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Evidence of Breakdown of Pneumococcal Polysaccharides *in Vivo* (40659)[1]

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Polysaccharides are being increasingly employed as vaccines for the prevention of serious bacterial infections because of their low toxicity and good immunogenicity in man. Pneumococcal polysaccharides are among the recent additions to this armamentarium. There is still much to be learned about the polysaccharides as immunogens, however, including the duration of the immunity they afford and the eventual metabolic fate of the antigens. Pneumococcal capsular polysaccharides (PCP) are known to be resistant to degradation *in vivo*. This resistance is evident from their prolonged persistence in reticuloendothelial tissues in studies of immunological tolerance (1). The persistence of polysaccharide in animals injected with large doses of PCP in tolerance studies, however, does not preclude the possibility that these antigens are slowly degraded *in vivo*. In an analogous situation, Janeway and Humphrey (2) have observed that "indigestible" D-amino acid polypeptides are metabolized in mice, but 22 times more slowly than the corresponding L-amino acid polypeptides. In the present studies, high-molecular-weight type 1 or type 8 PCP were injected into rats, and evidence of their breakdown was obtained by identifying low-molecular-weight derivatives of the antigens in the urine.

Materials and methods. Adult, male, Sprague–Dawley rats weighing an average of 400 g were used. Rats were fed 5% sucrose water for 2 h, after which they were placed in metabolic cages for 6 hr for collection of urine. Serum was obtained by bleeding from the tail vein.

Type 1 and type 8 PCP for injection (Lots 812632 and 812643F, respectively) were obtained from Eli–Lilly & Company, Indianapolis, Indiana. The antigens contained less than 2.5% contaminating protein, nucleic acid and C-substance.

Chromatography of PCP was performed with a 0.5-ml sample size on Sephadex G-200 with a 0.9 × 60-cm column equilibrated with 0.1 *M* Tris (Trishydroxymethylaminomethane)-HCL buffer (pH 8.0) and 0.02% Na azide. The void volume was measured with blue dextran (2×10^6 mean M_r), and columns were calibrated with dextrans T20 (M_r 20,000), T40 (M_r 40,000), and T70 (M_r 70,000) (Pharmacia, Inc., Piscataway, N.J.). Eluate fractions of 0.5 ml were collected at a flow rate of 4.5 ml/hr, and dextran in the eluate was determined by the method of Mokrasch (3). PCP in eluate fractions was detected by counterimmunoelectrophoresis (CIE), as described elsewhere (4). The sensitivity of this assay is 0.1 μg/ml. Type-specific rabbit antipneumococcal sera (Statens Seruminstitut, Copenhagen, Denmark) was used throughout. Precipitin lines were developed for 24–48 hr at 5°.

For gel-precipitin analysis of PCP, solutions containing antigen were dialyzed against distilled water at 5° in cellulose bags (retention size > 12,000 M_r, Arthur H. Thomas Co., Philadelphia, Pa.). Samples were concentrated 5- to 40-fold by lyophilization and reconstituted in normal saline. Double diffusion was carried out with plates coated with 1% agarose in barbital–barbital-sodium buffer (pH 8.2). Precipitin reactions were developed at room temperature and read at 72 hr. Immunoelectrophoresis was performed for 1.5 hr under the same electrical conditions as were used for CIE. Antiserum was added to troughs cut equidistant between the wells, and precipitin reactions were developed for 24–48 hr.

PCP, IgG, and IgM in rat kidney were evaluated by indirect immunofluorescence, as described in detail elsewhere (5). Briefly, tissue was quick frozen and sections of 4–5 μm were cut on a cryostat. Optimal dilutions of rabbit or goat antisera (Cappel Laboratories, Cochranville, Pa.) were applied for 15

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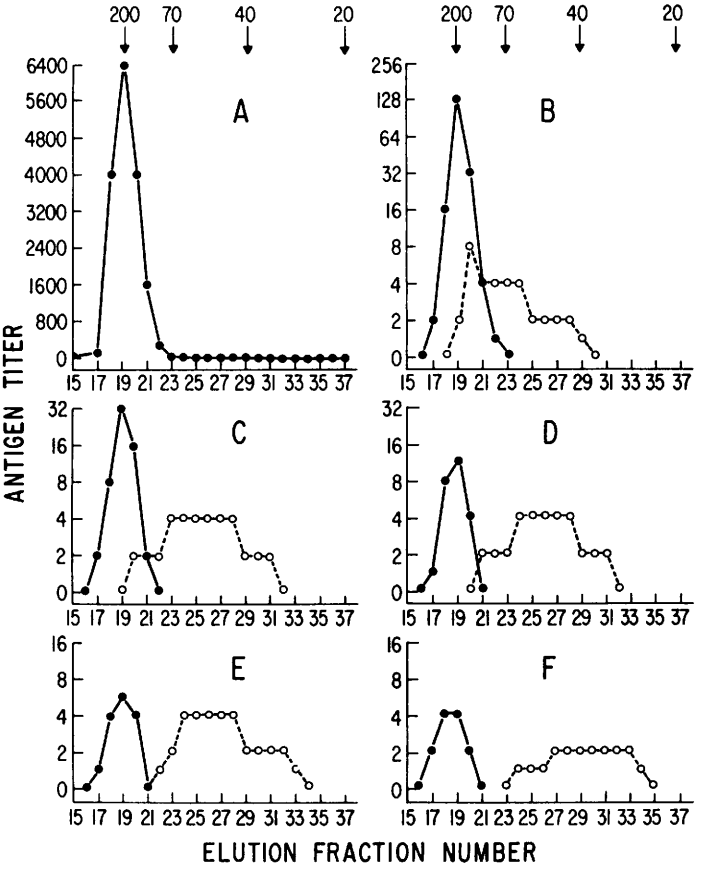

FIG. 1. Chromatograph of type 8 PCP on G-200 before injection (A) into rats and in serum (●) or urine (○) at 3 (B), 6 (C), 14 (D), 21 (E), and 35 (F) days after injection.

min at room temperature, followed by three washings of 15 min each with phosphate-buffered saline. Controls included the use of antipneumococcal sera of a heterologous type, normal goat, or rabbit sera, and the use of fluoresceinated antisera alone. Normal tissue controls were also employed.

Results. Type 8 PCP vaccine for use in rats was chromatographed on Sephadex G-200 in order to free it of low-molecular-weight polysaccharide. In this procedure, 1.0 mg of PCP was dissolved in 0.25 ml of normal saline and applied to a precalibrated column. Eluate fractions of 0.5 ml were collected and the antigen titer was determined by CIE. The bulk of the antigen eluted in the void volume of the column. Fractions representing high-molecular-weight PCP (≥70,000), as indicated by column markers, were pooled, lyophilized, and redissolved in 0.25 ml of sterile water. The pooled fractions were chromatographed a second time on Sephadex G-200. A typical pattern from this elution is indicated in Fig. 1A. The appropriate fractions were again pooled and lyophilized. The pooled antigen was dialyzed against phosphate-buffered saline (pH 7.2) and brought to a volume of 1.0 ml for injection. Titration by CIE of preparations of doubly chromatographed PCP indicated that ~80% of the initial PCP was recovered as high-molecular-weight antigen for injection.

Each of four rats were injected i.p. with ~800 μg of doubly fractionated type 8 PCP. Serum and urine were collected serially and chromatographed on Sephadex G-200 to measure molecular weight. Results were essentially identical for all animals. Data for a representative animal are depicted in Fig. 1. The mean molecular weight of the doubly fractionated PCP before injection was ≥200,000, with no detectable antigen of

<70,000 (Fig. 1A). Chromatographs of PCP in urine collected at 72 hr after injection (Fig. 1B) revealed small amounts of PCP in the molecular weight range of 40,000–70,000. The chromatograph of PCP in urine at 72 hr overlapped appreciably with the chromatograph of the PCP in the serum sample taken at 72 hr. There was progressively less overlap between chromatographs of serum and urine PCP in samples taken at subsequent intervals (Fig. 1C–1F). Serial samples of urine obtained over a 35-day period consistently contained PCP of <70,000 M_r. In contrast, PCP in the serum throughout this period was >200,000 M_r. Immunologic analysis of type 8 PCP in the urine showed that it differed from doubly fractionated type 8 PCP vaccine. In double immunodiffusion in agarose, urine PCP gave a reaction of partial identity with the vaccine (Fig. 2). In immunoelectrophoresis (Fig. 3), urine PCP displayed more rapid anodal migration than did vaccine PCP. PCP in serum obtained at 6, 14, and 21 days after injection was immunologically identical with vaccine PCP in double-immunodiffusion studies.

Additional studies were carried out using type 1 PCP rather than type 8 PCP. The antigen was doubly chromatographed on Sephadex G-200, after which it had a mean M_r of ≥200,000 and contained no antigen of <70,000 M_r. Each of six rats were injected i.p. with ~1200 μg. Results of chromatographs of the serum and urine of these animals are indicated in Table I. After injection, PCP appeared very promptly in the urine (as early as 3 hr in one spot check) where it persisted during 21 days of observation. Early

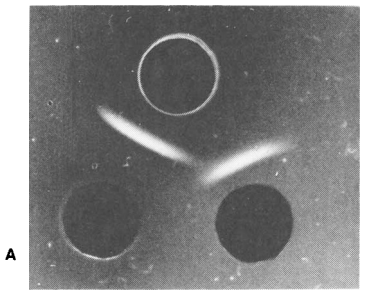

FIG. 2. Comparison of PCP in rat urine (A) and vaccine PCP (B) by use of double immunodiffusion. Urine PCP was obtained from a rat injected 12 days earlier with type 8 vaccine. The upper well was filled with type 8 rabbit antipneumococcal antibody.

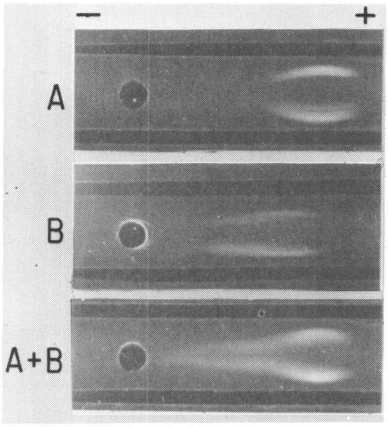

FIG. 3. Immunoelectrophoresis of rat urine PCP (A), vaccine PCP (B), or a mixture of urine and vaccine PCP (A+B). Urine PCP was obtained from a rat injected 12 days earlier with type 8 vaccine. Troughs were filled with type 8 antibody.

TABLE I. MOLECULAR SIZE OF PCP IN THE SERUM AND URINE OF SIX RATS INJECTED WITH ~1200 μg TYPE 1 PCP

Days	No. rats studied	Body fluid	Range of molecular size of PCP: ≥200,000 to 70,000	70,000 to 40,000	40,000 to 20,000
3	2	Serum	X		
		Urine	X	X	X
7	1	Serum	X		
		Urine	X	X	X
13	2	Serum	X		
		Urine		X	X
21	1	Serum	X		
		Urine		X	X

samples of urine contained PCP >70,000 M_r, but this relatively high-molecular-weight antigen disappeared from the urine by 13 days. As was observed with type 8 antigen, the urine of the injected animals consistently contained antigen of 20,000–70,000 M_r. Type 1 PCP in the serum was of high-mean-molecular weight (≥200,000) with no PCP of <70,000.

In a third study, rats were injected with 1500 μg of type 1 PCP, and renal tissue was evaluated with indirect immunofluorescence to determine the levels of PCP in the tissue over a 49-day period. Results are listed in Table II. Aggregates of polysaccharide were detected principally in the glomerular mesangium and to a lesser extent in renal capil-

TABLE II. RESULTS OF TESTS FOR ANTIGEN IN 20 RATS INJECTED WITH 1500 μg OF TYPE 1 PCP

Days	No. rats	No. with positive renal fluorescence			No. with PCP in urine	Serum PCP (μg/ml)
		PCP	IgM	IgG		
0	2	0	0	0	0	<0.1
1	2	2	0	0	2	128
2	2	2	0	0	2	80
3	2	2	1	0	2	68
6	2	2	1	0	2	24
10	3	3	1	0	3	—
14	2	2	0	0	2	4
28	3	1	0	0	3	2
49	2	0	0	0	0	1

lary endothelium. The amount of PCP in the kidneys increased during the first several days after injection and then declined. By 28 days, only one of three rats had detectable type 1 PCP in the kidneys, and two rats examined at 49 days had no PCP. The decrease in detectable PCP was not due to masking by specific antibody produced by the animals, because neither IgM nor IgG could be detected in sections of kidney obtained after 14 days.

Discussion. The present studies indicate that when high-molecular-weight PCP is injected i.p. into rats, antigen is excreted promptly in the urine. Some of this excreted antigen is of high molecular weight (i.e., >70,000), and because of its very rapid appearance in the urine probably represents PCP which passes unaltered through the kidneys. It has been reported that type 3 PCP with a mean M_r of 150,000 is excreted readily in the urine of mice after i.p. injection (6). Type 8 PCP (and probably type 1 PCP) resembles type 3 PCP in that it is a simple, unbranched molecular structure; this fact may explain why large molecules of the antigen are readily excreted by the kidney (6, 7).

Low-molecular-weight PCP (<70,000) is also present in the urine soon after injection of vaccine PCP, and this low-molecular-weight antigen remains detectable long after the larger-molecular-weight PCP has disappeared from the urine. It is possible, although unlikely, that this antigen originated as a trace contaminant in the injected PCP. If this were true one might expect that all of the low-molecular-weight PCP would have been rapidly excreted, but low-molecular-weight PCP was excreted for weeks after the higher-molecular-weight PCP (which certainly constituted the bulk of the injected material) had disappeared from the urine. Also, type 8 PCP in the urine showed only partial identity with vaccine PCP, and urine PCP showed significant differences from vaccine PCP in immunoelectrophoresis. Taken together, these observations strongly suggest that low-molecular-weight PCP in the urine is a degradation product of the injected antigen.

The conclusions from the present study are consistent with observations by Jones *et al.* (6) who found that mice injected with ^{125}I-labeled tyramine–type 3-polysaccharide excreted small amounts of ^{125}I-labeled tyramine–glucose in the urine, and the ^{125}I-labeled polysaccharide that was excreted in the urine did not bind type 3 antibody as well as the original antigen. These findings were interpreted by the authors as evidence of degradation.

Several investigators have documented that injected PCP gradually disappears from animal tissues. Stark (8, 9), for example, noted that there was a marked decrease with time in quantity of antigenic ^{14}C-type 1 PCP in the spleens of mice. Kaplan and Coons, who used immunofluorescence techniques (10), showed a gradual decrease in detectable PCP in tissues of mice injected with types 2 or 3 antigens. The present studies with immunofluorescence confirm their observations and indicate that the decline in detectable PCP is not due to masking by endogenously produced antibody. The findings offer further support for the concept that there is gradual degradation of PCP *in vivo*.

If the present observations are applicable to man, one would expect that pneumococcal polysaccharides would be subject to breakdown in vaccinated subjects. Because the immune response to PCP may depend on stim-

ulation of B lymphocytes by persisting polysaccharide (1), degradation of the antigens may be associated with waning of immunity. The stability of polysaccharide antigens *in vivo* appears to be an additional factor to consider in assessing polysaccharide vaccines.

Summary. Type-specific PCP vaccines (types 1 and 8) were fractionated twice on Sephadex G-200 to eliminate antigen of <70,000 M_r and the vaccines were injected i.p. into rats. PCP of <70,000 M_r was excreted in the urine within 72 hr after injection, and within 1 week most of the PCP in the urine was <70,000 M_r. PCP in the urine had increased electrophoretic mobility and showed only partial immunologic identity in comparison with the injected PCP. Deposits of PCP in the renal mesangium and endothelium declined with time and were eventually eliminated from the tissue. These data indicate that types 1 and 8 PCP are slowly degraded in rats.

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