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STUDIES ON IMMUNOLOGICAL PARALYSIS

II. THE DETECTION AND SIGNIFICANCE OF ANTIBODY-FORMING CELLS IN THE SPLEEN DURING IMMUNOLOGICAL PARALYSIS WITH TYPE III PNEUMOCOCCAL POLYSACCHARIDE

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SUMMARY

The immunocytoadherence (rosette) technique has been adapted to study the immune response to varying doses of type III pneumococcal polysaccharide (SIII). Syngeneic erythrocytes coated with SIII were incubated with washed spleen cell suspensions isolated 0·5–39 days after i.v. injection into CBA strain mice of immunogenic (0·5 or 5·0 μg) or paralysing (500 μg) doses of SIII; the immunizing or paralysing effect of these doses of SIII was confirmed by a passive haemagglutination technique. The number of rosette-forming cells per spleen was significantly increased above the background range in twenty-seven out of forty-two, twenty-four out of thirty-seven and forty-one out of forty-six animals injected with 0·5, 5·0 and 500 μg SIII, respectively. Not only was there a greater incidence of 'positive' values in the paralysed group, but the response was better maintained than in the immunized groups.

In view of the apparent lack of central inhibition in these experiments, paralysis with SIII was considered to be attributable to the antibody-neutralizing effect of persisting undegraded antigen. It is suggested that the phenomenon of polysaccharide paralysis may be the expression of more than one mechanism.

INTRODUCTION

During previous studies on the induction of immunological paralysis by type II pneumococcal polysaccharide (SII), it was found that a transient phase of weak immunity was detectable during the latter part of the 1st week and before the appearance of maximum paralysis (Siskind & Howard, 1966). The fact that this phenomenon was found only with relatively low paralysing doses of antigen is not surprising in view of the well-documented persistence of undegraded pneumococcal polysaccharide which remains available for interaction with antibody (Dixon, Maurer & Weigle, 1955; Stark, 1955; Siskind & Paterson, 1964). The important question arises as to what may be the real extent of this apparently feeble response which appears before the onset of paralysis, for a more substantial reaction

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could be largely submerged beneath an excess of reactive antigen. In effect, what had been detected previously by conventional means could well be analogous to the visible tip of an iceberg. A further incentive for pursuing this problem was the possibility that it might contribute towards explaining the differences which appear to exist between the induction of paralysis to polysaccharide and protein antigens respectively (Howard & Siskind, 1969). In particular, the apparent independence of paralysis induction of the extent to which polysaccharide is phagocytosed is clearly at odds with current ideas concerning the production of central, specific inhibition within a population of lymphocytes.

We have examined the immunological reactivity of spleen cells which had been taken from mice at varying periods after either paralysing or immunizing doses of polysaccharide (SIII) and freed from their antigen-charged environment by repeated washing. These cell suspensions were examined for antibody formation by an adaptation of the immunocytoadherence (rosette) technique described by Biozzi and his colleagues and by Zaalberg (Nota *et al.*, 1964; Zaalberg, 1964; Biozzi *et al.*, 1966; Biozzi, Stiffel & Mouton, 1967; Biozzi *et al.*, 1968). For the present experiments, erythrocytes were sensitized with SIII by the method of Askonas, Farthing & Humphrey (1960), a technique which has since been used extensively by Brooke (1966a, b) for haemagglutination studies in the mouse, although like him we were unable to achieve sensitization with SII. In view of the very weak immune response which is evoked in the mouse by these polysaccharides (Sercarz & Coons, 1959; Siskind, Paul & Benacerraf, 1967) and the relatively large natural background reactivity against sheep erythrocytes with the rosette technique, sensitized CBA strain mouse (rather than sheep) erythrocytes were used throughout satisfactorily in conjunction with syngeneic spleen cells.

The results obtained with this system have indicated strikingly that no central inhibition of the immune response is demonstrable at various times throughout a 4-week period of observation when spleen cells from mice given a paralysing dose (500 μg) of SIII are compared with cells from animals given an immunizing dose (0·5 or 5 μg).

MATERIALS AND METHODS

Mice

These were obtained from an inbred CBA colony maintained in the Department of Zoology, University of Edinburgh. Adult animals weighing 20–30 g were used throughout.

Polysaccharides

Type III pneumococcal polysaccharide (SIII) was generously given by Dr J. H. Humphrey (National Institute for Medical Research, Mill Hill, London, N.W.7). Type II pneumococcal polysaccharide (SII) was from a batch kindly prepared by Dr I. W. Sutherland and Dr J. F. Wilkinson (Department of General Microbiology, University of Edinburgh). Both preparations were dissolved in phosphate-buffered saline, pH 7·2 (PBS) and were injected intravenously into a tail vein in a volume of 0·5 ml.

Bacterial vaccines

Diplococcus pneumoniae type III (NCTC strain 7978) and type II (strain D39S) were grown overnight in the medium of O'Meara & Brown (1936) to provide a starter culture.

Of this, 0·2 ml was inoculated into 100 ml of the same medium which was then incubated for 7–8 hr at 37°C. Viable counts of approximately 10^9/ml were obtained. Formalin, 4·0 ml, was added to each culture to give a final concentration of 1·6% formaldehyde. The cultures were left for 24 hr at 4°C, washed three times with PBS and re-suspended to give a final concentration of 10^9/ml.

Induction of paralysis and immunity

A preliminary dose–response curve was carried out on the SIII preparation by challenging groups of mice which had been injected 8 days previously with various doses of SIII with 0·5 ml i.p. of a 10^6 dilution of a 14-hr culture in O'Meara & Brown's medium. Maximal immunization was obtained with dosages of 5 and 0·5 μg SIII, whereas the absence of survivors in the group receiving 500 μg indicated that this was a fully paralysing dose. These three dosages were subsequently used for the induction of immunity and paralysis, respectively.

Bleeding

Mice were bled from the retro-orbital venous plexus with a microhaematocrit capillary tube into: (a) plastic disposable tubes (internal diameter 0·5 cm, length 5 cm) for the separation of serum, and (b) 3·8% sodium citrate for preparation of erythrocyte suspensions.

Polysaccharide sensitization of erythrocytes

CBA red cells were washed four times in PBS prior to sensitization. To 10 ml of PBS were added first, 0·3 ml of pneumococcus type III culture filtrates (prepared according to the method of Askonas *et al.*, 1960) and then 0·1 ml of packed washed CBA erythrocytes. After 45 min incubation at 37°C, the cells were washed four times with 10-ml volumes of PBS and then resuspended as follows: (a) at a concentration of 1% (v/v) in PBS containing 0·5 mg/100 ml crystalline bovine plasma albumin (Armour) for haemagglutination assays, and (b) at a concentration of 30×10^6/ml in PBS for immunocytoadherence assays. Sham-sensitized red cell suspensions were prepared by similar incubation with 0·3 ml of normal O'Meara & Brown's medium.

Haemagglutination

Serial doubling dilutions of 0·1-ml volumes of mouse serum were made in disposable microhaemagglutination trays and 0·03-ml volumes of 1% SIII sensitized CBA erythrocyte suspension added to each well. Agglutination readings were made after 4 hr incubation at room temperature. The efficacy of sensitization was always tested by the inclusion of a titration of standard rabbit anti-SIII serum (Burroughs Wellcome) which gave a titre of 1024 in this system. The specificity of the reaction was confirmed by the demonstration that haemagglutination was inhibited by the addition of 100 μg SIII but remained unchanged after the addition of an equivalent amount of SII. It should also be mentioned that similar titres were obtained with SIII-sensitized sheep erythrocytes used instead of the CBA mouse cells, although in this instance the test sera were first absorbed with normal sheep erythrocytes.

Immunocytoadherence (rosette) technique

The method used was adapted from that described by Biozzi *et al.* (1967) for use with erythrocytes as antigen. Mice were killed by cervical dislocation and their spleens removed and dissociated individually in loose-fitting ground glass homogenizers. The cell suspension obtained was filtered successively through nylon gauze, a coarse mesh stainless steel sieve and finally a fine mesh stainless steel sieve. A count of the total nucleated cell yield from each spleen was made at this stage. The cells were then washed four times in 10-ml volumes of PBS to remove free polysaccharide as far as possible. Any fibrin clots which appeared during this process were removed with a Pasteur pipette. The cells were finally dispersed by passing through a 25 SWG needle, counted and adjusted to a final concentration of 12×10^6/ml. Of this cell suspension, 0·5 ml was placed in a glass tube (0·9 cm internal diameter, 7·5 cm length) together with 0·5 ml SIII sensitized CBA erythrocyte suspension (concentration 30×10^6/ml) and 0·05 ml pooled normal CBA mouse serum. The tubes were sealed with a silicone rubber bung, centrifuged at 150 *g* for 7 min to sediment the cells and incubated at 4°C for 90–120 min. The cells were then re-suspended by horizontal rotation for 10 min at 16 rev/min on a wheel of 24 cm radius.

Tests on each spleen were set up in duplicate. Approximately 50,000 nucleated spleen cells (equivalent to a total volume of 9 mm^3 of mixture) were scored for rosettes in haemocytometers by three observers working at the same time. Total nucleated cell counts were also made, so that the results could be expressed as rosette-forming cells (RFC) per 10^6 nucleated cells. By reference to the total spleen cell count obtained on primary isolation, a value for the total number of RFC per spleen was calculated.

RESULTS

Passive haemagglutination titration after varying doses of SIII

Titrations for anti-SIII activity by the passive haemagglutination technique were performed on sera obtained from individual mice at intervals varying between 12 hr and 39 days after injection of three standard doses of SIII: 0·5, 5 and 500 μg. The results are expressed graphically in Fig. 1. The results on sixty-four sera obtained from mice immunized with 0·5 μg SIII show that antibody is already detectable within 12 hr, that maximum titres are obtained within 4 days and that the response begins to wane towards the end of 1 month. Sera obtained from forty-eight mice immunized with 5 μg revealed a somewhat slower initial rise to reach maximal levels around day 6. In this group, on the other hand, antibody levels were well maintained when observations were discontinued after day 39. By contrast, the paralysing effect of a dose of 500 μg SIII is immediately obvious. Out of eighty-nine sera tested, seventy-six were entirely devoid of haemagglutinating activity, seven gave marginal titres of 1 : 2–1 : 4, whilst the remaining six alone gave titres greater than 1 : 4. The incidence of these weak positive sera appeared to be greater from the end of the 2nd week. Haemagglutinating activity in such sera could be removed either by absorption with 50% (v/v) of packed type III pneumococci or by the addition of SIII (concentration 100 μg/ml). Out of forty-six sera from untreated mice, forty-two were negative, three were positive at 1 : 2 and one positive at 1 : 4 dilution.

Immunocytoadherence after varying doses of SIII

Although the background number of RFC in the spleens of untreated control mice was

found to be very low, some degree of variability was apparent between thirty such mice which were used as controls throughout this investigation. When these numbers of total RFC per spleen were plotted logarithmically, they fitted closely to a normal distribution.

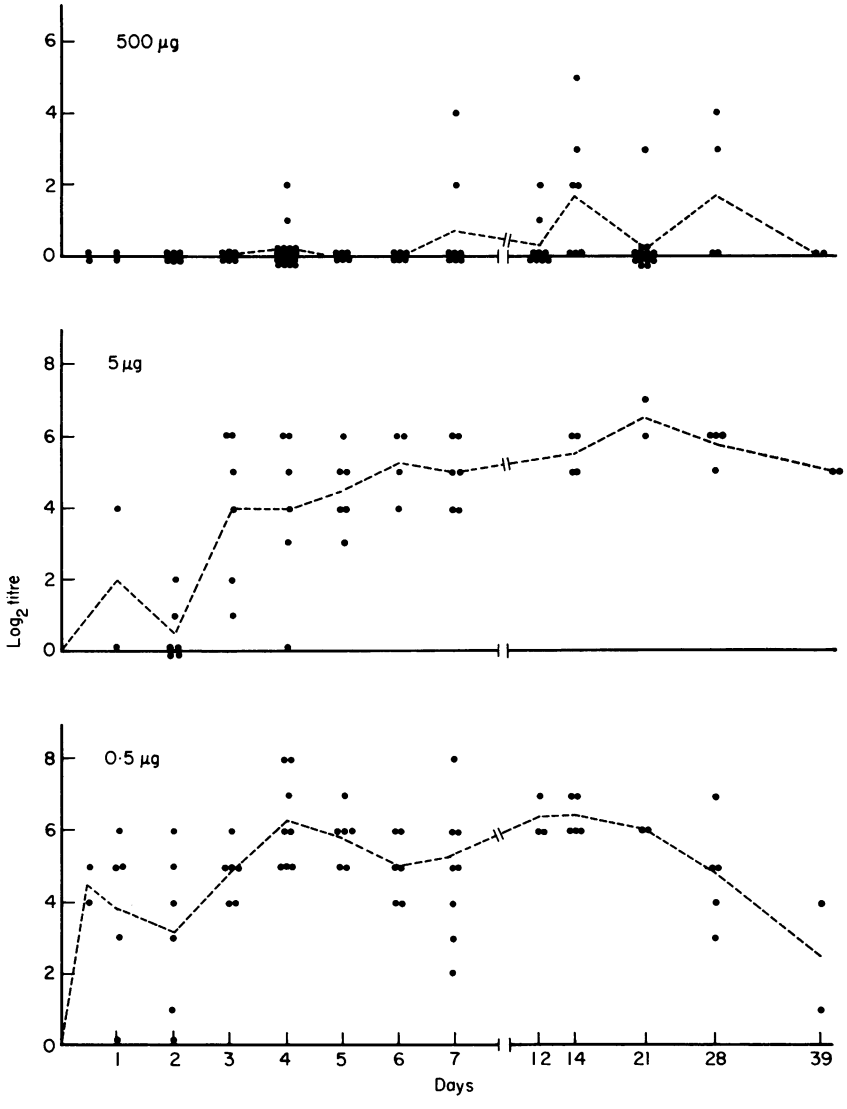

FIG. 1. Anti-SIII haemagglutination titres on individual mice at varying intervals after i.v. injection of 0·5, 5 and 500 μg SIII.

The geometric mean value obtained was 5630 and the range given by ±2 standard deviations calculated on log transformed data 1850–17,000. With one exception (which was below the lower limit) all background control values of RFC per spleen fell within this range.

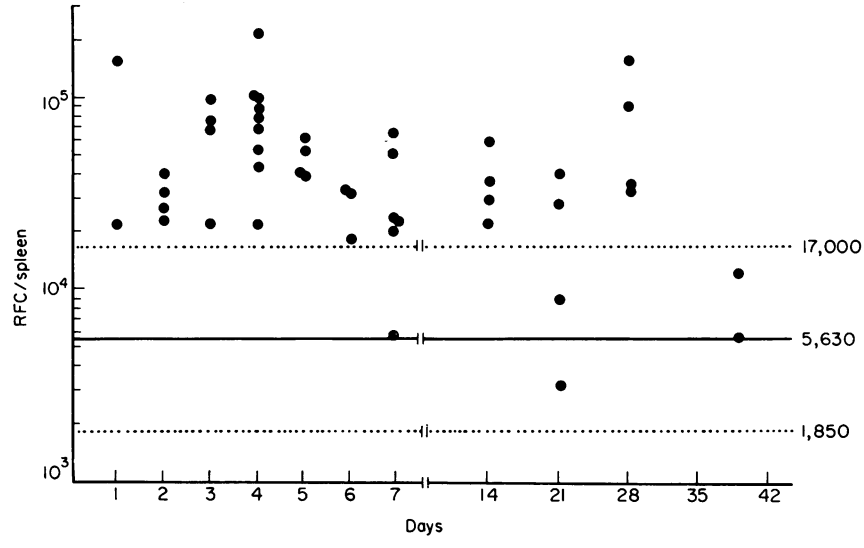

FIG. 2. Anti-SIII rosette-forming cells in the spleen at varying intervals after i.v. injection of 500 μg SIII. Each point represents an individual mouse. The horizontal lines indicate the mean ± 2 SD on log transformed data from thirty untreated animals.

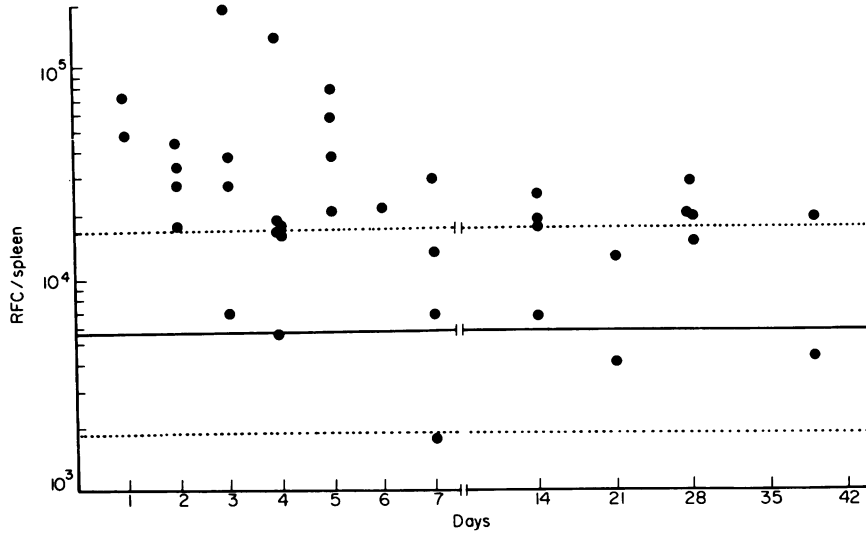

Fig. 3. As Fig. 2, but after 5 μg SIII.

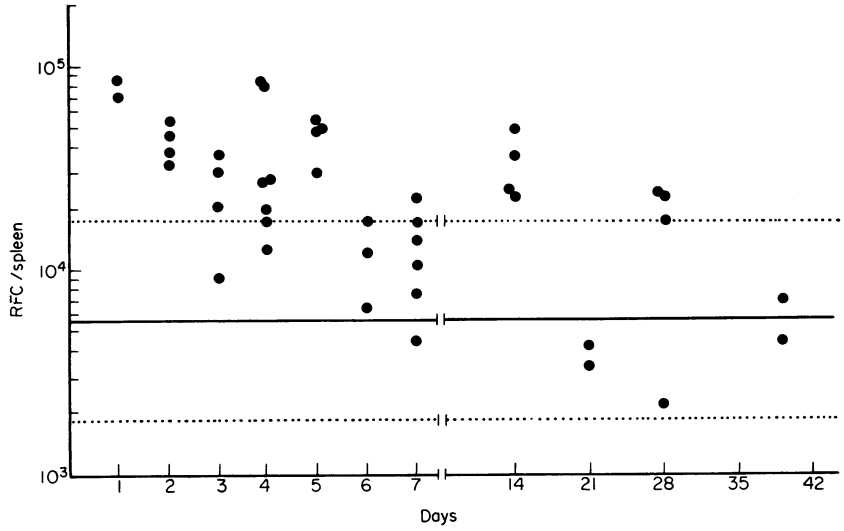

Fig. 4. As Fig. 2, but after 0·5 μg SIII.

TABLE 1. The geometric mean number of rosette-forming cells per spleen and per 10^6 nucleated spleen cells at various times after i.v. injection of paralysing (500 μg) and immunizing (5 and 0·5 μg) doses of SIII

Days after i.v. injection of SIII	Geometric mean total No. of RFC/spleen			Geometric mean No. of RFC/10^6 nucleated spleen cells		
	500 μg	5 μg	0·5 μg	500 μg	5 μg	0·5 μg
1	58,600 (2)*	58,300 (2)	76,700 (2)	330†	382	413
2	29,900 (4)	28,700 (4)	43,800 (4)	198	207	271
3	57,900 (4)	33,400 (4)	21,200 (4)	352	231	137
4	72,000 (9)	20,200 (6)	28,000 (7)	359	136	188
5	47,600 (4)	42,300 (4)	43,600 (4)	493	325	292
6	27,000 (3)	21,000 (1)	10,900 (3)	209	170	84
7	24,400 (6)	8,100 (4)	10,100 (6)	183	74	88
14	34,000 (4)	14,800 (4)	31,100 (4)	230	98	185
21	13,500 (4)	6,900 (2)	3,700 (2)	94	40	25
28	63,200 (4)	19,600 (4)	12,000 (4)	387	153	86
39	8,200 (2)	8,900 (2)	5,600 (2)	52	85	49
Untreated controls		5,630 (30)			45·3	

* Figure in parentheses indicates number of observations per group.

† Refers to the same group described under total No. rosette-forming cells (RFC) per spleen.

The individual values for numbers of RFC per spleen on groups of mice injected with 500, 5 and 0·5 μg SIII are shown in Figs. 2–4. The geometric mean of these individual values together with the corresponding mean number of RFC/10^6 nucleated spleen cells for the various times after injection of SIII is given in Table 1. The spleen of twenty-seven out of forty-two mice immunized with 0·5 μg SIII and twenty-four out of thirty-seven immunized with 5 μg SIII showed total RFC counts per spleen which were greater than 2 SD (standard deviations) above the geometric mean control value. In both cases, the mean values had attained maximum levels within 1 day. In spite of the considerable scatter of the individual values, a pattern of regression is apparent in both Figs. 3 and 4. Similar tests on the spleens of forty-six mice which had received the paralysing dose of 500 μg SIII showed that in forty-one instances the total RFC count per spleen was greater than 17,000 (the upper limit of the normal range). Reference to the figures and Table 1 indicates that, not only was there a greater incidence of values above the upper limit of the normal range, but that the response was better maintained than in the two immunized groups. Particularly striking are the high values obtained with four mice 28 days after injection of 500 μg SIII. The limited number of animals investigated at day 39, however, showed low values in all three groups.

TABLE 2. Inhibition of rosette-formation by the addition of SIII or by the use of sham-sensitized erythrocytes (rosette-forming cells/10^6 spleen cells)

SIII-sensitized erythrocytes	Sham-sensitized erythrocytes	SIII-sensitized erythrocytes + 100 μg SIII/ml
440	40	80
300	40	—
180	30	40

A similar general picture emerged on consideration of the data in terms of RFC/10^6 spleen cells (instead of total number per spleen) as indicated in Table 1. However, examination of the log transformed control data indicated a more irregular distribution than was obtained from a similar analysis in terms of RFC per spleen. In view of this and the fact that RFC may be diluted in the presence of other cellular proliferation, representation of total counts per whole organ is probably more meaningful.

The specificity of this assay was confirmed in two ways. Firstly, rosette formation was compared using sham-sensitized CBA erythrocytes instead of SIII-sensitized cells. Secondly, the effect of adding 100 μg SIII to the spleen cell suspension 15 min before mixing with SIII-sensitized erythrocytes was also examined. The results in Table 2 show clearly that the high experimental values of RFC/10^6 spleen cells obtained after injection of 500 μg SIII were reduced to background levels by either procedure. Furthermore, these results imply that the low background levels found in this system are not necessarily indicative of anti-SIII specific cells.

Thus, although the haemagglutination data confirmed that 500 μg of the SIII preparation

is an effective dose for the induction of immunological paralysis, an examination by the immunocytoadherence technique of well-washed spleen cell suspensions from these animals revealed that they contained initially as many (and subsequently more) antibody-forming cells as did animals given immunizing doses.

DISCUSSION

The validity of the rosette technique as a basis for assaying the specific cellular response to antigen has been firmly established by the extensive investigations of Biozzi and his colleagues (Biozzi *et al.*, 1966, 1967, 1968). Using the sheep erythrocyte system, the majority of RFC have been identified as large lymphocytes or blast cells with only a minority of plasma cells and small lymphocytes (Pavlovsky, Biozzi & Binet, 1967). The applicability of the technique to non-particulate antigens has previously been demonstrated with dinitrophenol-sensitized erythrocytes (Biozzi *et al.*, 1967) and the present results indicate the usefulness of this method for studying the immune response to pneumococcal polysaccharide. The specificity of the reaction was affirmed both by the lack of effect using sham-sensitized erythrocytes and by the inhibition produced by prior introduction of polysaccharide into the reacting cell mixture. As might be anticipated, the specific cellular response to SIII detected in the spleen was small compared with the analogous results which have been reported using sheep erythrocytes as antigen (Biozzi *et al.*, 1966, 1967). Although the background number of RFC in untreated animals was one order of magnitude lower in the present series, the maximum number of RFC above background which was detected following SIII administration was at best 5% of the number attained following immunization with sheep erythrocytes.

Baker *et al.* (1966) and Baker & Landy (1967) recently reported a variation of the rosette technique using bentonite particles coated with SI. Their background levels ($13–17/10^6$ spleen cells) and maximum levels after immunization ($400/10^6$ cells) are in general agreement with the present data. They noted briefly that high doses of SI suppressed full development of RFC by about 50%. This apparent difference from the results reported here might be explicable by variations in technique, such as the use of formalin-fixed, rather than viable, spleen cells. On the other hand, the extreme brevity of the induction period preceding the peak of an immune response to SI, which was recorded by Baker & Landy (1967) has been confirmed by the present experiments using SIII. The implication that little or no cell proliferation occurs in these responses was also proposed recently by Paul *et al.* (1967). They found that although SIII would induce a short-lived secondary response when given late after primary immunization with type III pneumococci, it failed to stimulate [^{3}H]-thymidine uptake when added to cells from immunized animals *in vitro*. Inability of purified pneumococcal polysaccharides to stimulate division of antigen-sensitive cells could explain the minimal antibody levels which they elicit.

The main feature of the present investigation is the finding that throughout a 4-week period of observation, as many RFC were detected in the spleens of mice which had been paralysed with 500 μg SIII as in the spleens of animals which had been immunized with 0·5 or 5 μg. This unequivocal result implies that with this dose of SIII and for the duration of 1 month, the resultant state of immunological paralysis could not be attributed to central inhibition of antibody formation and must have been determined in some other way. Experiments of Dixon *et al.* (1955) and Stark (1955) established beyond any reasonable

doubt that if antibody were to be formed in polysaccharide paralysed animals, it would be rapidly inactivated by the ready availability of undegraded reactive antigen. It was suggested in the previous paper (Howard & Siskind, 1969) that antigen remains free to react for prolonged periods, because it is chronically released from the depots contained within macrophages which lack enzymes to catabolize it. The alternative of intracellular polysaccharide reacting with circulating antibody seems less likely. Dixon and his colleagues (Dixon *et al.*, 1955) found that passively transferred ^{131}I-labelled anti-SIII combined with SIII persisting in paralysed animals, was rapidly catabolized and non-protein bound ^{131}I excreted. Clearly, if antibody is formed, a dynamic situation could exist whereby polysaccharide would function as a treadmill for its removal as antigen–antibody complexes which will be phagocytosed, antibody broken down and polysaccharide re-emerge intact to repeat the process. Although the existence of a potential efferent block was clearly established some time ago, in recent years it has been considered unlikely to play any major part in determining polysaccharide paralysis, in view of results from two different experimental approaches which have supported the idea that the phenomenon is basically similar to all other forms of tolerance in being attributable to a mechanism of central inhibition.

Support for the latter concept in polysaccharide paralysis was first provided by Sercarz & Coons (1959) who applied a fluorescent antibody technique to sections of spleen 14 days after injection of SII. Whereas they were able to detect a small and highly variable number of antibody forming cells when mice had been immunized with 1 μg (about 1% of the equivalent number which could be found after immunization with BSA), such cells could not be found in mice which had been paralysed with 500 μg. The apparent discrepancy between this result and those reported here may well be attributable to the persistence of free polysaccharide in the spleen. The greater sensitivity of the rosette technique and the ability to work with washed cell suspensions argue in favour of acceptance of the present findings. The second line of approach has been the studies on adoptive transfer carried out by Brooke & Karnovksy (1961) and by Neeper & Seastone (1963) who worked with SII and SI respectively. Firstly, both groups found that 2 days after the intraperitoneal transfer of spleen cells from three paralysed donors, mice showed no immunity to homologous bacterial challenge, although similar cell transfers from immunized donors were effective. It seems not unlikely that these results were attributable to antigen transfer, for although Siskind & Paterson (1964) were also able to obtain a similar result on adoptive spleen cell transfer to syngeneic mice, they found that recipients of heat-killed cells from tolerant donors died upon pneumococcal challenge while recipients of one-tenth the dose of killed cells were protected upon subsequent challenge. This result implies that sufficient polysaccharide was transferred with the spleen cells to induce paralysis. The strongest support for the existence of central inhibition in this system is provided by essentially similar experiments performed by Brooke & Karnovsky (1961) and Neeper & Seastone (1963) who found that paralysed mice appeared to be immunized adoptively by the intraperitoneal transfer of large doses of spleen cells from immunized mice 2 days before bacterial challenge. Owing to the use of histoincompatible animals, it was necessary to challenge by the same route only 2 days after the injection of cells from three spleens into the peritoneal cavity, at a time when the transferred cells might not have dispersed. We have, however, successfully repeated this experiment using SII, CBA inbred mice and an 11-day interval between cell transfer and challenge (unpublished). On the other hand, no circulating antibody

could be detected when similar large doses of spleen cells were transferred from immunized mice to animals paralysed with SIII.

However, these positive results do not in themselves constitute formal proof of central inhibition, for in view of the large doses of immune cells transferred ($4–5 \times 10^8$) and the minimal paralysing doses used by previous workers and ourselves, antibody formation could be sufficiently boosted to overcome the neutralizing block provided by persisting polysaccharide. The data of Siskind & Paterson (1964) indicate that the relatively small amount of passively transferred antibody required to protect paralysed mice is consistent with this interpretation.

Many of the individual features of polysaccharide paralysis are undoubtedly more readily explicable in terms of continuous neutralization of antibody than they are by forcing a strict analogy with unresposiveness to protein antigens. In particular, the irrelevance of the route of injection (Felton *et al.*, 1955), the simple cumulative effect of repeated injections (Howard & Siskind, 1969), the lack of effect of pre-immunization with SSS and the relatively small effect with bacterial hyperimmunization in shifting the paralysis threshold level (Siskind & Howard, 1966; Howard & Siskind, 1969), the apparent independence of paralysis induction on the extent of polysaccharide uptake by macrophages (Howard & Siskind, 1969), and the fact that loss of paralysis with time passes directly into a phase of spontaneous immunity (Siskind, Paterson & Thomas, 1963), are but some of the features which accord well with an efferent block by an antigen which is only very slowly eliminated.

The present lack of central inhibition observed in paralysis with SIII taken together with previous failure to induce low-zone tolerance with SII (Siskind & Howard, 1966; Howard & Siskind, 1969) raises the question: 'Do pneumococcal polysaccharides differ fundamentally from proteins in their ability to render a lymphocyte population tolerant?' It would be premature to overstress the present limited results in terms of any widespread applicability, for the use of other antigenic types of polysaccharide, more massive dosages or perinatal recipients might reveal a significant suppression of RFC formation. Furthermore, as release of antibody from the RFC in paralysed animals has yet to be demonstrated, a block at this level has not been formally excluded. It is also conceivable that tolerance in the orthodox sense may develop in the present system after a longer period than the 1 month which we have studied. Unfortunately, it is not possible to attach any relevant meaning to a fall in the number of RFC at this later stage, for it also occurs in immunized animals where circulating antibody persists. Moreover, a minority of mice showed signs of release from paralysis as judged by the haemagglutination data. Although the original work of Felton and his colleagues (1955) indicates that polysaccharide paralysis can persist in mice for 15–18 months, periods of only 2–4 months have been reported by some other workers using comparable dosages (Siskind *et al.*, 1963; Batshon, Baer & Shaffer, 1963; Neeper, 1964; Wu & Trice, 1967). Should SIII require a period of more than 1 month to produce central inhibition of lymphoid tissue *in vivo*, this would constitute a far greater period of time than is required by protein antigens. Adoptive transfer studies by Golub & Weigle (1967) indicated clearly that unresponsiveness to human γ-globulin required an induction period of 4 days and Mitchison (1968) has found that the time required for other protein antigens is considerably shorter still.

Although the existence of an antibody-neutralizing mechanism in polysaccharide paralysed mice has not been doubted, it has latterly been considered to play no significant

causal role in determining the unresponsive state of the whole animal. The present results indicate that this is not invariably so and that paralysis by these antigens may be the outward reflection of more than one process. In particular, it would appear that the extent to which a central inhibition component can be held responsible for polysaccharide unresponsiveness requires further examination.

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REFERENCES

ASKONAS, B.A., FARTHING, C.P. & HUMPHREY, J.H. (1960) The significance of multiple antibody components in serum of immunized rabbits. *Immunology*, **3**, 336.

BAKER, P.J., BERNSTEIN, M., PASANEN, U. & LANDY, M. (1966) Detection and enumeration of antibody-producing cells by specific adherence of antigen-coated bentonite particles. *J. Immunol.* **97**, 767.

BAKER, P.J. & LANDY, M. (1967) Brevity of the inductive phase in the immune response of mice to capsular polysaccharide antigens. *J. Immunol.* **99**, 687.

BATSHON, B.A., BAER, H. & SHAFFER, M.F. (1963) Immunologic paralysis produced in mice by *Klebsiella pneumoniae* type II polysaccharide. *J. Immunol.* **90**, 121.

BIOZZI, G., STIFFEL, C. & MOUTON, D. (1967) A study of antibody-containing cells in the course of immunization. *Immunity, Cancer and Chemotherapy*, pp. 103–131. Academic Press, New York.

BIOZZI, G., STIFFEL, C., MOUTON, D., BOUTHILLIER, Y. & DECREUSEFOND, C. (1968) A kinetic study of antibody producing cells in the spleen of mice immunized intravenously with sheep erythrocytes. *Immunology*, **14**, 17.

BIOZZI, G., STIFFEL, C., MOUTON, D., LIACOPOULOS-BRIOT, M., DECREUSEFOND, C. & BOUTHILLIER, Y. (1966) Etude du phenomène de l'immuno-cyto-adherence au cours de l'immunisation. *Ann. Inst. Pasteur*, **110**, 7.

BROOKE, M.S. (1966a) A hemagglutination test and a specific depolymerase for studying immunologic paralysis in mice. *J. Immunol.* **96**, 358.

BROOKE, M.S. (1966b) Studies on the induction, specificity, prevention and breaking of immunologic paralysis and immunity to pneumococcal polysaccharide. *J. Immunol.* **95**, 364.

BROOKE, M.S. & KARNOVSKY, M.J. (1961) Immunological paralysis and adoptive immunity. *J. Immunol.* **87**, 205.

DIXON, F.J., MAURER, P.H. & WEIGLE, W.O. (1955) Immunologic activity of pneumococcal polysaccharide fixed in the tissues of the mouse. *J. Immunol.* **74**, 188.

FELTON, L.D., KAUFFMANN, G., PRESCOTT, B. & OTTINGER, B. (1955) Studies on the mechanism of the immunological paralysis induced in mice by pneumococcal polysaccharides. *J. Immunol.* **74**, 17.

GOLUB, E.S. & WEIGLE, W.O. (1967) Studies on the induction of immunologic unresponsiveness. II. Kinetics. *J. Immunol.* **99**, 624.

HOWARD, J.G. & SISKIND, G.W. (1969) Studies on immunological paralysis. I. A consideration of macrophage involvement in the induction of paralysis and immunity by type II pneumococcal polysaccharide. *Clin. exp. Immunol.* **4**, 29.

MITCHISON, N.A. (1968) Immunological paralysis induced by brief exposure of cells to protein antigens. *Immunology*, **15**, 531.

NEEPER, C.A. (1964) Mechanisms of immunologic paralysis by pneumococcal polysaccharide. III. Immunologic paralysis in relation to maturation of the immunologic response of mice. *J. Immunol.* **93**, 860.

NEEPER, C.A. & SEASTONE, C.V. (1963) Mechanisms of immunologic paralysis by pneumococcal polysaccharide. I. Studies of adoptively acquired immunity to pneumococcal infection in immunologically paralyzed and normal mice. *J. Immunol.* **91,** 374.

NOTA, N.R., LIACOPOULOS-BRIOT, M., STIFFEL, C. & BIOZZI, G. (1964) L'immuno-cytoadherence: une methode simple et quantitative pour l'etude '*in vitro*' des cellules productrices d'anticorps. *C.R. Acad. Sci.* (*Paris*), **259,** 1277.

O'MEARA, R.A.Q. & BROWN, A.M. (1936) On growing the pneumococcus with a view to the production of cultures of standard maximal density, virulence and polysaccharide content. *J. Path. Bact.* **42,** 679.

PAUL, W.E., SISKIND, G.W., BENACERRAF, B. & OVARY, Z. (1967) Secondary antibody responses in haptenic systems: cell population selection by antigen. *J. Immunol.* **99,** 760.

PAVLOVSKY, S., BIOZZI, G. & BINET, J. (1967) Etude au microscope eléctronique du phenomène de l'immuno-cyto-adherence au cours de la reponse immunitaire. *J. Microsc.* **6,** 74a.

SERCARZ, E. & COONS, A.H. (1959) Specific inhibition of antibody formation during immunological paralysis and unresponsiveness. *Nature* (*Lond.*), **184,** 1080.

SISKIND, G.W. & HOWARD, J.G. (1966) Studies on the induction of immunological unresponsiveness to pneumococcal polysaccharide in mice. *J. exp. Med.* **124,** 417.

SISKIND, G.W. & PATERSON, P.Y. (1964) A bioassay for estimation of pneumococcal polysaccharide in unresponsive (paralyzed) mice. *J. Immunol.* **93,** 489.

SISKIND, G.W., PATERSON, P.Y. & THOMAS, L. (1963) Induction of unresponsiveness and immunity in newborn and adult mice with pneumococcal polysaccharide. *J. Immunol.* **90,** 929.

SISKIND, G.W., PAUL, W.E. & BENACERRAF, B. (1967) The use of radioactively labelled SIII to study the blood clearance and the immune response of mice to pneumococcal polysaccharides. *Immunochemistry,* **4,** 455.

STARK, O.K. (1955) Studies on pneumococcal polysaccharide. II. Mechanism involved in production of 'Immunological Paralysis' by type I pneumococcal polysaccharide. *J. Immunol.* **74,** 130.

WU, W.G. & TRICE, K.L. (1967) Influence of cellular factors on immune unresponsiveness induced by *Klebsiella pneumoniae* capsular antigen. *J. Bact.* **94,** 896.

ZAALBERG, O.B. (1964) A simple method for detecting single antibody forming cells. *Nature* (*Lond.*), **202,** 1231.