibodies with time after immunization could be observed. It is of interest that the globulin fraction from normal rabbit serum binds as much as 41% of 1 ng and 21% of 2 ng of S III. Whether this represents a saturatable nonspecific precipitation or the presence of a very small amount of anti-S III antibody in normal rabbit serum cannot be stated.

Because S III is a polyvalent antigen, studies of the kinetics of dissociation of S III*–anti-S III complexes would be expected to amplify any differences which might exist between antibody populations with respect to their binding affinities for S III. The results of such dissociation studies of antibody from three individual rabbits at three different times in their immune response are shown in Fig. 4. Indeed, relatively larger differences are noted in these experiments than were seen in the equilibrium binding studies described above. In two instances, the dissociation rate of postboost antibody was clearly slower than that of the preboost antibody, but this was not so in the third case. No definite pattern of change was observed when antibodies produced early in the immune response were compared with those produced 9 months later, but before boosting. This data demonstrates that some heterogeneity of binding exists in anti-

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bodies against this very simple antigen, but that no apparent cell selection has occurred either during the primary or the secondary anti-S III immune response. This is in marked contrast to what is observed with multideterminant

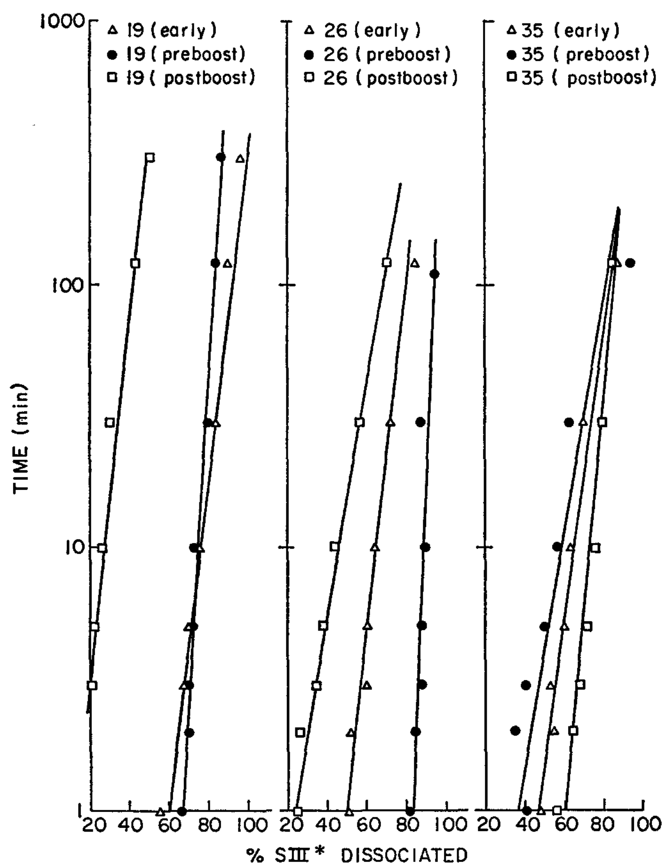

FIG. 4. Dissociation kinetics of anti-S III-S III* immune complexes prepared as described in methods from sera of rabbits 19, 26, and 35, obtained shortly after primary immunization with Pn III, 9 months later (immediately prior to secondary immunization with S III) and 7 days after secondary immunization with S III.

A large excess of unlabeled S III was added to preformed complexes at time 0 and the amount of S III* which dissociated was determined as described in the text.

protein antigens or hapten protein conjugates (13). The conclusion can also be made that successful anamnestic antibody responses to S III in rabbits initially immunized with type III pneumococci cannot be explained by the stimulation of an enlarged population of high affinity cells.

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B. Progressive Depletion of Anti-S III Antigen-Sensitive Cell Population as a Consequence of Repeated Boosting.—

Each of the 10 rabbits in this experiment exhibited a marked primary anti-S III antibody response to a killed Pn III vaccine and, 8 months later, a significant anamnestic anti-S III response to an intravenous injection of 0.5 mg of S III. (Fig. 5, Table I). Prior to the secondary injection with S III, the mean anti-S III antibody concentration was 0.02 mg/ml while 7 days after secondary

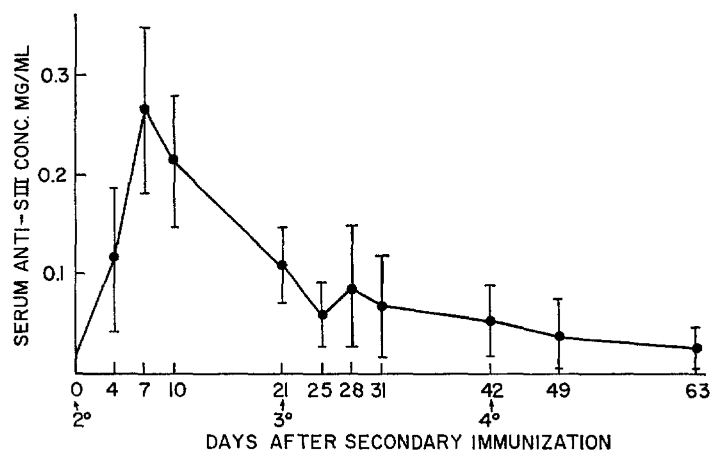

FIG. 5. Sequential anamnestic antibody responses to 500 μg of S III injected intravenously into rabbits immunized with Pn III 8 or 9 months earlier. Arrows indicate the times of secondary, tertiary, and quaternary immunization. The points are the mean responses of the group and the brackets delimit ± 1 standard deviation.

immunization it was 0.25 mg/ml (Fig. 1). On the other hand, only 2 of 10 rabbits produced a tertiary antibody response and only 1 of 9 produced a quaternary response to the same repeated dose of S III (Table I). In no case was the tertiary response of greater magnitude than the secondary response in the same animal. The single quaternary response which was obtained was the smallest of any of the "successful" anamnestic responses (Table I).

Examination of the preboost antibody levels suggests that it is highly unlikely that the suppressive effect of circulating antibody is responsible for the results obtained. In 3 cases, quaternary immunization elicited little or no anamnestic response despite the fact that the preboost anti-S III antibody concentrations were equal to or lower than those present just prior to successful secondary or tertiary immunization in the same animal.

DISCUSSION

Secondary responses can be elicited by S III in rabbits immunized several months previously with killed type III pneumococci[1] (7). No significant change

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in the character of the anti-S III antibody produced during this interval or following the anamnestic challenge, either as to molecular size, light chain mobilities, or antigen-binding properties could be demonstrated. Thus, the selective proliferation of a group of high affinity cells cannot account for the time dependence of the anamnestic antibody response to S III. It seems more probable that the time required to prepare the animals for a secondary response to S III allows for the accumulation of a sufficient number of specific memory cells. These experiments demonstrate also that repeated attempts to elicit such S III specific anamnestic antibody responses become progressively less success-

TABLE I

Sequential Immunization with S III

Animal No.	Anti-S III antibody (mg/ml)					
	Secondary		Tertiary		Quarternary	
	0*	7	0	7	0	7
76	**0.02**‡	**0.11**	0.03	0.01	0.02	0.02
77	**0.01**	**0.21**	**0.08**	**0.13**	0.05	0.06
78	**0.01**	**0.28**	0.12	0.05	0.05	0.02
79	**0.04**	**0.16**	**0.12**	**0.22**	0.14	0.13
80	**0.03**	**0.29**	0.13	0.16	0.06	0.01
83	**0.02**	**0.43**	0.18	0.12	0.08	0.05
84	**0.01**	**0.28**	0.13	0.04	0.03	0.01
86	**0.02**	**0.31**	0.10	0.12	0.05	0.02
87	**0.01**	**0.30**	0.11	0.07	**0.00**	**0.02**
89	**0.02**	**0.27**	0.08	0.03	0.02	—§

* 0 and 7 refer respectively to serum antibody concentrations (mg/ml) just prior to and 7 days after secondary, tertiary, or quaternary immunization with S-III.

‡ Boldface pairs of numbers indicate the occurrence of an anamnestic antibody response.

§ This animal died before the completion of the experiment.

ful and that when successful, the sequential responses decrease in magnitude until specific immunologic unresponsiveness to S III is established. These findings strongly suggest that the pool of "memory" cells present as a result of initial immunization is progressively depleted by exposure to the polysaccharide.

The response of immune rabbits to S III is therefore very similar in quality (although quantitatively at a different level) to the response of normal nonimmune mice or rabbits. Type specific pneumococcal polysaccharides are known to readily induce a state of immunologic tolerance in mice and rabbits, although a low level of antibody synthesis is observed after very small doses of antigen (1–5). It is of interest that even when tolerogenic doses are injected in mice, cells forming anti-polysaccharide antibody are nevertheless detected. Thus it has recently been shown by Howard et al. (14) that following a tolerogenic dose

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of pneumococcal polysaccharide as many antibody forming cells can be detected in the spleen as are found after an optimal immunizing dose of this antigen. During induction of tolerance to S III some circulating antibody can also be detected after antigen injection (15) and cells capable of transferring protection to a challenge with virulent organisms are present but only for a brief time (16).

Therefore, there is a considerable degree of similarity in the behavior of antigen sensitive cells in the primary response and of the more numerous "memory" cells in the anamnestic response to the pneumococcal polysaccharide. Both cell types respond to S III by differentiating into antibody forming cells rather than by forming proliferating clones; as a consequence the pool of S III responsive cells becomes exhausted and a state of tolerance is achieved.

One may next inquire whether this behavior is a property peculiar to pneumococcal polysaccharides or whether it characterizes the response to other antigens as well. Although it is by no means established, several other substances may well have similar immunological properties, for example: poly-D-amino acid polymers (17) and vinyl polymers (18). If true, this suggests that this is a property of poorly metabolized antigenic determinants when injected without suitable carriers. According to this interpretation, one biological function of the carrier molecule would be to provide a proliferative stimulus to amplify and prolong the antibody response of the hapten specific cells to the haptenic material. The role of the haptenic group would simply be the selection of appropriate antigen sensitive cells through an interaction with cell associated antibody (19, 20). The carrier, directly or indirectly, would determine that the interaction of hapten with cell associated antibody results in proliferation of specific cells as well as in differentiation to antibody formation.

Some additional aspects of the response to pneumococcal polysaccharides observed in these experiments and previously reported by others (5, 9) are also worthy of comment: (*a*) The antibodies specific for these rather simple polymeric molecules are considerably more homogeneous than those produced against more complex multideterminant protein antigens and hapten-protein conjugates, with respect to their binding affinity and the electrophoretic mobility of their dissociated L chains. Characteristically identical L chain electrophoretic patterns were observed with anti-S III antibodies obtained from different rabbits and at different times in immunization. This limited heterogeniety was also observed with antibodies elicited by anamnestic immunization with the polysaccharide alone. These antibodies did not differ in L chain mobility or binding affinity from those initially stimulated by the whole organisms, indicating that in this system the carrier, which is essential for a proliferative response, does not participate appreciably in the specificity of the anti-S III antibodies. (*b*) In contrast with the character of the antibody formed in response to proteins or hapten protein conjugates which changes in affinity

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with time, the relative affinity of the anti-S III antibodies did not change appreciably with the time of immunization, indicating that the selection and stimulation of cells bearing anti-S III antibodies of progressively higher affinity does not occur in this system. In this respect, pneumococcus polysaccharides behave like the ordered DNP conjugate of a polypeptide of L-lysine and DL-alanine studied by Richards et al. (21, 22).

How can one account for these differences? The reduced heterogeneity of anti-S III antibodies as compared with conventional anti-hapten antibodies is probably explained by the homogeneous character of the S III polymeric determinant and by the genetic constitution of the animals. The absence of change in antibody binding affinity with time may result in part from the limited heterogeneity in binding affinity of the postulated cell associated antibody (19, 20). From such a relatively homogenous cell population little selection is possible. The considerable increase in binding affinity with time observed with protein antigens must depend upon the initial heterogeniety of the antigen sensitive cell population from which continuous selection does occur. Another factor must also be considered is the relative resistance of the polysaccharide to enzymatic degradation. This is also the case for poly-D-amino acids and for the synthetic antigen studied by Richards et al. (21, 22) which contained alternative D- and L-alanyl residues. If the increase in antibody binding affinity with time after immunization observed with protein antigens is explained by the selection and proliferative stimulation of specific cells by antigen then higher affinity cells can only be preferentially selected if the concentration of antigen in the lymphoid tissues decreases. If the antigen cannot be efficiently metabolized, its effective concentration cannot be expected to change significantly with time and to thus bring about the selection of cells producing higher affinity antibody.

SUMMARY

Rabbits immunized with killed type III pneumococci respond to anamnestic challenge by type specific polysaccharide (S III) with the synthesis of anti-S III antibody if a long interval is allowed to elapse between primary and secondary immunization. A study of the anti-S III antibody produced early and late in the immune response revealed no change in molecular class, banding pattern of dissociated light chains, or S III binding characteristics as measured under equilibrium conditions or by study of dissociation kinetics utilizing radioiodinated *p*-OH-benzyl-S III.

Sequential booster injections of S III into rabbits primarily immunized with whole organisms 8 or 9 months earlier led to a progressive decrease in the number of animals showing successful anamnestic responses and in the magnitude of those responses.

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It is concluded that S III depletes the antigen sensitive cell population in the secondary response largely because of its limited ability to stimulate sustained proliferation by such cells.

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