POLYSACCHARIDE IN DELAYED HYPERSENSITIVITY

I. Pneumococcal Polysaccharide as Inducer and Elicitor of Delayed Reactivity in Guinea Pigs

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The role of polysaccharides as antibody inducers has been appreciated for many years, but there has been some doubt as to their capacities as immunogens for delayed hypersensitivity. If they are active in this respect, the capacity is less clearly evident than is that of proteins or of contact inducing agents.

For example, in the test devised by Tillet and Francis (1) for patients with lobar pneumonia, the intradermal injection of purified pneumococcal polysaccharide derived from the infecting type of organism gave rise to reactions of the immediate type, although the pneumococcus is known to induce delayed reactivity to its protein constituents (1, 2). In work with blood group substances, Holborow and Loewi (3) found antibodies to be produced against the sugar components, while delayed reactivity, which was also seen, was attributed to the peptide moiety of these substances. Similar conclusions were reached using chondromucoprotein as antigen (4). Jankovic and Waksman (5) also concluded that delayed hypersensitivity induced by the saccharide–peptides of human erythrocytes may have been in part directed against the peptide structure. Barker and coworkers (6) with polysaccharide from *Trichophyton* concluded that it participated in antibody-mediated reactions, the protein of the organism in delayed ones.

In 1963 it was demonstrated that mono- and disaccharides coupled to protein carrier induced delayed reactivity elicitable by the same hapten attached to different carriers, although homologous conjugates were best for the purpose (7). Several reports describe the induction of delayed reactivity to polysaccharides, while presumably obviating the possible role of associated peptides. Thus, Knight et al. (8–10) described the induction of delayed reactivity in guinea pigs to polysaccharides of *Histoplasma capsulatum* and *Blastomyces dermatitidis* by sensitization with whole fungi and testing with appropriate polysaccharide preparations, but these contained 4–5% nitrogen. Freund et al. (11) induced aspermatogenesis in guinea pigs with testicular extract

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composed largely of polysaccharide, but containing about 4% N and giving positive biuret and ninhydrin reactions. Rowlands, Crowle and Russe (12) found positive reactions to a polysaccharide derived from *Candida albicans* in mice immunized with heat-killed fungi, and Campbell earlier (13) made similar observations in rabbits sensitized with *Ascaris* and tested with a highly purified polysaccharide derived from this worm. In the case of *Mycobacterium tuberculosis*, despite a long history of evidence indicating that tuberculin reactivity is directed against its protein constituents, Baer and coworkers (14–17) have more recently detailed experiments implicating a polysaccharide extract in this respect, also, in guinea pigs sensitized with whole organisms. However, some protein nitrogen was present in this preparation, and there was cross reactivity between the polysaccharide and protein fractions used. Azuma and coworkers (18) with a nitrogen-free polysaccharide isolated from culture filtrates of the tubercle bacillus have been able to find only anaphylactic reactivity elicitable in guinea pigs sensitized by bacilli.

Recently, Crowle and Hu (19) have described induction and elicitation of delayed reactions to dextran in the mouse, and Battisto et al. (20) have established such reactivity to this polymer in certain strains of guinea pigs. These last works in fact come closest to the only evidence extant that pure polysaccharides may be able to induce the delayed hypersensitive state. The evidence on the whole is very scant that polysaccharides can induce delayed reactivity, and sufficiently irregular in respect to elicitation of reactions as to have led to the suggestion (21) that saccharides may be relatively ineffective in delayed reactions, possibly because they lack sufficient binding energy to interact effectively with the antibody (or antibody-like substance) responsible for such reactions.

In the present report, a pneumococcal capsular polysaccharide (type II) was used to induce and elicit delayed hypersensitive reactivity in guinea pigs. This was employed in the face of several discouraging reports. Freund and Bonanto (22) in 1944 had incorporated pneumococcal polysaccharide (type not stated) in adjuvant containing mycobacteria in efforts to induce antibodies or hypersensitivity in rabbits. Their failure may have reflected the relative inability of this species to respond immunologically to such polysaccharides (23–25). Failure with type III pneumococcal polysaccharide in guinea pigs was reported by Maurer and Mansmann (26) with small inducing doses (10 μg) which could have been an insufficient quantity for sensitization. However, in the work reported here, we also failed with this polysaccharide in amounts of 100 μg. Tremaine (2) could not detect delayed reactivity to capsular polysaccharide in the corneas of rabbits passively sensitized with lymphoid cells from donors previously immunized with intact pneumococci.

The present work was instigated primarily by an interest in the question of mechanistic basis for delayed hypersensitive reactivity. We think that delayed reactivity of the "tuberculin type" eventuates under certain circumstances of exposure of an animal to antigen, while another expression of cellular reactivity (termed "Jones-Mote" type) comes about under other circumstances of exposure to antigen as an early phase of antibody synthesis (27–31). The bases for this distinction have been observational, including the time of appearance of the reactive state, its duration, and the gross and microscopic characteristics

of dermal reactions (30–34). It was thought that if a polysaccharide were capable of inducing antibodies but lacked the capacity to induce persisting delayed reactivity of the tuberculin type, it would be interesting to observe its possible role in the induction and elicitation of early-appearing Jones-Mote reactivity. If the latter were in fact an aspect of the evolution of the conventional antibody response, the polysaccharide should participate in this. We turned to PnIIS as a saccharide known to be free of nitrogen-containing sugars (35, 36) and hence amenable to testing for possible protein contaminants. We found that this substance per se is immunogenic for guinea pigs, inducing antibodies in low but detectable quantities in most of them, and giving rise to striking levels of delayed reactivity and to excellent instances of Jones-Mote reactivity. This report deals with studies of the homogeneity and composition of the polysaccharide employed, and with the results of its use as a stimulus to the induction of these various reactivities as determined by skin tests, by tests for inhibition of macrophage migration, by serum and cell transfers, by serologic tests, and by histological correlations.

Materials and Methods

Animals.—Random-bred guinea pigs, 250–350 g, were used for sensitization and skin testing, and as the source of cells for macrophage migration tests. Animals of 250–300 g were used for passive cutaneous anaphylactic (PCA) tests. Albino Wistar rats, 200–250 g, were used as recipients of guinea pig serum for heterologous PCA tests.

Antigens.—Pneumococcus type II capsular polysaccharide (PnIIS) was obtained from Lederle Laboratories, Division of American Cyanamid, Pearl River, N.Y., courtesy of Dr. W. S. Hammond. The sample received had an N content of 0.10%. In an effort to reduce this, the sugar was repeatedly precipitated with 95% ethanol and propanol, followed by shakings in chloroform and butanol (35–37). It was then precipitated with barium acetate, reprecipitated with sodium acetate and isopropanol, centrifuged at 10,000 rpm and retreated with 95% ethanol (35, 36). The eventual product contained 0.04% nitrogen as an irreducible minimum. In view of the fact that PnIIS does not contain amino sugars, it was important to determine whether this might represent peptide nitrogen, which in turn could be responsible for some of the biologic activities to be described. The polysaccharide was subjected to the following tests and procedures.

Antigenic homogeneity was determined by double diffusion tests in agar against rabbit anti-PnII sera from animals vaccinated with killed organisms; these showed single lines of precipitate. The polysaccharide was also subjected to electrophoresis on slides in Noble agar at pH 8.6, 30 ma, for 2–5 hr. Against rabbit anti-pneumococcal type II serum, only one precipitation arc formed.

The *composition* of the sample of PnIIS was assayed by several methods:

Methyl Pentose Analysis.—Rhamnose accounts for about 50% of the PnIIS molecule (38), which is composed of L-rhamnose, D-glucose, and D-glucuronic acid in the ratio 3:1:2. Quantitative tests for this sugar (39) were carried out simultaneously with control samples of rhamnose dried to constant weight. 10 mg samples of PnIIS and rhamnose were hydrolyzed with H_2SO_4, treated with freshly prepared cysteine HCl reagent, and measured for absorption at 396 mμ, correcting for glucose absorption on the basis of its equivalent absorption peaks at 396 and 430 mμ. Similar untreated samples served as blank controls for reading in the Beckman DB spectrophotometer. Rhamnose was found to comprise 50% of the total weight.

Thin Layer Chromatography in Silica Gel.—10 mg of PnIIS was treated with 2 N H_2SO_4, heated at 100°C. for 3 hr, and neutralized to pH 7 with saturated $Ba(OH)_2$. The salts were centrifuged off, the samples evaporated to 0.5 ml, and 10 λ quantities were spotted on gel plates (2 parts 0.1 M boric acid and 1 part silica gel G. mixed in a blender), with solutions of glucose, rhamnose, and glucuronic acid as controls. The spotted plates were placed in butanol–acetic acid (4:1) solvent for 5 hr, then sprayed with a solution of aniline (1%) and diphenylamine (1%) in acetone, and dried with a heat gun. Only the appropriate sugars were found.

The *nitrogen content* of the polysaccharide had been determined by micro-Kjeldahl analyses of three 10 mg samples to be 0.04%. The following analyses were made to determine its source:

Fluorometry.—The possible presence of aromatic amino acids was sought with a Turner Model 111 Fluorometer, using 0.01–10 mg samples in 1 ml. Reference proteins were included as controls. This method is sensitive to 0.001 μg of tyrosine. There was no detectable result above background.

Silica Gel Chromatography for Amino Acids.—Three 10 mg samples of polysaccharide were hydrolyzed with 2 N H_2SO_4 at 100°C. The hydrolysates along with solutions of known amino acids and amino sugars were spotted on Eastman Chromogram No. 6060 sheets in volumes ranging from 20–40 microliters of solution containing 40 mg PnIIS/ml (up to 1.6 mg polysaccharide per test, of which the N could represent about 4 μg of peptide). The chromatograms were developed with a mixture of butanol, acetic acid, and water (4:1:1) for 3 hr, then dried and sprayed with ninhydrin reagent. This method can detect as little as 0.01 μg glycine and 0.1 μg proline. When a mixture of constituent sugars of PnIIS and ammonium salts (NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$) were chromatographed, color developed in the same region as in the experimental analysis of the PnIIS, suggesting that the N in this preparation derived from ammonia absorbed from the air by the acidic polysaccharide.

Tests for Free Amino Groups were made on 10 mg hydrolyzed samples of the polysaccharide with 2,4,6-trinitrobenzene 1-sulfonic acid (40). Readings for absorbance at 340 mμ showed values corresponding to a possible average content of 1.4 μg of amino acids.

Enzymatic treatment of the PnIIS with peptidases will be described under experimental results.

Pneumococcal type III capsular polysaccharide was obtained from Dr. Michael Heidelberger. It was used in skin and serological tests as a control for the possible presence of non-type-specific bacterial substances in the PnIIS preparation which might have escaped the purification procedures.

Vaccination Procedures.—PnIIS was administered to guinea pigs intradermally in saline or subcutaneously in incomplete or complete Freund's adjuvant (Difco). Varying concentrations of polysaccharide in 0.1 ml of saline were mixed with 0.1 ml of adjuvant.

Skin tests were performed at 7, 14, or 21 days after the single sensitizing injection, using usually 100 μg of polysaccharide in 0.1 ml of saline in the flank. Readings were made at 4, 12, 24, and 48 hr.

Antisera.—Several rabbit anti-PnII sera were used for immunodiffusion tests. One lyophilized sample[1] was selected as a standard positive control in carrying out various serological tests with experimental guinea pig sera.

Serological Tests.—Guinea pigs were bled at various intervals just before, or in some cases after, skin testing. Antibodies were detected by these procedures:

(*a*) Double diffusion in agar, using wells of 0.01 ml capacity spaced 2 mm between circumferences. Concentrations of PnIIS used varied between 0.1 and 0.0001 mg.

(*b*) Passive cutaneous anaphylaxis (PCA) tests were carried out in guinea pigs (for detection of γ_1 antibodies (41–43)), and in heterologous animals (Wistar albino rats) for the detection of γ_1 and γ_2 antibodies (42, 43) with 0.1 ml quantities of undiluted serum. In the same recipi-

[1] Obtained from the Communicable Disease Center, Atlanta, Ga.

ents, a nonspecific rabbit serum (anti-ribonuclease) and a positive control rabbit serum (anti-PnII) were used. After 4 hr, Evans blue dye was injected intravenously; if no dye leakage occurred by 5 min, 5 or 10 mg of PnIIS in 1 ml of saline was injected intravenously. Reactions were read at ½ hr.

(*c*) Micro-complement fixation tests were done with sheep red cells labeled with radioactive chromium (44). Lysis was measured by the release of radioactivity in mixtures of test serum, rabbit hemolysin, and complement.

Tests were done with 0.05 ml samples of heat inactivated serum added to 1 μg PnIIS in 0.025 ml in an ice bath. To this was added 0.05 ml of guinea pig serum containing two 50% hemolytic doses of complement for the sheep cell system employed. The mixture was incubated at 37°C for 1 hr with occasional shaking, and left overnight at 4°C. Control tubes contained known positive (rabbit) serum; normal inactivated guinea pig serum; antigen plus complement; test serum plus complement; and serum alone. Sheep cells collected in citrated Alsever's solution were washed 3 times in Veronal-buffered saline (pH 7.3) containing 1% gelatin. To 0.1 ml packed cells, about 50 μCi of ^{51}Cr were added (^{51}sodium chromate, Abbott Laboratories, North Chicago, Ill.). The mixture was incubated at 37°C for 1 hr with continuous gentle agitation, and left overnight at 4°C. The cells were then washed 5 times with buffer and made up to a 5% suspension.

The incubated test mixtures each received 0.025 ml of the red cell suspension mixed with rabbit hemolysin. Tubes were reincubated at 37°C for 1 hr; 1 ml of cold buffer was added, and the tubes were centrifuged at 2000 rpm under refrigeration. Sedimens and supernatants were counted in a well-type gamma sensitive scintillation counter. This method can detect probably 0.003 μg antibody N (44).

Macrophage inhibition tests were carried out by a modification of the procedure of David et al. (45). Guinea pigs received 10 ml heavy mineral oil i.p. 4 days before cell removal. The peritoneal cavities were washed with 20 ml Hanks' solution containing 1% normal guinea pig serum and in each ml 10 units of preservative-free heparin, 50 μg streptomycin, and 50 units of penicillin. Cells were washed 3 times in this solution without heparin in a refrigerated centrifuge at 500 rpm for 5 min. Differential counts were made; these usually showed about 60–68% macrophages, 20% (± 10%) lymphocytes and some polymorphonuclear cells. Cells were suspended 0.05 ml packed volume per 1 ml medium. Approximately 2.5×10^6 cells were taken up in capillary tubes (1.3–1.5 mm inside diameter, × 75 mm). The filled tubes were sealed at one end with paraffin and centrifuged at 400 rpm for 5 min, providing packed cell columns about 1–2 mm long. The capillaries were cut at the cell-fluid interface, and six such tubes were laid in a Falcon disposable tissue culture dish (60 mm diameter, 12 mm deep) and attached with stopcock grease. These were overlaid with 4 ml of medium consisting of 199 Earle's salt base plus 15% normal guinea pig serum, 200 μg bovine serum albumin (BSA)/ml with antibiotics (penicillin, 50 units; streptomycin and neomycin, 50 μg of each), and containing 25 μg PnIIS/ml. The plates were incubated in 95% air–5% CO_2 for 24 hr at 37°C. The supernatant was then removed and the dish allowed to dry. The areas of cell migrations were projected to 7 × onto paper of constant weight with a photographic enlarger, the outlines of the migrating cell borders were drawn, and these areas were cut out and weighed. The percentage of inhibition of migration was calculated for each cell suspension by comparing the mean of the migration in the presence of antigen to that in the absence of antigen.

Cell and Serum Transfers.—Sensitized guinea pigs were treated as follows: On the 7th, 14th, or 21st day after sensitization, 10–12 animals of each group were bled, and mineral oil-induced peritoneal exudates were harvested. The cells were pooled and washed 3 times, counts were made, and $3–4.3 \times 10^8$ cells in 2.5 ml of Hank's solution containing 1% normal guinea pig serum were injected intravenously into each of 2 or 3 normal recipients. 15 min–1 hr later, the animals were given skin tests with the usual dose of PnIIS.

Serum of each group were also pooled and within 4–6 hr of bleeding, 20 ml were injected

i.p. into each of 2–4 normal recipients. Again skin tests were done at ¼–1 hr after the injections had been made.

RESULTS

Immunologic Responses of the Guinea Pig to PnIIS

100 μg[2] of PnIIS was given to guinea pigs in incomplete or complete Freund's adjuvant, subcutaneously in the groin. Skin tests were made at 7, 14, and 21 days in separate animals with the same dose of polysaccharide. Readings were at 4, 24, and 48 hr. The average of reactions from several experiments are shown in Table I and Figs. 1 and 4.

These results indicate that reactions early (7 days) after sensitization appear essentially the same in both groups, with no indications of immediate (anaphylactic or Arthus) responses up to 4 hr, with zeniths at 24 hr and decline by

TABLE I

Skin Reactions in Guinea Pigs at 7, 14, and 21 Days after Subcutaneous Sensitization with 100 μg of PnIIS in Incomplete or Complete Freund's Adjuvant, and Tested with 100 μg of PnIIS*

Days	Number of animals	4 hr	24 hr	48 hr
Incomplete Freund's adjuvant				
7	33	0.2, 0‡	9.4, 0.4	3.9, 0.03
14	14	6.3, 0.33	10.8, 0.6	0.7, 0
21	28	17.5, 1.3	8.9, 0.3	0.5, 0
Complete Freund's adjuvant				
7	34	0.6, 0	10.2, 0.6	5.4, 0.2
14	15	7.5, 0.57	17.1, 1.07	4.0, 0.18
21	27	12.9, 0.8	21.8, 1.4	10.6, 0.6

* One test per animal.

‡ First number, mm diameter; second number, estimated mm thickness.

§ Control readings in normal guinea pigs given skin tests at the same times have been subtracted. For 42 animals, the means were: 4 hr.: 6.9, 0.4; 24 hr.: 1.4, 0.1; 48 hr.: 0.1, 0.02.

48. As shown in Table II, antibodies were not detectable in either adjuvant group by homologous and heterologous PCA tests (presumably detecting γ_1 and γ_2 antibodies) but microcomplement fixations tests were positive, usually at low levels, in 8 of 14 sera in the incomplete Freund's group, and in 5 of 14 in the complete Freund's group. On the basis of serologic behavior, we judge this to be IgM. Thus, guinea pigs sensitized with PnIIS in incomplete or complete Freund's adjuvant showed delayed reactivity in the absence of detectable immediate skin responses and, in 15 of 28 cases, in the absence of antibodies detectable by PCA or microcomplement fixation tests. The skin sites in both groups had the appearance of Jones-Mote reactions in that they showed con-

[2] Quantities as small as 20 μg were found to elicit responses; 10 μg were insufficient.

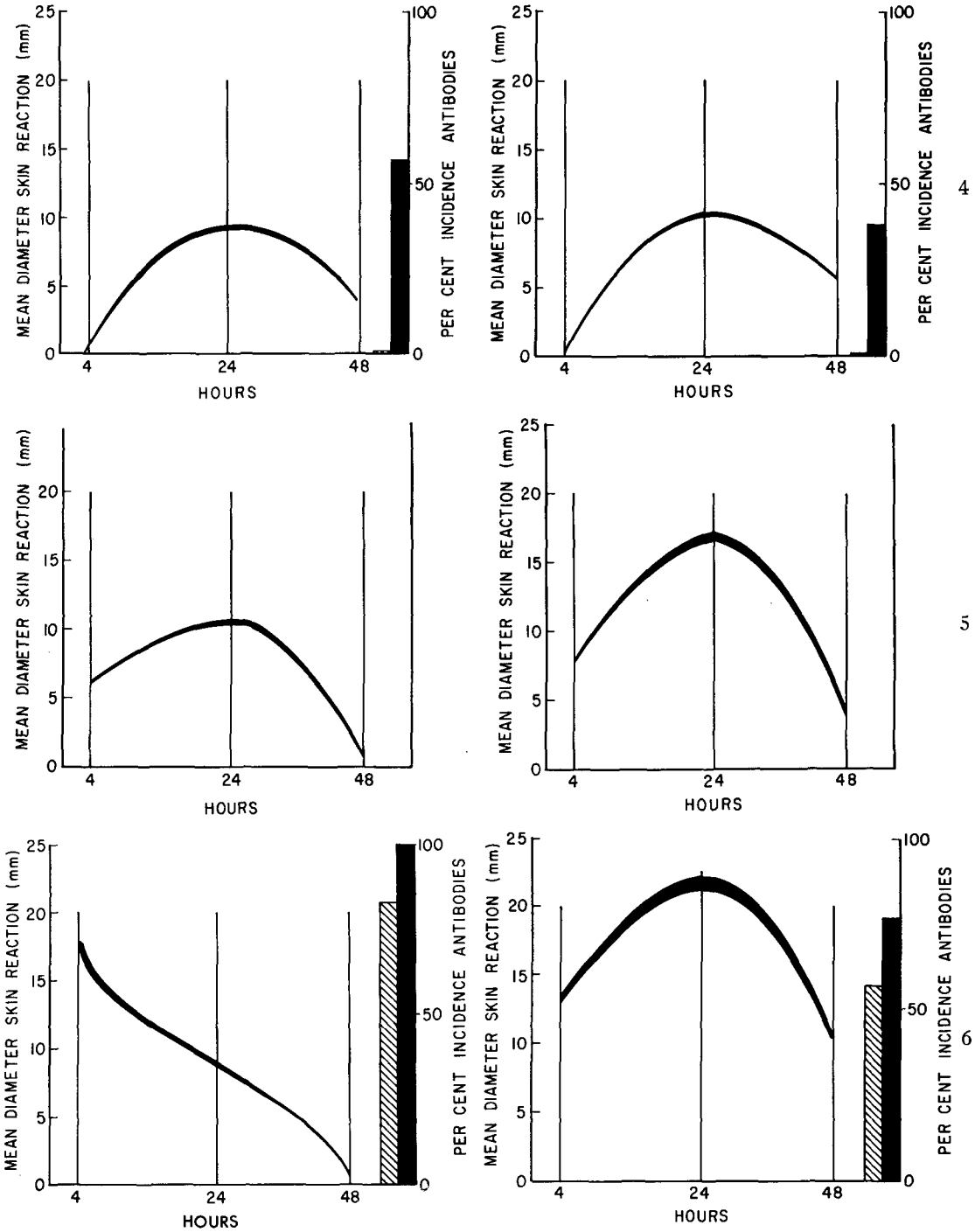

FIG. 1. PnIIS in water/oil emulsion—7 day reactions; hatched lines, antibodies determined by PCA ($\gamma 1$ and $\gamma 2$); solid bar, antibodies determined by C.F. ($\gamma 2$ or γM). FIG. 2. PnIIS in water/oil emulsion—14 day reactions. FIG. 3. PnIIS in water/oil emulsion—21 day reactions. FIG. 4. PnIIS in complete Freund's adjuvant —7 day reactions. FIG. 5. PnIIS in complete Freund's adjuvant—14 day reactions. FIG. 6. PnIIS in complete Freund's adjuvant—21 day reactions.

siderable erythema with very moderate induration, as indicated in Table I and by the thickness of the plotted lines in Figs. 1 and 4.

In the incomplete adjuvant group, 27 of the 33 animals showed these reactions at 24 hr, and 6 of these persisted to 48 hr. In the complete adjuvant group, 28 of the 34 animals reacted at 24 hr, but more (13/28) persisted to 48 hr. The incidence of antibodies detectable by microcomplement fixation test was somewhat lower in the complete adjuvant group. There is no suggestion that by the 7th day the presence of mycobacteria in the adjuvant mixture had intensified the immunologic response to this polysaccharide in any way.

By contrast, animals of both groups tested later, at 21 days after sensitization, developed reactions which differed markedly for the two groups (Table I, Figs. 3 and 6). Again, almost all animals in each group adhered to the pattern

TABLE II

Antibodies vs. PnIIS in Guinea Pigs Sensitized Subcutaneously with 100 μg in Incomplete or Complete Freund's Adjuvant

Days	Homol. PCA (γ_1)	Heterol. PCA (γ_1, γ_2)	C.F.* (γ_2, IgM)
Incomplete Adjuvant			
7	0/14	0/9	8/14
21	10/12	n.d.‡	12/12
Complete Adjuvant			
7	0/14	0/14	5/14
21	8/14	n.d.	10/13

Serum samples were taken before skin tests were applied.

* C.F., microcomplement fixation tests.

‡ n.d., not done.

shown by the average values. Early responses of Arthus type (at 4 hr) occurred in both groups (27/28 in incomplete; 25/27 in complete adjuvant) but at a somewhat higher level in those treated with the incomplete adjuvant. Again, there was a higher proportion of positive serologic responses in the incomplete adjuvant group (Table II, Fig. 3). A striking distinction between developing reactions in the two groups was seen in succeeding hours after test; whereas those in the incomplete adjuvant group fell off progressively to 24 hr and had disappeared by 48 hr, reactions in the complete adjuvant group increased in size and induration to 24 hr, and persisted, at a somewhat lower level, through 48 hr (in 24/27). These results are similar to those reported by us earlier (31) with protein antigen, and indicate that the mycobacterial component of complete adjuvant leads to a type of delayed reactivity to accompanying antigen which differs from the delayed responsiveness induced by the same antigen in the absence of this component of adjuvant. The latter (Jones-Mote) reactivity fails to persist beyond a rather brief period after a sensitizing injection;

depending upon the magnitude of the stimulus, it is followed by Arthus reactivity as seen here, or if the stimulus has been a small one, it may be succeeded by loss of all detectable dermal reactivity.

It might be inferred that the continuing and increasing delayed reactivity seen in animals sensitized with immunogen in complete mycobacterial adjuvant depends upon some kind of potentiation of the antigenic stimulus by the myco-

TABLE III

Skin Reactions in Guinea Pigs at 14 Days after Subcutaneous Sensitization with 100 μg of PnIIS in Incomplete or Complete Freund's Adjuvant and Tested with 100 μg of PnIIS

	Incomplete adjuvant			Complete adjuvant		
Type of reaction	4 hr	24 hr	48 hr	4 hr	24 hr	48 hr
Negative	0	0	0			
	0	1.5, 0.1	0			
Arthus*	5.1, 0	0	0			
	21.1, 1.6	1.5, 0.1	0			
Delayed	1.0, 0	7.5, 0	0	2.0, 0.1	12.5, 1.4	10.0, 0.5
	0	3.5, 0.1	0	3.0, 0.1	12.5, 0.4	5.0, 0.5
	1.0, 0	6.5, 0.1	0	2.0, 0	13.5, 0.4	0
	2.0, 0	13.5, 0.4	0	1.0, 0	11.5, 0.4	10.0, 0.2
	2.0, 0	19.5, 0.9	0	0	12.5, 0.4	7.0, 0.5
	2.0, 0	21.5, 1.1	0	4.0, 0.6	16.5, 1.4	10.0, 0.5
				4.0, 0.1	12.5, 0.4	8.0, 0.2
Arthus* and delayed	15.0, 1.6	17.5, 0.1	0	6.0, 0.6	14.5, 0.9	0
	14.0, 1.1	21.5, 1.9	7.0, 0	6.0, 0.1	18.5, 0.9	0
	12.0, 0.6	19.5, 1.4	5.0, 0	7.0, 0	18.5, 0.4	0
	13.0, 1.1	19.5, 1.9	0	11.0, 0.1	19.5, 1.4	0
				14.0, 1.6	21.5, 1.9	0
				16.0, 2.1	27.5, 2.4	12.0, 0.5
				16.0, 1.6	21.5, 1.9	0
				20.0, 2.1	22.5, 1.4	0

Notes as in Table I.

* Arthus reactivity at 4 hr arbitrarily considered to be 5 mm or greater.

bacteria, but such an inference is not supported by these results, for in the present case immediate (Arthus) reactivity was more striking at 21 days in the incomplete Freund's group, and there was a more general induction of antibodies in that group also.

The foregoing interpretation of unfolding events at 1 and 3 wk is supported by the results of tests done at 14 days after sensitization. Unlike the uniformity of profiles of developing reactions at 7 and 21 days, at this point after sensitization reaction types were mixed. The averages of reactions in the two groups

(Table I, Figs. 2 and 5) show the appearance of Arthus reactivity in both, and persistence of delayed (24 hr) reactivity in both also, the latter more marked in the complete Freund's group. However, animals showed marked individual differences in the incomplete adjuvant group (Table III). Of 14 guinea pigs, 2 had become nonreactive, 6 continued to respond with delayed (Jones-Mote) reactions, 4 showed combined Arthus and delayed reactions, and 2 had apparently lost the delayed component and responded with Arthus reactions only. In the complete Freund's group of 14 animals there was also some indication of "transitional" reactions, but in this group all animals had delayed responses, either of the 24 hr kind, or of the more persisting "tuberculin" type, with or without Arthus reactivity (Fig. 5). None showed the tendency to lose delayed reactivity. Thus, whereas animals in the incomplete adjuvant group were on their way toward losing (Jones-Mote) delayed reactivity while in some cases acquiring Arthus reactivity, the guinea pigs sensitized with polysaccharide in complete adjuvant were acquiring more intense delayed reactivity as well as Arthus reactivity. Observations at 21 days, already described, show these two trends closer to their end points.

Enzyme Treatment of PnIIS

As described under Methods, the PnIIS used for these studies showed itself in various tests to be antigenically homogeneous and of appropriate sugar composition, and its small content of N can apparently be ascribed to absorption of ammonia from the environment. However, in order further to obviate the possibility that some peptide might be residual in the sensitizing doses of polysaccharide used (100 μg of PnIIS could contain a maximum of 0.25 μg of peptide on the basis of N content), the polysaccharide was subjected sequentially to trypsin, pronase, carboxypeptidase A, carboxypeptidase B, and leucine aminopeptidase, for their possible influences upon immunogenicity and antigenicity.

15 mg of PnIIS were dissolved in 3 ml of phosphate-buffered saline, pH 8.2, containing 0.15 mg of trypsin, and allowed to stand for 24 hr at room temperature. Next, the same amount and volume of pronase was added, and the same period of incubation was allowed. After this, the mixture was heated at 82°C (water bath) for 20 min to inactive this enzyme, which had been found in preliminary tests to inactivate subsequently added enzymes. These were carboxypeptidase A, carboxypeptidase B, and leucine aminopeptidase, each added in the same quantities for the same incubation periods. After the final enzyme treatment the 15 ml of mixture was again heated as above. The same sequence of enzymes was tested in parallel against casein; chromatographic examination of the end products in this case indicated that complete digestion had taken place.

The treated polysaccharide was now tested for its capacities to induce and elicit reactions. For induction, 0.1 ml quantities of the preparation (containing 100 μg of treated PnIIS) was injected subcutaneously into 36 guinea pigs, 18 in incomplete and 18 in complete Freund's adjuvant. At the same time, 36 animals received the untreated polysaccharide in the same way. Skin tests were done at 7 or 21 days as usual, one test per animal. The test consisted of

100 μg of native PnIIS in one flank and the same amount of enzyme treated polysaccharide in the other flank of animals sensitized with native PnIIS. Those sensitized with enzyme treated material were tested only with native polysaccharide, in order to avoid complications introduced by possible sensitivity to the enzymes themselves.

TABLE IV

Skin Reactivities in Guinea Pigs Induced with 100 μg of Enzyme Treated PnIIS and Elicited with 100 μg of Native PnIIS

Days	4 hr	24 hr	48 hr
Incomplete adjuvant			
7*	0	13.0, 0.5	7.7, 0.03
21‡	13.3, 1.1	9.8, 0.3	1.0, 0.03
Complete adjuvant§			
7*	0	10.9, 0.6	2.8, 0.1
21‡	11.9, 1.2	16.8, 1.1	10.3, 0.45

Notes as in Table I.
* 9 animals.
‡ 8 animals.
§ 9 animals for each testing time.

TABLE V

Skin Reactivities in Guinea Pigs Induced with 100 μg of Native PnIIS, and Elicited with 100 μg of Native and Enzyme-Treated PnIIS

Days	Tested with	4 hr	24 hr	48 hr
Incomplete adjuvant				
7‡	PnIIS	0	11.2, 0.45	3.5, 0
	PnIIE*	0	7.5, 0.35	1.7, 0.05
21§	PnIIS	8.4, 0.65	6.8, 0.3	1.0, 0
	PnIIE*	8.8, 1.4	2.8, 0.1	0
Complete adjuvant				
7‡	PnIIS	0	7.3, 0.35	4.6, 0.03
	PnIIE*	0	3.0, 0.25	1.0, 0
21‖	PnIIS	8.7, 1.0	21.7, 1.3	12.0, 0.3
	PnIIE*	8.3, 0.75	19.5, 1.1	12.2, 0.3

Notes as in Table I.
* Enzyme treated.
‡ 8 animals.
§ 6 animals.
‖ 4 animals.

The results (Tables IV and V) show that the enzyme treatment did not significantly detract from the polysaccharide as a sensitizing or eliciting agent. Sensitizing activity is shown in Table IV; groups of guinea pigs sensitized with enzyme-treated PnIIS in incomplete or complete Freund's adjuvant were tested 7 or 21 days later with native polysaccharide. The chronologic response

curves were of the types and degrees seen in animals sensitized with native polysaccharide. The obverse experiment is shown in Table V, where sensitization was done with native polysaccharide, and the animals were simultaneously tested on opposite flanks with native and enzyme-treated material. Again, the responses were parallel in kind; some tendency for apparently smaller responses to the enzyme-treated material at 24 hr after testing may depend upon the fact that these values were arrived at by subtracting average readings made in 10 control animals tested at the same time. Whereas native polysaccharide never produced local irritation persisting for 24 hr, the enzyme treated material showed a degree of this.

Again related to the question of possible responses to a nonpolysaccharide contaminant of the PnIIS preparation, skin tests were made with PnIIIS at

TABLE VI

Macrophage Inhibition Tests, with PnIIS As Antigen*

Sensitization	Test after sensitization days	Number of tests	Average % inhibition‡
Normal controls	—	12	2
PnIIS, 100 μg in w/o§, subcutaneously	7	20	17.5
	21	8	9
PnIIS, 100 μg in Freund's adjuvant, subcutaneously	7	15	22
	21	13	32

* 25 μg PnIIS/ml medium.

‡ Compared with cells not exposed to antigen.

§ w/o, water/oil emulsion.

7 and 21 days in groups of guinea pigs sensitized with PnIIS in incomplete and complete adjuvant. The thought was that if a non–type-specific proteinaceous substance of the pneumococcus accounted for the reactions seen, this might accompany the polysaccharide obtained from an organism of another antigenic type. 11 animals tested at 7 days, and 9 at 21 days, responded well in the usual patterns to PnIIS, but showed no reactions above control levels to PnIIIS.

Macrophage Inhibition Tests

The basis for the reaction of inhibition of macrophage motility by appropriate antigen appears to be either a mediator of unknown nature produced by lymphoid cells (46) or a cytophilic antibody (47, 48). Despite the still cryptic significance of this reaction in respect to elucidating the nature of delayed reactivity, we think that it has been helpful for present purposes. The results shown in Table VI are recorded as average degrees of inhibition of migration of 24 hr packed cell preparations made from the peritoneal exudates of guinea

pigs sensitized in the manners indicated, which were the same as used for preparing animals for skin testing.

The first horizontal column shows that the concentration of PnIIS used had no influence on the behavior of cells of normal animals, as compared with cells in the absence of antigen. The remaining columns indicate average extents of inhibition of cells from variously sensitized animals at 7 and 21 days. At 7 days after sensitization the results are similar whether cells have come from animals given antigen in incomplete or complete Freund's adjuvant. At 21 days, however, there is a marked distinction between the two groups. Al-

TABLE VII

Transfer of Reactivities Induced by PnIIS in W/O and in Complete Freund's Adjuvant at 7, 14, and 21 Days after Sensitization*

Sensitization	7 days				14 days				21 days			
	Number of g. pigs	4 hr	24 hr	48 hr	No. g. pigs	4 hr	24 hr	48 hr	Number of g. pigs	4 hr	24 hr	48 hr
Cell transfers:												
PnIIS w/o	2	1.3,	2.5,	0	2	0,	2.5,	0	2	0,	0,	0
PnIIS CF	2	0,	9.0,	0	2	0,	5.0,	0	4	0,	9.7,	4.2
Serum transfers:												
PnIIS w/o	3	5.0,	0,	0	2	0,	0,	0	3	0,	0,	0
PnIIS CF	2	0,	0,	0	3	0,	0,	0	3	0,	0,	0
Actively sensitized:												
PnIIS w/o	4	0,	8.2,	0	3	0.3,	0,	0	3	8.0,	1.3,	1.7
PnIIS CF	3	0,	15.3,	7.3	2	0,	17.5,	5.0	4	10.3,	24.2,	16.0

Skin test readings—diameters in mm, with normal control readings subtracted.

* w/o, water/oil emulsion.

though as noted before, Arthus reactivity is just as apparent in the incomplete as in the complete adjuvant group, and the incidence of antibodies is higher in the former, the macrophages of these animals have lost to a marked degree the tendency to be hobbled by exposure to antigen. In contrast, the cells of the complete Freund's group show a significant increase of this effect.

Transfer of Reactivities by Cells and Serum

Transfers were carried out at 7, 14, and 21 days after the sensitizing injections with PnIIS in incomplete or complete Freund's adjuvant. Direct skin tests of some of these animals were done at the same time. The results, shown in Table VII, support those derived from preceding experiments in suggesting differences in pathways of response depending upon the presence or absence of mycobacteria accompanying the sensitizing dose of PnIIS.

All transfers were positive in both groups at 7 days and to some degree at 14 days. By 21 days, however, all four recipients of cells from donors sensitized

with antigen in mycobacterial adjuvant showed good delayed reactions at levels somewhat higher than at the earlier times after sensitization. In contrast, no significant reactivity was transferred to the three recipients from the water/oil emulsion sensitized group. This provides evidence again of increasing cellular reactivity in the mycobacterial adjuvant group at 3 wk, when that in the water-in-oil group had vanished. It bears repeating that this distinction occurs even though the latter animals show a higher incidence and titer of antibodies than the former at this time.

Serum in large quantities (20 ml per recipient) failed to transfer the 7 day or subsequent delayed reactivities from either group. It was surprising that the immediate (4 hr) reactivity so apparent by direct tests in the water-in-oil group at 21 days was not passively transferred. However, circulating antibodies, as indicated in a preceding section, seemed not to attain good concentrations in either group at best.

Histologic Observations

Skin test sites were examined after 4, 12, 24, and 48 hr, from guinea pigs sensitized with PnIIS in incomplete and complete Freund's adjuvants and tested at 7 and 21 days.

The sites from the incomplete adjuvant group at 7 days showed mononuclear cell infiltration restricted to the dermis; at 21 days, many of the cells appearing at the test site had developed into plasmacytes by 48 hr. By contrast, the 21 day reactions in the Freund's group revealed a larger component of small lymphocytes among the infiltrating mononuclears, epidermal invasion by such cells was common, and there were relatively few plasmacytes in the site even by 48 hr. These findings were consistent with those that we have described previously with another antigenic system (30, 32).

DISCUSSION

This report deals with two questions concerning delayed hypersensitive reactivity. One has to do with the role of polysaccharide immunogens as inducers and elicitors of such reactivity; the second with the question whether the delayed reactive state may be heterogeneous from the standpoint of reasons for occurrence, in one instance depending upon a cellular responsiveness which represents an early phase of antibody synthesis (termed Jones-Mote reactivity); in another, determined by a cell-bound effector of unknown kind, and revealing itself as the persisting reactivity of "tuberculin type."

It has become apparent from several recent studies that polysaccharides may act as elicitors of delayed hypersensitive reactivity when this has been induced by immunogen of which the sugar is a moiety. The evidence that a pure saccharide may be an inducer of the delayed reactive state is more restricted, although this has been shown to occur (e.g., 20). In the present case, the type specific polysaccharide of *Diplococcus pneumoniae* type II, freed of nitrogen that could be a constituent of peptides, was found to be an excellent inducer

of delayed hypersensitivity in random-bred guinea pigs, when administered in complete Freund's adjuvant. Its capacity to induce antibodies under these circumstances was very modest, as judged by homologous and heterologous PCA tests and microcomplement fixation tests.

This immunogen was then found to lend itself well to a study of the second point; the existence of Jones-Mote reactivity as a distinct type of cellular hypersensitivity, different from that of tuberculin type. It was soon seen that administration of PnIIS without the mycobacterial component of Freund's adjuvant led to the early (7 day) advent of delayed reactivity which manifested itself by flat erythematous reactions with minor induration, reaching a maximum at 24 hr and usually disappearing by 48 hr, and with histologic characteristics somewhat different from those of reactions of the tuberculin type (30, 32). This reactive state, as has been described before (30–32) tended to disappear by 2 or 3 wk, to be succeeded by Arthus reactivity.

Skin tests at 21 days revealed striking gross and histologic differences in the reactions elicited in the two groups. These differences were seen also in macrophage inhibition tests, which showed, at 21 days, a disappearance of cellular reactivity from the Jones-Mote group, and an intensification of this reactivity in the complete adjuvant-sensitized group with developing reactivity of tuberculin type. Analogously, attempted transfers of reactivities by serum and cells from animals of the two groups at 7 and 21 days revealed that by the latter time, only cells from the complete Freund's group successfully transmitted reactivity, despite evidence that both groups had received an equally intense stimulus as judged by occurrence of antibodies and Arthus reactivity.

SUMMARY

A highly purified pneumococcal polysaccharide (Type II SSS) is a very efficient inducer of delayed hypersensitivity in random-bred guinea pigs. The cellular reactivity induced by this polysaccharide administered subcutaneously in complete Freund's adjuvant is of "tuberculin type"; it increases in intensity with time after the sensitizing injection, as judged by skin tests, the macrophage inhibition reaction and transfer of reactivity by peritoneal exudate cells.

By contrast, the cellular reactivity induced by this immunogen in the absence of mycobacterial adjuvant has the characteristics of "Jones-Mote" reactivity. It is best seen at about 1 wk after sensitization; the reactions are characteristically little indurated and show histologic differences from tuberculin type responses; and the reactive state begins to disappear by 2–3 wk, with the accession of Arthus reactivity. This type of delayed reactivity may be related to an early phase of antibody synthesis.

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