STUDIES ON THE INDUCTION OF IMMUNOLOGICAL UNRESPONSIVENESS TO PNEUMOCOCCAL POLYSACCHARIDE IN MICE*

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Immunologic unresponsiveness (paralysis) to pneumococcal polysaccharide, first described in detail by Felton (1–5), represents a simple, easily manipulated model for the study of the unresponsive state. It is now generally agreed that paralysis to polysaccharides and unresponsiveness to protein antigens are both expressions of the same basic biologic mechanisms (6). Current evidence supports the concept that the different examples of unresponsiveness all represent a specific, central depression of antibody synthesis and not a binding of antibody by excess antigen (7–18). In contrast to protein antigens, pneumococcal polysaccharide appears not to be readily metabolized by mice and therefore to have a very long half-life in these animals. Possibly as a consequence of the prolonged persistence of antigen, paralysis to polysaccharides is readily induced in adult animals by a single injection of relatively small amounts. This is in contrast with the extended course of injections generally required to obtain unresponsiveness to protein antigens.

A number of experiments were undertaken in order to examine in detail the induction phase of paralysis to pneumococcal polysaccharide. Specifically, an attempt was made to answer the following questions: (*a*) What is the quantitative effect of immunization upon subsequent induction of unresponsiveness? (*b*) Can a transient phase of immunization be demonstrated prior to induction of unresponsiveness? (*c*) Does "low dose" paralysis, such as recently described by Mitchison (19) for bovine serum albumin, exist for pneumococcal polysaccharide? (*d*) Is intense nonspecific stimulation of lymphoreticular tissues per se capable of altering induction of unresponsiveness or of terminating an established state of unresponsiveness as has been suggested by previous work (20–24)?

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Results of experiments designed to answer these questions are presented in this communication.

Materials and Methods

Mice.—Adult, male, Swiss Webster mice weighing 20 to 25 g were used throughout.

Polysaccharide.—Type II pneumococcal polysaccharide (Squibb lot 27276) (SII) was kindly provided by Dr. C. M. MacLeod (Department of Medicine, New York University Medical Center). This preparation was used in all experiments with one exception which is indicated in the text. In this instance Type II polysaccharide prepared by Dr. Paul. D. Hoeprich (Department of Medicine, University of Utah College of Medicine) was used.

Bacteria.—Diplococcus pneumoniae Type II (strain D39S) and Type III (strain A66) were used to challenge mice. The pneumococci were kindly provided and cultured by Miss Marjorie Krauss (Department of Medicine, New York University Medical Center). Cultures were incubated for 18 hr at 37°C, in neopeptone beef heart infusion broth containing approximately 0.5% defibrinated rabbit blood.

A formalinized, heat-killed suspension of *Corynebacterium parvum* (strain 936B) was generously given by Dr. G. Biozzi (L'Hôpital Broussais, Paris).

Treatment of Mice.—Appropriate doses of SII were injected, intraperitoneally, in 0.5 ml of phosphate-buffered saline (PBS) (0.15 M NaCl, 0.01 M potassium phosphate buffer, pH 7.5) as indicated in the text.

C. parvum was administered intravenously in a dose of 0.5 mg (dry weight) contained in a volume of 0.25 ml of PBS.

Mice were challenged by a single intraperitoneal injection of 0.5 ml of an appropriately diluted overnight culture of fully virulent, recently mouse passaged pneumocci. Mice were observed for deaths occurring over a period of 5 days. Culture dilutions were prepared in nutrient broth. The dilution used for challenge of experimental mice was 10^{-7}. In every experiment, groups of normal mice, serving as virulence controls, received 10^{-7}, 10^{-8}, and 10^{-9} dilutions of the overnight culture. Based upon deaths among the virulence controls, pneumococcal challenges (10^{-7} dilution) were found to generally contain between 10 and 100 lethal doses. Any experiment was discarded if the challenge did not fall within these limits of virulence. Virulence controls receiving the 10^{-7} dilution died, without exception, in all experiments. These have been omitted from the tables for the sake of brevity. It should be emphasized that all experiments were designed so that groups of animals to be compared were challenged simultaneously with the same bacterial suspension. Details of individual experiments are indicated in the footnotes to the appropriate tables.

EXPERIMENTAL RESULTS

Induction of Paralysis in Immunized Mice.—The effect of immunization with SII upon the subsequent response of mice to varying dosages of SII is indicated in Table I. From the response of normal mice, also included in this table, it is clear that maximal protection (immunization) follows the intraperitoneal injection of 0.05 μg of the SII preparation 1 wk before challenge. With this polysaccharide preparation and with the Swiss Webster mice used approximately 50% protection was consistently the maximum attainable. As the dose of polysaccharide was increased above 0.05 μg the degree of protection decreased progressively until, with 100 μg of antigen, little or no protection was detected. From previous work (1–5, 25) it is known that animals receiving these higher

doses of polysaccharide are subsequently incapable of responding to an optimal immunogenic dose. In all experiments, therefore, such a decrease in protection at relatively high dose levels has been taken to signify immunological paralysis (unresponsiveness or tolerance). Progressively poorer protection was also observed with doses of SII below 0.05 μg, but such animals are, of course, capable of responding further to optimal immunogenic doses of polysaccharide (see Table V).

If the response of preimmunized animals is compared with that of normal animals, it is apparent that the maximum degree of immunization attained is identical in both groups. This lack of any obvious "booster" response is consistent with classical observations using purified polysaccharide antigens (26) On the other hand, in contrast to previous observations (4, 27, 28) (see Discussion), no elevation of the paralysis threshold was observed. In fact, with 0.5, 2, and 10 μg SII, preimmunization appeared to facilitate subsequent induction of unresponsiveness. It should be noted that, in order to assure confidence in the differences observed, large groups of mice (60 to 100) were used over the more critical part of the dose range.

TABLE I

*Effect of Previous Immunization upon the Response of Mice to SII**

Dose SII	Result of challenge No. survived/No. challenged (per cent survival)	
	Normal	Preimmunized
μg		
100	1/30 (3)	1/29 (3)
25	11/60 (18)	7/60 (12)
10	14/58 (24)	7/60 (12)
2	35/91 (39)	24/98 (25)
0.5	24/60 (40)	17/60 (28)
0.05	9/19 (47)	9/20 (45)
0.005	8/20 (40)	8/20 (40)
0.0005	4/20 (20)	8/20 (40)
0.00005	4/44 (9)	22/45 (49)
0	0/30 (0)	50/104 (48)

* Preimmunized with 0.5 μg SII intraperitoneally. 7 days later given varying doses of SII and challenged with virulent Type II pneumococci after a further 7 days.

Desensitization of Immunized Mice.—It was of interest to determine the amount of polysaccharide required to bind all of the available antibody present in an immunized mouse and to compare this with the amount required to induce paralysis. From Table II it is clear that immunized mice can be rendered nearly fully susceptible to pneumococcal challenge by the intraperitoneal injection of

0.5 μg of SII, 1 day previously. In fact, even 0.05 μg was partially effective. Thus, normally highly immunogenic doses of polysaccharide are sufficient to bind most available antibody in maximally immunized mice. That is, induction of paralysis requires far more antigen than is required for simple desensitization. Furthermore, by comparison with Table I, it is clear that this desensitization is, as expected, quite transient when compared with the long-lasting state of paralysis. That even 100 μg of SII failed to completely desensitize may be the result of continuing antibody synthesis following rapid phagocytosis of the injected polysaccharide.

TABLE II

*Desensitization of Immunized Mice with SII**

Dose of SII	Result of challenge
	No. survived/No. challenged (per cent survival)
μg	
100	4/34 (12)
25	3/33 (9)
10	2/35 (6)
2	4/35 (11)
0.5	4/35 (11)
0.05	12/34 (35)
0.005	19/34 (56)
0.0005	17/34 (50)
0.00005	14/25 (56)
0	20/40 (50)
Normal mice	0/20 (0)

* Mice were immunized with 0.5 μg SII. 14 days later they received varying doses of SII and were challenged 1 day later with virulent Type II pneumococci.

Demonstration of Immunization Prior to the Appearance of Unresponsiveness.— While it is clear from the results of previous workers (7–18) that fully paralyzed mice are not producing antibody, it appeared possible that an early and transient phase of antibody synthesis might occur prior to the establishment of complete unresponsiveness. We have, therefore, examined in detail the immune status of mice, at daily intervals, immediately following doses of polysaccharide ranging from 0.005 to 100 μg. In order that all the mice in any one experiment should be challenged with the same bacterial suspension groups of 5 to 20 mice were injected with the varying doses of antigen 1, 2, 3, 4, 5, 7, and 9 days prior to challenge. Initial experiments indicated that any early phase of weak immunity could be detected 4 to 7 days after injection of 10 and 25 μg of antigen. These observations were subsequently made consistently on replicate experiments.

The cumulative data are presented in Table III. It is apparent from this table that with paralyzing doses of SII (10 and 25 μg) an early phase of immunization can be detected. It is further clear that with the lowest doses of SII studied (0.05 and 0.005 μg), the onset of protection was 1 day earlier than with 0.5 or 2 μg.

In view of the small magnitude of the effects observed, large groups of animals were employed at critical dose levels and, in addition, the entire experiment was repeated using a second preparation of SII polysaccharide (Table IV). Although minor quantitative differences between these two experiments are apparent, the essential qualitative findings presented in Table III were substantiated.

TABLE III

Induction of Immunization and Unresponsiveness to SII

Dose of SII	Results of challenge [No. survived/No. challenged (per cent survival)]						
	Days before challenge*						
	1	2	3	4	5	7	9
μg							
100	0/15 (0)	0/15 (0)	2/35 (6)	0/25 (0)	1/35 (3)	3/34 (9)	0/10 (0)
25	1/45 (2)	3/45 (7)	4/74 (5)	9/64 (14)	11/74 (15)	12/74 (16)	2/44 (5)
10	1/15 (7)	0/15 (0)	3/45 (7)	5/35 (14)	9/45 (20)	9/44 (21)	3/40 (8)
2	1/15 (7)	0/15 (0)	2/35 (6)	8/25 (32)	12/35 (34)	12/35 (34)	2/10 (20)
0.5	0/15 (0)	0/15 (0)	2/35 (6)	10/25 (40)	14/34 (41)	15/35 (43)	4/10 (40)
0.05	0/15 (0)	1/15 (7)	7/35 (20)	12/25 (48)	20/35 (57)	17/35 (48)	5/10 (50)
0.005	0/15 (0)	0/15 (0)	12/35 (34)	7/23 (30)	10/34 (28)	17/35 (48)	5/10 (50)

* Mice received doses of SII at indicated number of days before challenge with virulent Type II pneumococci. Of 40 normal mice challenged along with experimental mice, none survived challenge.

It was necessary to exclude the unlikely possibility of increased nonspecific resistance being responsible for the apparent transient immunity following high doses of polysaccharide. For this purpose three groups of 20 normal mice were injected with 25 μg SII, 3, 5, and 7 days prior to challenge with approximately 20 lethal doses of Type III pneumococci. All 60 animals died. Thus, nonspecific protection appears not to be responsible for the early immunity in Tables III and IV. Furthermore, it seems likely that the degree of early protection observed is a highly dampened reflection of the immune response as the excess of antigen available would certainly be expected to combine with a major part of any antibody formed (see Table II and reference 15).

Unsuccessful Attempt to Obtain "Low Dose" Paralysis.—It has been shown recently by Mitchison (19) that it is possible to obtain specific immunologic

unresponsiveness to protein antigens by repeated injections of subimmunogenic doses. Similar results have been obtained by several workers (29–31) using different antigens. As indicated in Table V, repeated injections of 5×10^{-6} μg SII failed either to immunize or to impair the response of mice to an immunogenic dose of polysaccharide. A 10-fold higher dose (5×10^{-5} μg), shown to be

TABLE IV

Induction of Immunization and Unresponsiveness to SII

Dose of SII	Results of challenge [No. survived /No. challenged (per cent survival)]					
	Days before challenge*					
	1	2	3	4	5	7
μg						
25	0/10 (0)	0/10 (0)	1/20 (5)	4/20 (20)	2/20 (10)	0/20 (0)
10	0/10 (0)	1/10 (10)	3/19 (16)	2/20 (10)	0/20 (0)	1/20 (5)
5	0/10 (0)	0/10 (0)	1/20 (5)	2/20 (10)	4/20 (20)	0/20 (0)
2	0/10 (0)	0/10 (0)	3/20 (15)	2/20 (10)	5/20 (25)	2/20 (10)
0.5	0/10 (0)	1/10 (10)	2/20 (10)	9/20 (45)	5/20 (25)	3/20 (15)
0.05	0/10 (0)	0/10 (0)	3/20 (15)	12/20 (60)	10/20 (50)	8/20 (40)
0.005	0/10 (0)	0/10 (0)	7/20 (35)	—	8/20 (40)	6/20 (30)

* Mice received the dose of polysaccharide indicated at varying numbers of days prior to challenge with virulent Type II pneumococci. The Hoeprich preparation of SII was used for this experiment only. Of 15 normal mice challenged along with experimental mice, none survived challenge.

TABLE V

*Effect of Repeated Injections of Small Doses of SII on Immunologic Status**

Dose of SII	Duration of injection	Result of challenge No. survived/No. challenged (per cent survival)	
		No further treatment	Immunizing dose of SII
μg	wk		
0	—	0/5 (0)	16/20 (80)
5×10^{-6}	3	1/19 (5)	15/19 (79)
5×10^{-6}	6	0/17 (0)	17/18 (95)
0	—	0/10 (0)	12/20 (60)
5×10^{-5}	3	5/19 (26)	12/19 (63)
5×10^{-5}	6	2/17 (12)	10/17 (59)

* Mice received injections of SII twice weekly for the indicated number of weeks. 4 days after the last injection half of each group received an immunizing injection of SII and 1 wk later all mice were challenged with virulent Type II pneumococci. In the first set of experiments indicated in the table, the immunizing dose used was 0.5 μg SII while with the second set of experiments, 0.2 μg SII was used.

feebly immunogenic in Table I, induced weak immunity upon repeated injection but failed to cause any change in response to subsequent immunization. Thus, no evidence for a "low dose" zone of paralysis, analogous to that described by Mitchison (19), was elicited with this polysaccharide antigen.

Effect of Lymphoreticular Stimulation upon Response to SII.—Previous workers have demonstrated termination of immunologic unresponsiveness and/or increased resistance to its induction by treatment with agents known to cause lymphoreticular stimulation (20–24). *Corynebacterium parvum* has recently been shown to cause massive proliferation of RE cells (32), to have a

TABLE VI

Effect of C. Parvum on Immunologic Response of Mice to SII

Dose of SII	Result of challenge No. survived/No. challenged (per cent survival)		
	Normal mice	*C. parvum* before SII*	*C. parvum* simultaneously with SII‡
μg			
100	1/15 (7)	0/15 (0)	0/9 (0)
25	2/15 (13)	2/15 (13)	0/10 (0)
10	6/26 (23)	8/24 (33)	3/10 (30)
2	8/24 (33)	17/25 (68)	3/9 (33)
0.5	10/25 (40)	16/23 (70)	4/10 (40)
0.05	18/25 (62)	20/24 (83)	7/10 (60)
0.005	12/25 (48)	17/24 (71)	—
0.0005	2/15 (13)	4/16 (24)	2/10 (20)
0.00005	1/15 (7)	2/15 (13)	1/10 (10)
0	0/20 (0)	—	—

* Mice given varying doses of SII intraperitoneally 1 wk after *C. parvum* intravenously. Mice were challenged with virulent Type II pneumococci 1 wk after SII.

‡ Mice received *C. parvum* intravenously and SII intraperitoneally 1 to 2 hr later. Mice were challenged with virulent Type II pneumococci 1 wk later.

potent adjuvant effect on immunization (33) and to lead to a marked increase in the number of splenic cells synthesizing antibody to a concomitantly administered antigen (34). The effect of pretreatment with this agent on the response of mice to SII polysaccharide was studied and the results are presented in Table VI. The greater amplitude of the dose response curve following pretreatment with *C. parvum* is consistent with the previously described adjuvant effect of this material (33). Despite this increase in antibody formation seen even on the paralysis side of the dose response curve, there does not appear to be any significant shift in the tolerance induction threshold. When given simultaneously with SII, *C. parvum* was without any detectable effect.

C. parvum was also tested for its ability to terminate an established state of

unresponsiveness to SII polysaccharide. Table VII indicates that *C. parvum*, administered 1 wk after polysaccharide, and 2 wk before challenge, was totally ineffective in terminating paralysis.

TABLE VII

*Failure of C. parvum to Terminate Paralysis**

Dose of SII	Result of challenge No. survived/No. challenged (per cent survival)	
	Normal mice	C. parvum treated
μg		
100	1/10 (10)	1/10 (10)
25	2/15 (13)	2/15 (13)
10	7/25 (28)	6/25 (24)
0.5	6/15 (40)	6/15 (40)
0.05	7/15 (47)	8/14 (57)
0.005	4/15 (27)	4/14 (29)
0.0005	0/15 (0)	3/15 (20)
0.00005	1/15 (7)	0/15 (0)
0	0/15 (0)	0/10 (0)

* Groups of mice received doses of SII as indicated. 1 wk later half of each group received *C. parvum* intravenously. All mice were challenged with virulent Type II pneumococcic 3 wk after receiving SII.

DISCUSSION

It is clear from the data presented that paralysis to a polysaccharide antigen can be readily induced in previously immunized mice. In fact, preimmunization may facilitate slightly the induction of unresponsiveness. Thus, in the system studied, immune cells are no more difficult to render tolerant than are normal cells. Several previous workers have demonstrated that tolerance to various antigens could be induced in previously immunized animals (4, 27–29, 35–42). Generally, these studies did not permit detailed quantitative assessment of the relative tolerance induction thresholds of normal and immune animals. Dresser (27), however, using bovine gamma globulin, has reported that comparable doses of antigen produced a lower degree or lack of tolerance in immunized as compared with normal recipients. Makela and Mitchison (28), using a cell transfer system and a protein antigen, presented evidence that relatively high concentrations of antigen are required to paralyze cells from previously immunized donors. The data referred to on the response curves for normal cells, however, came from studies using intact animals and are, therefore, not strictly comparable. Felton et al. (4) reached a similar conclusion for a polysaccharide antigen from a rather limited series of observations. On the other hand, our own studies with polysaccharide, based upon detailed dose-

response curves utilizing large numbers of animals, have failed to support this contention. Considering the available evidence it appears reasonable that the modulation of tolerance induction in preimmunized animals is very likely the result of alterations in the cellular distribution and subsequent catabolism of antigen as a result of the presence of antibody. The rate of elimination from the animal of a polysaccharide antigen, which is rapidly phagocytozed but not metabolized, may be less affected by the presence of antibody than is the rate of elimination from the host of proteins which once phagocytozed are readily metabolized.

It has been shown here that a transient period of weak immunity can be detected prior to establishment of complete unresponsiveness. Several previous workers (19, 43–45), using protein or cellular antigens, have observed the development of immunity prior to, or simultaneously with, the development of unresponsiveness. In contrast, Michie and Howard (46) failed to detect any immune phase prior to the onset of tolerance induced with high doses of histocompatibility antigens, despite the fact that the neonatal animals used were shown to be capable of giving an immune response to lower doses of antigen. The failure of Sercarz and Coons (8, 13) to demonstrate any antibody-forming cells in fully paralyzed mice suggests that the initial antibody-forming cells soon cease to produce detectable antibody. Whether this early antibody formation is important in the induction of unresponsiveness, as has been suggested by Rowley and Fitch (43–45), and possibly indicated by the greater ease of paralyzing immune mice reported here, cannot be said at present.

Our failure to induce tolerance with repeated subimmunogenic doses of polysaccharide would appear to represent a distinction from the outcome following similar treatment with certain protein antigens. A "low dose" zone of paralysis, of the type first described in detail by Mitchison (19) has also been observed by several other workers (27, 29–31) using a variety of different proteins. Although it was originally suggested by Mitchison that detection of "low dose" paralysis might be restricted to antigens of low immunogenicity, the reported ability of Nossal and Ada (29) to elicit this phenomenon with flagellin, a highly immunogenic protein, suggests that this limitation need not apply rigorously. The inability of a polysaccharide antigen to produce a comparable "low zone" paralysis cannot be satisfactorily explained, but may be related to differences in the doses of antigen used with polysaccharide as compared with proteins. For example, the maximally immunogenic dose of polysaccharide is, on a weight basis, approximately 10^6-fold below the optimal immunizing dose of bovine serum albumin as reported by Mitchison. In fact, on a weight basis, amounts of antigen which induce "low dose" paralysis with protein antigens would tend to produce "high dose" paralysis with polysaccharide antigens. It is well known that large polysaccharide molecules are readily phagocytozed (47). With the nanogram subimmunogenic doses of antigen used, clearance by phagocytosis

should be highly efficient and thus the amount of antigen remaining free to interact with cells susceptible to tolerance induction would be exquisitely small.

It has been reported that an established state of immunological unresponsiveness can be terminated by injection of complete Freund's adjuvant (21), endotoxin (20), or low dose X-irradiation (23, 24). In some cases these procedures can also render normal animals more resistant to induction of unresponsiveness (21, 22). These agents have in common a stimulatory action upon the lymphoreticular system. It appeared likely that the ability to alter immunologic unresponsiveness in this manner might be a general property of any agent capable of producing lymphoreticular hyperplasia. *C. parvum* which, as previously discussed, is known to cause massive proliferation of both macrophages and lymphoid cells (32) was shown here to give the expected adjuvant effect upon anti-SII antibody formation (33) but failed completely to alter a preexisting state of unresponsiveness. Thus, neither lymphoid hyperplasia nor increased phagocytic capacity are, in and of themselves, sufficient to terminate paralysis to a polysaccharide antigen. When administered prior to SII, *C. parvum* resulted in an increase in the amplitude of the immune response throughout the dose response curve. Despite this adjuvant effect upon the immune response, seen even in the paralytic zone of the dose-response curve, no elevation in the threshold dose for tolerance induction was seen. One possible explanation for this finding is that the induction of polysaccharide paralysis is related to the concentration of antigen present, rather than to the amount of antigen per immunologically competent cell. The fact that the increased phagocytic capacity resulting from *C. parvum* treatment did not alter induction of unresponsiveness suggests either that there is a nonphagocytosable fraction of the SII preparation which is crucial for tolerance induction, or that phagocytosis of antigen does not play any significant role in tolerance induction. We have previously shown (15) that throughout the course of paralysis some SII is present which is capable of binding (in vivo) passively administered antibody. This polysaccharide disappears with a biologic half-life of 50 days and might represent that fraction of antigen which is nonphagocytosable and is responsible for induction and maintenance of paralysis. This possibility is consistent with the findings of Dresser (31) and of Frei et al. (48) using protein antigens.

SUMMARY

1. Comparison of dose-response curves indicated that preimmunized animals were slightly more susceptible to the induction of immunological paralysis with pneumococcal polysaccharide than were normal mice. The results also indicated that the paralysis threshold was unaltered by preimmunization.

2. Transient desensitization of immunized mice could be achieved by an amount of polysaccharide far less than that required to induce paralysis.

3. A transient phase of weak immunity was detected prior to the onset of paralysis when induced by relatively low paralyzing doses of polysaccharide.

4. No "low dose" zone of paralysis (analogous to that obtainable with certain protein antigens) could be elicited with pneumococcal polysaccharide.

5. Massive proliferation of lymphoreticular tissues induced by *Corynebacterium parvum* failed to raise the threshold for paralysis induction, but amplified the immune response over the entire dose-response curve. Similarly, *C. parvum* failed to abrogate an established state of paralysis. The results suggest that the induction of polysaccharide paralysis is related to the concentration of antigen in the animal and is not modified by the number of immunologically competent cells.

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BIBLIOGRAPHY

1. Felton, L. D., Active immunization of white mice by a non-polysaccharide and probably non-protein derivative of the pneumococcus, *J. Immunol.*, 1932, **23,** 405.
2. Felton, L. D., and Ottinger, B., Pneumococcus polysaccharide as a paralyzing agent on the mechanism of immunity in white mice, *J. Bact.*, 1942, **43,** 94.
3. Felton, L. D., The significance of antigen in animal tissues, *J. Immunol.*, 1949, **61,** 107.
4. Felton, L. D., Kauffman, G., Prescott, B., and Ottinger, S., Studies on the mechanism of the immunological paralysis induced in mice by pneumococcal polysaccharides, *J. Immunol.*, 1955, **74,** 17.
5. Felton, L. D., Prescott, B., Kauffman, G., and Ottinger, S., Pneumococcal antigenic polysaccharide substances from animal tissues, *J. Immunol.*, 1955, **74,** 205.
6. Chase, M. W., Immunologic tolerance, *Ann. Rev. Microbiol.*, 1959, **13,** 349.
7. Gitlin, D., Monckelberg, F., and Craig, J. M., The nature of "immunological paralysis" induced by pneumococcus polysaccharides, *Am. J. Dis. Child.*, 1958, **96,** 496.
8. Sercarz, E. and Coons, A. H., Specific inhibition of antibody formation during immunological paralysis and unresponsiveness, *Nature*, 1959, **184,** Suppl. 14, 1080.
9. Brook, M. S., and Karnovsky, M. J., Immunological paralysis and adoptive immunity, *J. Immunol.*, 1961, **87,** 205.
10. Gitlin, D., Monckelberg, F., and Janeway, C. A., Effects of pneumococcus capsular polysaccharides on the degradation of rabbit antibodies in mice, *J. Immunol.*, 1961, **86,** 627.
11. Friedman, H., Transfer of antibody formation by spleen cells from immunologically unresponsive mice, *J. Immunol.*, 1962, **89,** 257.
12. Neeper, C. A., and Seastone, C. V., Mechanisms of immunologic paralysis by pneumococcal polysaccharide. I. Studies on adoptively acquired immunity to pneumococcal infection in immunologically paralyzed and normal mice, *J. Immunol.*, 1963, **91,** 374.
13. Sercarz, E. E., and Coons, A. H., The absence of antibody producing cells during unresponsiveness to BSA in the mouse, *J. Immunol.*, 1963, **90,** 478.

14. Dutton, R. W., The effect of antigen on the proliferation of spleen cells suspension from tolerant rabbits, *J. Immunol.*, 1964, **93,** 814.
15. Siskind, G. W., and Paterson, P. Y., A bioassay for the estimation of pneumococcal polysaccharide in unresponsive (paralyzed) mice, *J. Immunol.*, 1964, **93,** 489.
16. Dietrick, F. M., and Weigle, W. O., Immunologic unresponsiveness to heterologous serum proteins induced in adult mice and transfer of the unresponsive state, *J. Immunol.*, 1964, **92,** 167.
17. Stastny, P., Persistence of acquired tolerance in cells transferred to an antigen-free environment, *J. Immunol.*, 1964, **92,** 626.
18. Friedman, H., Adoptive tolerance to shigella antigen in irradiated mice receiving spleen cell transplants from unresponsive donors, *J. Immunol.*, 1965, **94,** 352.
19. Mitchison, N. A., Induction of paralysis in two zones of dosage, *Proc. Roy. Soc. London, Ser. B*, 1965, **161,** 275.
20. Brooke, M. S., Conversion of immunological paralysis to immunity by endotoxin, *Nature*, 1965, **206,** 635.
21. Neeper, C. A., and Seastone, C. V., Mechanisms of immunologic paralysis by pneumococcal polysaccharide. II. The influence of non-specific factors on the immunity of paralyzed mice to pneumococcal infection, *J. Immunol.*, 1963, **91,** 378.
22. Paraf, A., Fougereau, M., and Bussard, A., La Tolérance acquise et la tolérance naturelle à l'égard de substances antigéniques défines, C.N.R.S. Sumposium, Paris, 1963, 97.
23. Fefer, A., and Nossal, G. J. V., Abolition of neonatally-induced homograft tolerance in mice by sublethal X-irradiation, *Tran. Bull.*, 1962, **29,** 445.
24. Makela, O., and Nossal, G. J. V., Accelerated breakdown of immunological tolerance following whole body irradiation. *J. Immunol.*, 1962, **88,** 613.
25. Siskind, G. W., Paterson, P. Y., and Thomas, L., Induction of unresponsiveness and immunity in newborn and adult mice with pneumococcal polysaccharide, *J. Immunol.*, 1960, **90,** 929.
26. Heidelberger, M., MacLeod, C. M., Kaiser, S. J., and Robinson, B., Antibody formation in volunteers following injection of pneumococci or their type-specific polysaccharides, *J. Exp. Med.*, 1946, **83,** 303.
27. Dresser, D. W., Specific inhibition of antibody production. I. Protein-overloading paralysis, *Immunology*, 1962, **5,** 161.
28. Makela, O., and Mitchison, N. A., Effect of antigen dose on the response of adoptively transferred cells, *Immunology*, 1965, **8,** 549.
29. Nossal, G. J. V., and Ada, G. L., Recognition of foreign-ness in immune and tolerant animals, *Nature*, 1964, **201,** 580.
30. Thorbecke, G. J., and Benacerraf, B., Personal communication.
31. Dresser, D. W., Specific inhibition of antibody production. II. Paralysis induced in adult mice by small quantities of protein antigen, *Immunology*, 1962, **5,** 378.
32. Halpern, B. N., Prévot, A. R., Biozzi, G., Stiffel, C., Mouton, D., Morard, J. C., Bouthillier, Y., et Decreuesefond, C., Stimulation de l'activité phagocytaire du système réticuloendothélial provoquée par *Corynebacterium parvum*, *J. Reticuloendothelial Soc.*, 1964, **1,** 77.
33. Neveu, T., Branellac, A., et Biozzi, G., Proprietés adjuvantes de *Corynebacterium*

parvum sur la production d'anticorps et sur l'induction de l'hypersensibilité retardée envers les proteines conjuguées, *Ann. Inst. Pasteur*, 1964, **106,** 771.

34. Biozzi, G., Stiffel, C., et Mouton, D., Liacopoulos-Briot, M., Decreuesefond, C. et Bouthillier, Y., *Ann. Inst. Pasteur*, 1966, in press.
35. Glenny, A. T., and Hopkins, B. E., Duration of passive immunity, *J. Hyg.*, 1924, **22,** 208.
36. Sercarz, E., and Coons, A. H., *in* Mechanisms of Immunological Tolerance (M. Hasek, A. Lengerova, and M. Vojtiskova, editors), Prague, Czechoslovak Academy of Science, 1960, 73.
37. Brent, L., and Gowland, G., Induction of tolerance of skin homografts in immunologically competent mice, Nature, 1962, **196,** 1298.
38. Dorner, M. M., and Uhr, J. W., Immunologic tolerance after specific immunization, *J. Exp. Med.*, 1964, **120,** 435.
39. Dorner, M. M., and Uhr, J. W., The induction of immunological tolerance after specific antibody formation, *Ann. New York Acad. Sc.*, 1964, **120,** 424.
40. Frey, J. R., Gileick, H., and DeWeck, A., Immunological tolerance induced in animals previously sensitized to simple chemical compounds, *Science*, 1964, **144,** 853.
41. Makinodan, T., Hopper, I., Sado, T., Capalbo, E. E., and Leonard, M. R., The suppressive effect of supraoptimal doses of antigen on the secondary antibody-forming response of spleen cells cultured in cell-impermeable diffusion chambers, *J. Immunol.*, 1965, **95,** 466.
42. Cohn, M., and Thorbecke, G. J., Specificity of reaction to antigenic stimulation in lymph nodes of immune rabbits. II. Suppression of local morphologic reaction to alum precipitated bovine serum albumin by intraperitoneal injections of soluble bovine serum albumin in neonatal rabbits, *J. Immunol.*, 1964, **93,** 629.
43. Rowley, D. A., and Fitch, F. W., Homeostasis of antibody formation in the adult rat, *J. Exp. Med.*, 1964, **120,** 987.
44. Rowley, D. A., and Fitch, F. W., The mechanism of tolerance produced in rats to sheep erythrocytes. I. Plaque-forming cell and antibody response to single and multiple injections of antigen, *J. Exp. Med.*, 1965, **121,** 671.
45. Rowley, D. A., and Fitch, F. W., The mechanism of tolerance produced in rats to sheep erythrocytes. II. The plaque-forming cell and antibody response to single and multiple injections of antigen begun at birth, *J. Exp. Med.*, 1965, **121,** 683.
46. Michie, D., and Howard, J. G., Transplantation tolerance and immunological immaturity, *Ann. New York Acad. Sc.*, 1962, **99,** 670.
47. Kaplan, M. H., Coons, A. H., and Deane, H. W., Localization of antigen in tissue cells. III. Cellular distribution of pneumococcal polysaccharides types II and III in the mouse, *J. Exp. Med.*, 1950, **91,** 15.
48. Frei, P. C., Benacerraf, B., and Thorbecke, G. J., Phagocytosis of the antigen, a crucial step in the induction of the primary response, *Proc. Nat. Acad. Sc.*, 1965, **53,** 20.