

ANTIBODY FORMATION IN VOLUNTEERS FOLLOWING
INJECTION OF PNEUMOCOCCI OR THEIR
TYPE-SPECIFIC POLYSACCHARIDES*

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In previous studies on the immunization of human beings with type-specific polysaccharides of pneumococcus (1, 2) the serological evidence of an immune response has been based exclusively on relative methods such as mouse protection or the agglutinin or precipitin titers of sera of the vaccinated individuals, owing to the lack of any method suitable for estimating the very small quantities of antibody ordinarily present in human sera. Recently, however, modifications have been introduced by which quantitative absolute methods for the estimation of antibody nitrogen (3, 4) were made applicable to amounts as small as a few micrograms (5, 6). Essential changes were the use of a more sensitive method for measuring antibody protein, allowance of sufficient time for small quantities of specific precipitates to separate from the viscous serum medium, and use of relatively much serum (about 4 ml. per determination) when necessary. The present paper deals with data obtained by the new method with the sera of (a) a group of medical students injected with Type I and Type II pneumococci, (b) three groups injected with small amounts of Type I and Type II specific polysaccharides, and (c) a group injected with Types I, II, and V specific polysaccharides. A comparison of the results with the mouse-protective titers of representative sera is given in the following paper (7).

As the study of the initial prevaccination sera progressed it was found that few contained more than traces, at most, of antibodies to the type-specific polysaccharides of Types I, II, III, IV, and V pneumococci. When the sera were tested with Type VII polysaccharide, however, most of them reacted, many quite strongly. On closer scrutiny it developed that the sample of Type VII polysaccharide used contained roughly one-third of its weight of the C substance of pneumococcus (8) and that the greater portion of the precipitates obtained with the crude material was due to the reaction of the C substance with anti-C present in almost all of the sera.

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Owing to the widespread occurrence of antibodies reactive with C substance in normal sera it became necessary to remove these prior to estimation of the type-specific response due to vaccination, since almost all samples of type-specific polysaccharides contain C substance as an impurity and analytical results would otherwise tend to be too high. This must be borne in mind in any evaluation of the earlier portions of the new data, which were obtained before the necessity of a preliminary absorption with C substance became evident.

Materials, Subjects, and Methods

Types I and II Pneumococci.—Formalized young cultures in broth were centrifuged, washed with saline and twice with acetone (9), and dried *in vacuo*. The bacteria of each type were resuspended as evenly as possible in sterile saline and decanted from large particles. The cell count was about 100×10^9 per ml. After removal of aliquot portions for estimation of the polysaccharide content of the vaccines, 1 per cent by volume of 1 per cent merthiolate¹ was added. 2.0 ml. aliquot portions of each vaccine were incubated at 37°C. in closed 25 ml. flasks with 3 ml. of saline and 2.6 ml. of N NaOH. After 3 days the clear solutions were neutralized with N HCl and made up to 25 ml. with saline and water so that the final solutions would be approximately isotonic. Aliquot portions of each were added to measured quantities of homologous antipneumococcus horse serum which had been calibrated (10) with similarly treated specific polysaccharide, the amounts chosen being such that antibody remained in excess. From the quantity of nitrogen precipitated the amount of specific polysaccharide in the aliquot could be read from the calibration curve of the serum (10), and the quantity per milliliter of vaccine calculated. In this way it was found that 10 billion of the Type I pneumococci, the dose of vaccine given to group 1, contained 0.03 mg. S I² while 10 billion of the Type II pneumococci contained 0.04 mg. S II.³

Specific Polysaccharides of Pneumococcus.—The type-specific polysaccharides used were prepared essentially as already described (11), after preliminary ultrafiltration (12), except those used in group 5 which were isolated by the phenol method (13). Samples of C substance (8) were derived either from pneumococcus types other than those used for immunization or from pneumococci in the R (Dawson S) phase. Since blood group A substance originating in the culture media (14) might tend to concentrate in the relatively crude C fraction two samples were tested for A activity by the method of inhibition of hemagglutination.⁴ One sample was roughly one-tenth as active as a purified A substance, the other was entirely inactive. Since the amounts of C substance used for absorption were far below the order of magnitude required for the precipitation of antibody by A substance (15), and since the amounts of anti-C found appeared unrelated to the blood group of the subject, it is probable that the anti-C values given in the tables are rarely augmented by precipitation of anti-A.

Subjects.—Medical student volunteers, with no previous history of allergy, were studied. The writers are greatly indebted to these men and women, who submitted cheerfully to the injections and to the inconveniences of repeated bleedings despite the rigors of an accelerated medical program. Hearty thanks are also due to the many members of the staff of the Presbyterian Hospital who assisted with the injections and bleedings.

Many of the injections were followed by local reactions, of which few were severe. Several general reactions were also encountered.

Group 1, 13 subjects: Received two intracutaneous injections, 3 days apart, totalling 10 billion each Type I and Type II pneumococci containing 0.03 mg. S I² and 0.04 mg. S II².

¹ Manufactured by Eli Lilly & Company, Indianapolis, Indiana.

² S I = specific polysaccharide of Type I pneumococcus, S II = specific polysaccharide of Type II pneumococcus, etc.

³ The tests were kindly carried out by Dr. Elvin A. Kabat.

Group 2, 13 subjects: Received two intracutaneous injections, 5 days apart, of the washed copper precipitate from a solution of S I and S II. It is doubtful whether the entire amounts intended, 0.03 mg. S I and 0.04 mg. S II for comparison in particulate form with the vaccine, were actually injected, as the gummy material tended to stick in the syringes.

Group 3, 11 subjects: Received two intracutaneous injections, 6 days apart, of polysaccharide solution, totalling 0.05 mg. each of S I and S II.

Group 4, 9 subjects: Were treated like those of group 3, save that the polysaccharides were injected subcutaneously in two doses, 8 days apart.

Group 5, 20 subjects: Received 0.06 mg. each of S I, II, and V, injected subcutaneously in two doses, 3 days apart.

Analysis of Sera.—The first analyses of group 1 and group 2 sera were made with the aid of the micro-Kjeldahl method (3, 4), but use of this method was discontinued because the quantities of antibody nitrogen found were usually little greater than the analytical error involved. Most of the analyses were accordingly carried out by application of a modified Folin-Ciocalteu method (5, 6).

All analyses were run under conditions favoring sterility, owing to the long period required, but 1 per cent by volume of 1 per cent merthiolate¹ was added to the materials as an additional precaution. In order to avoid the uncertainties due to human complement (17), this was first removed from the sera by admixture of triplicate 3 to 4.5 ml. portions with 1 ml. of a dilution of rabbit anti-egg albumin serum containing about 0.4 mg. of antibody nitrogen and 1 ml. of a 0.9 per cent saline solution of crystalline egg albumin (Ea, containing about 0.04 mg. of Ea N). This was found preferable to heat inactivation, which usually destroyed at least a portion of the antibody in sera of low content. The Ea-anti-Ea precipitations were carried out at room temperature and allowed to stand until the precipitates flocculated (18), after which the tubes were left in the ice box for 48 hours and centrifuged in the cold.

Aliquot portions of the supernatants were set up as follows, in sterile, tapered, Pyrex centrifuge tubes of about 7.5 ml. capacity:—Three portions were measured out, one serving as a blank. To the other two, depending upon the intensity of a preliminary test with 1 ml. of serum, 0.005 to 0.02 mg. of pneumococcus C substance was added in a volume of 0.1 or 0.2 ml. The contents of the tubes were thoroughly mixed with a thin sterile glass rod or by rapid twirling. After capping with sterile rubber caps the tubes were placed in a water-bath at 37°C. for one-half to one hour and were then allowed to stand 8 days in the ice box, with occasional twirling. Precipitates often formed overnight in the strongest sera, but most of the weaker sera remained clear and required several days before the first small flocules separated. When precipitates formed in 24 to 48 hours after addition of only 0.005 or 0.01 mg. of polysaccharide repeated analyses were often avoided by adding an additional 0.01 mg., mixing as before, and allowing the tubes to stand for 8 more days. The tubes were finally centrifuged in the cold. A second absorption with 0.005 to 0.01 mg. of C substance was usually carried out on the supernatants, especially when relatively large precipitates had formed. The precipitates and blanks were washed in the cold in the usual way (3, 4) with chilled saline, stirring well, or twirling vigorously to ensure maximum disintegration of the precipitate and thorough mixing. When a stirring rod was used, it was rinsed with a little chilled saline. Supernatants from the washings were often recentrifuged in order to recover any precipitate carried over, and usually the washings from duplicates were combined in larger centrifuge tubes. A third washing was usually given all tubes. Precipitates were dissolved in a few drops of 0.1 N sodium hydroxide, using a hand lens if necessary to make sure that the sometimes glassy precipitate was completely dissolved. The solution of the traces of precipitate recovered on recentrifugation was divided approximately equally between the two duplicates. The volumes were then made up with water to a calibration mark (usually 2.5 ml.) in the tubes, or the solutions were transferred to 5.0 or 10.0 ml. measuring flasks when the precipitates were relatively heavy. Two ml. portions of the duplicate solu-

tions of dissolved precipitate were analyzed for nitrogen according to reference 5, with use of the Folin-Ciocalteu reagent and copper, and measurement of the blue color against that of the blank in a Coleman spectrophotometer. One hundredth to 0.04 mg. (10 to 40 micrograms) of antibody nitrogen in the aliquot proved most satisfactory, and in this range, successive determinations usually varied by only a few thousandths of a milligram. The tyrosine color value of the antibody was checked by direct comparison of the colorimetric with the micro-Kjeldahl method.

The supernatant sera from the anti-C analyses, including the blank, were carefully drained into sterile centrifuge tubes, such as those suggested above. To the duplicates from the anti-C estimations were then added amounts of type-specific carbohydrate, depending upon the result of a preliminary test with the supernatant of the 1 ml. of serum used in the previous preliminary test. Usually, 0.005 to 0.05 mg. sufficed. The analyses were carried out in the same manner as those for anti-C and supernatants were treated with more specific polysaccharide if the precipitates were relatively large. In occasional sera precipitates formed almost as quickly as in hyperimmune rabbit sera and analyses could be carried out by the micro-Kjeldahl method, or as described above with as little as 1 ml. of serum. Analyses were carried out with S I and S II successively, in either order, and in the sera of group 5, with S V, as well.

Although this procedure is laborious and time-consuming it is accurate,⁴ yields reproducible values, and is well adapted to the analysis of large numbers of sera, as the work may be planned so that analyses on sets of four to six sera become due each day.

In the earlier analyses S II was added after removal of the complement, and the analysis with S I was run last. In a number of instances, analyses were run in the reverse order as well. Examples are given in the tables, and it will be noted that the order of analysis was immaterial. After removal of anti-C became routine, the order of analysis was usually with C, S I, S II, and S V.

Occasional sera, for reasons which are not yet apparent, required repeated absorptions with small quantities of C or S of one type or another for removal of all of the antibody in question. A single addition of a larger quantity of polysaccharide did not suffice.

Numerous comparisons were made of heat inactivation and precipitation with Ea-anti-Ea for removal of the complement in the sera. In all instances except that of 78, (Table III, footnote) heat inactivation resulted in marked reduction in the amount of precipitable antibody, possibly because of more or less complete formation of non-precipitating complexes of antibody with albumin such as those noted on heating antisera to 75°C. (19). As most of these analyses were run before anti-C was estimated, only a later comparison, equally typical, is given in the protocol.

Pooled Sera of Various Bleedings

Antibody estimated	Micrograms antibody N per 4 ml.		
	Complement removed by		
	Ea-anti-Ea	Heat, 56°C., 30 min.	Heat, 56°C., 50 min.
Anti-C.....	36	2	0
Anti-I.....	8	3	0
Anti-II.....	10	2	0

In another pool anti-C was reduced from 14 to 6 and 0, respectively; in a third, from 57 to 30 and 22.

⁴ Cf. comparisons with the micro-Kjeldahl method in Tables II and III. Also, 85 to 95 per cent of antibody added to normal serum was recovered analytically (unpublished experiments).

TABLE I
Comparison of Antibody Levels in Human Beings after Intracutaneous Injections of Types I and II Pneumococci or Their Specific Polysaccharides

Subject No.	Anti-bodies to	Micrograms antibody N per 4 ml. serum										
		Bleeding No.										
		0* Before	1 3 wks.†	2 6 wks.	3 3 mos.	4 5 mos.	5 6 mos.‡	6 12 mos.§	7 14 mos.	8 2 yrs.**	9 3 to 4 mos. later	
Group 1	8	C									23	29
		I	0	1	0	1	2	1	3	2	2	0
		II	0	+	+	7	7	8	3	5	3	7
		15	C							28	14	20
		I	0	82	91	64	61	60	38	44	30	33
		II	0	39	56	44	41	34	25	21	11	18
		19	C							16	29	29
		I	0	29	24	24	25	22	15	8	4	11
		II	0	142	144	118	100	88	<57††	57	43	49
				2½ mos.	4 mos.	5 mos.‡	10½ mos.§	12½ mos.	23 mos.**	3 to 4 mos. later		
Group 2	32	C						47	57	52		
		I	0	0	4	6	3	1	4	3		
		II	6	3	3	3	7	3	1	5		
		29	C					24	44	37		
		I	36	40	31	30	27	14	13	11		
		II	22	27	21	25	25	18	<14	21		
		31	C					26	70	62		
		I	29	19	7	12	30††	4	8	5		
		II	51	43	33	30	9††	7	6	13		

Initial values for group 1 in part by micro-Kjeldahl method; data for first 4 bleedings of sera 8, 32 probably too low, owing to use of too much S I and S II. No anti-II was found in 32; after heat inactivation. In 15, 2 years after last analysis above, anti-I, 17.

* Before vaccination.

† After second injection.

‡ 2½ to 4 weeks after skin-testing with 0.01 mg. each S I and S II. Positive tests were irregular.

§ Followed by reinjection, subcutaneously, of 0.025 to 0.05 mg. each S I and S II.

|| Two months after reinjection.

** 10 months after reinjection. Again injected subcutaneously with 0.04 mg. of each polysaccharide.

†† Values out of line, reason unknown.

‡‡ Original value 33. Repeated after 2.5 years, <57. Two absorptions were carried out; a third was indicated.

As shown by comparative analyses on 78₄, with and without 0.5 per cent phenol, this preservative can be used even though the final nitrogen estimations are made with the Folin-

TABLE II
Comparison of Antibody Levels in Human Beings after Subcutaneous or Intracutaneous Injections of Specific Polysaccharides of Types I and II Pneumococcus

Subject No.	Anti- bodies to	Micrograms antibody N per 4 ml. serum						
		Bleeding No.						
		0 Before	1 3 to 4 wks.	2 2½ mos.	3 8 mos.*	4 10 mos.	5 20-23 mos.†	6 1 to 3 mos. later
Group 3 42	C					40		
	I	0	1	3	0	0		
	II	0	8	10	9	3		
43	I	0	37	37	26‡			
	II	2	<77	103	58‡			
38	C				61	67	92	
	I	0	17	24	4	5	5	
	II	7	121	142	132	137	15‡‡	
Group 4 51	C					25	16	21
	I	0	9	11	20	3	0	3
	II	0	10	10	6	2	2	6
56	C					48		
	I	11	106§	<154	149	101		
	II	0	(17)	16	16	8		
53	C					18**	12	37
	I	0	20	21	30	7**	5	10
	II	0	247††	312††	251††	237**	140	155

* Followed by reinjection, subcutaneously, of 0.025 mg. each S I and S II.

† Followed by subcutaneous reinjection of 0.04 mg. each S I and S II.

‡ Repetition gave anti-C, 44; anti-I, 13; anti-II, 54; anti-VII, 11.

§ By micro-Kjeldahl.

|| Colorimetric analysis 1.5 years later gave anti-C 20; anti-I, 104; anti-II, 13.

** A sample from which complement was removed by heat inactivation instead of by Ea-anti-Ea gave anti-C, 18; anti-I, 5; anti-II, 187.

†† Anti-II N for bleedings 1, 2, and 3 by micro-Kjeldahl, 268, 305, and 244 respectively. After 1.5 years a colorimetric analysis of bleeding 3 showed anti-C, 23; anti-I, 7; anti-II, 199.

‡‡ Anti-VII in bleedings 3, 4, and 5: 2, 11, and 2, respectively.

Ciocalteu phenol reagent. The washings recommended suffice for quantitative removal of phenol as well as of non-specific proteins. This applies equally to merthiolate,¹ which also gives a blue color with the phenol reagent.

TABLE III
Antibody Levels in Human Beings after Subcutaneous Injections of Specific Polysaccharides of Types I, II, and V Pneumococcus

Subject No.	Anti-bodies to	Micrograms antibody N per 4 ml. serum						
		Bleeding No.						
		0* Before	1 2 wks.†	2 6 wks.	3 5½ mos.‡	4 8 mos.	5 15½ mos.‡	6 4-5 mos. later
61	C				49	45	63	77
	I	0	0	1	1	10	0	2
	II	0	0	0	11	1	1	0
62	V	0	0	0	8	2		
	C					9	7	12
	I	0	54§	(56)		50	19	18
63	II	0	81	79		45	25	23
	V	0	10	10		7		
	C					18	18	29
64	I	0	25	26		12	6	3
	II	0	1?	24		9	6	6
	V	1	24	24		5		
65	C				32	26		
	I	4	67	122	104	93		
	II	1	33	53	21	22		
66	V	0	45	56	47	40		
	C				11	12	20	
	I	0	16	15	8	17	9	
67	II	0	33	49	38	38	27	
	V	1	11	16	4	3		
	C				47	64	53	69
68	I	0	18	27	20	15	11	9
	II	0	67	82	62	50	31	35
	V	1	24	39	22	22		10
69	C				25	17	19	
	I	0	33	32	15	31	15	
	II	0	(16)	14	3	6	8	
70	V	1	(28)	18	3	2		

* Pool containing 0.75 ml. each 62-82: anti-C, 23; anti-VII, 9.

† After second of injections 3 days apart.

‡ Followed by reinjection of 0.05 mg. each S I and S II (after bleeding 3, except Nos. 65, 68, 75, and 76). Reinjection after bleeding 5: same amounts, except that No. 80 received 0.05 mg. S V in addition.

§ After 5 months, repetition on a smaller sample gave: anti-C, 5; anti-I, 49; anti-II, 63; anti-V, 6.

TABLE III—Continued

Subject No.	Anti-bodies to	Micrograms antibody N per 4 ml. serum						
		Bleeding No.						
		0° Before	1 2 wks.†	2 6 wks.	3 5½ mos. †	4 8 mos.	5 15½ mos†	6 4-5 mos. later
69	C				9	12	22	27
	I	0	30	32	19	20	10	15
	II	1	41	46	32	31	19	25
	V	0	6	13	2	0		
70	C				37	43	54	67
	I	0	22	25	14	12	13	14
	II	0	36	34	24	26	31 ?	28
	V	0	14	24	7	3		
71	C				13	15		
	I	0	29	36	20	43		
	II	5	39	34	38	36		
	V	1	30	36	10	8		
72	C				15	24	38	57
	I	1	18	17	7	13	4	5
	II	0	3	18	27	36	21	22
	V	0	11	31	(8)	4		
73	I	0	39	46				
	II	0	72	74				
	V	1	12	15				
74	C				50	41	70	67
	I	1	18	23	12	27	9	9
	II	2	64	(83)	88	76	48	53
	V	0	15	23	11	8		
75	C				47	63	44	45
	I	2	12	17	14	33	11	10
	II	5	12	14	3	9	15	(13)
	V	2	12	15	7	8		
76	C				97		109	
	I	3	135	196	156		76	
	II	0	59	71	43		29	
	V	1	34	39	9			
78	C				18	18‡	37	31
	I	2	69	74	58	46	41	41
	II	2	35	42	34	25	20	20
	V	0	49	60	37	33	20	5

‡ In order C, I, II, V. Order C, V, II, I gave 19, 45, 22, and 51, respectively. This serum, contrary to most others, showed no marked change in either order after removal of complement by heat inactivation instead of by Ea-anti-Ea.

TABLE III—*Concluded*

Subject No.	Anti-bodies to	Micrograms antibody N per 4 ml. serum						
		Bleeding No.						
		0 ^o Before	1 2 wks.†	2 6 wks.	3 5½ mos.‡	4 8 mos.	5 15½ mos.‡	6 4-5 mos. later
79	C				43	75	69	73
	I	0	7	7	14	9	2	4
	II	0	14	28	29	26	19	>19
	V	1	28	36	35	30	19	
80	C				12	25	32	34
	I	6	72	63	73	54	45	39
	II	1	37**		18	12	11	10
	V	1	129	136	166	143††	97	<113
81	C				17	15	26	24
	I	1	15	21	17	14	10	10
	II	1	23	29	12	12	10	
	V	0	1	2	5	1		
82	C				74	41	63	68
	I	3	14	16	7	10	4	3
	II	1	67	78	50	44	24	24
	V	1	74	79	57	51	24	

** In order II, V, I. Order V, II gave anti-V, 139; anti-II, 31.

†† By micro-Kjeldahl: 146.

Analytical Data

Group 1: In Table I are given values for antibody nitrogen in 4 ml. of serum of volunteer 8, the poorest antibody former in the group, No. 15, the best producer of antibody to Type I, and No. 19, the best for Type II. Averages for the group are to be found in Table IV.

Group 2: Data on three subjects, corresponding to those selected from group I, are also given in Table I. Averages in Table IV.

Groups 3 and 4: Data on the poorest antibody former in the group and the best for each type are given in Table II. Averages in Table IV.

Group 5: Since the analyses on the sera of this group were carried out after the second bleeding with removal of anti-C before measurement of the type-specific antibody response, it is believed that the data reflect with some accuracy the wide variety of the response of human beings to simultaneous immunization with several polysaccharides. They are therefore given in full in Table III. Averages in Table IV.

TABLE IV
Mean Values of Antibody Nitrogen

Subjects	Anti- bodies to	Micrograms antibody N per 4 ml. serum									
		Bleeding No.									
		0 Before	1 3 wks.	2 6 wks.	3 3 mos.	4 5 mos.	5 6 mos.*	6 12 mos.	7 14 mos.†	8 2 yrs.‡	9 3 mos. later
Group 1	C								25	38	46
	I	0	26	31	24	21	22	19	10	9	13
	II	0	56	55	44	40	35	27	20	19	22
			3 wks.	2½ mos.	4 mos.	5 mos.*	10½ mos.	12½ mos.†	23 mos.	26-27 mos.§	
Group 2	C							40	64	76	
	I		13	13	9	10	14	4	6	6	
	II		20	22	19	18	18	10	7	13	
			3-4 wks.	2½ mos.	8 mos.	10 mos.†	20-23 mos.				
Group 3	C	21**				27	40††				
	I	1	15	18	17	8	6††				
	II	1	37	45	44	33	18††				
			3-4 wks.	2½ mos.	8 mos.	10 mos.†	20-23 mos.††	1-5 mos. later			
Group 4	C	23**				28	25	24††			
	I	1	27	35	34	20	8	11††			
	II	0	58	69	58	50	43	47††			
			2 wks.	6 wks.	5½ mos.§§	8 mos.	15½ mos.§§	20-21 mos.			
Group 5	C	23			35	31	44	49***			
	I	1	33	29	33	29	17	13***			
	II	1	31	28	31	29	20	21†††			
	V	1	25	22	26	22					

* 2.5 to 4 weeks after skin tests with 0.01 mg. S I and S II.

† 2 months after reinjection with S I and S II.

‡ 8 subjects, reinjected after bleeding.

§ 3 to 4 months after second reinjection, 6 subjects.

|| 5 subjects.

** Separate analysis on several pooled sera.

†† 4 subjects.

‡‡ Followed by second reinjection.

§§ Followed by reinjection with S I and S II only.

||| Separate analysis on pool of all sera; subsequent analysis for anti-VII, 9 µg.

*** 14 subjects.

††† 13 subjects.

DISCUSSION

Quantitative analyses have already been presented on the antibody content of the sera of patients shortly after recovery from pneumococcal pneumonia (6). The present data on the human response to vaccination with pneumococci or their type-specific polysaccharides were obtained concurrently, and, in their larger subject material and longer period of observation, supplement and extend the still all too small body (6, 15, 20) of analytically exact data on the immune response of human beings.

Perhaps the most surprising result of the investigation, although an inherently reasonable one, was the constant finding of antibody to the C substance of pneumococcus in normal and immunized subjects, as already noted (6). In general, the prevaccination bleedings contained little or no antibodies to the specific polysaccharides of Types I, II, III, and V pneumococci. When, however, Type VII polysaccharide was added, most of the sera gave appreciable precipitates and it was found that the reaction was usually due, in large measure, to the high C content of the preparation of Type VII polysaccharide used. Analyses with C isolated from other types and from pneumococci in the Dawson S phase showed that the C-anti-C reaction behaved as a true antigen-antibody interaction in that the serum component occurred in the globulin fraction (6). It was therefore unlike that observed in the sera of patients in the acute febrile phase of numerous diseases, in which C reacts with an albumin-like protein temporarily present (8a, 21). Another remarkable feature of the C-anti-C reaction was the comparative constancy, for any one subject, of the values of anti-C over periods as long as a year or more. This is shown in the values for anti-C given in the tables; indeed, certain of the volunteers might actually have been identified solely by the anti-C content of their sera. Such variations as may be noted are doubtless due in part to the greater uncertainty of the earlier analytical estimations, and in part to the unfortunate use, in some of the analyses, of C derived from formalinized cultures. Such preparations appeared to give higher results than the others, possibly owing to condensation of C molecules into a formaldehyde-C polymer capable of co-precipitating other globulins with the true antibody. Possibly, also, the preparations varied with respect to their content of undegraded Forssman antigen (8d, e). The results did not appear to be influenced by blood group A substance, which contaminated some of the C preparations (see experimental part).

A possible explanation of the apparent state of equilibrium existing in most of the subjects with respect to the C antigen may lie in the more or less constant occurrence of pneumococci in one phase or another in the nose and throat (22). Human beings would then be under a continuing stimulus due to this somatic antigen common to all pneumococci, and each person would react characteristically and with the individual variability noted in other animals (*cf.* 6, 23).

Up to the time anti-C was removed before estimation of the type-specific response, all of the preliminary, or 0 bleedings, had been analyzed and many of the subsequent ones as well. All of these postvaccination values, therefore, are probably too high by variable amounts difficult to evaluate, but depending upon the often appreciable quantity of C substance in the sample of type-specific polysaccharide used. The magnitude of these errors was indicated in some instances, recorded in the tables, by later analyses in which the anti-C was first removed, but it is by no means certain that the full difference recorded was always due to the prior removal of the anti-C, since the sera, although kept sterile and in the cold, might have deteriorated during storage. The question also arises why so many of the sera in the 0 bleedings gave no precipitates with S I and S II in spite of their often high anti-C content and the presence of traces of C in the specific polysaccharides. It is likely that the traces of C substance present in these analyses would give rise to precipitates only in exceptional normal sera, whereas in the sera after immunization, small amounts of C-anti-C complexes, which might not separate by themselves, would probably co-precipitate with the insoluble S-anti-S formed.

The features of the type-specific antibody response can be outlined by first considering the data for each group of subjects.

Group 1: Two separate intracutaneous injections were given, totalling 10 billion each of pneumococcus Type I and Type II containing 0.03 mg. of S I and 0.04 mg. of S II. In Table I are given the antibody contents of the sera of various bleedings of selected subjects in micrograms per 4 ml. of serum, 4 ml. being the average quantity used for each blank and determination in duplicate. Data are given for subject 8, the poorest reactor in the group, 15, the best producer of antibodies to the specific polysaccharide of Type I pneumococcus (anti-S I), and 19, the highest in anti-S II. It will be noted that the antibody content of the sera reached the maximum within 3 to 6 weeks, and remained almost constant for about 5 months, in general agreement with others (1, 2). The behavior of the sera of the other members of the group was similar.⁵ After 5 months the subjects were given intracutaneous skin tests of 0.01 mg. S I on one arm and 0.01 mg. of S II on the other, but the intensity of the immediate and 24 hour reactions bore no relation to antibody levels in the sera, in agreement with references 1 *e*, 2 *a*. It might have been expected (1) that these tests would have acted as booster doses and increased the antibody level, but the next bleeding, No. 5, 2½ to 4 weeks later, showed no such effect, nor was there an increase in the average values for the entire group (Table IV). At the end of another 6 months, one year after the original injections, the anti-

⁵ Full data on the sera of the intermediate members of groups 1 to 4 are on file in a final report to the Pneumonia Commission, Army Epidemiological Board, but are omitted here in order to conserve space, as it is believed that the figures for group 5, given in full, are more accurate and lead to the same conclusions. Copies of the data for groups 1 to 4 will be lent upon request.

body levels had dropped to two-thirds to one-half of their original values. At this time the students were reinjected subcutaneously with 0.025 to 0.05 mg. of S I and S II, and again there was no increase in the antibody content of the sera. Instead, the slow decrease continued. At the end of the second year a few subjects were reinjected a second time, but only a very slight rise in antibody content occurred.

Group 2: In order to make the conditions of a comparison of polysaccharide with intact pneumococci as close as possible, an attempt was made to inject the polysaccharides in particulate form in the same quantities as in the bacterial vaccines. The insoluble copper salts were used for this purpose, but as the precipitates were gummy and tended to stick in the syringes, most of these subjects probably received less than 0.03 mg. of S I and 0.04 mg. of S II. Possibly for this reason the average antibody formation was less than in group 1 (Tables I and IV). Results of skin-testing and reinjection were, on the whole, negative, as in group 1.

Groups 3 and 4 (Table II): In group 3, 0.05 mg. each of S I and S II in solution in phenolized saline was injected intracutaneously, while in group 4 the same amounts were injected subcutaneously. Antibody values were somewhat higher in group 4 than in group 3, but the difference was scarcely greater than might be expected between two small groups. Subcutaneous injection of the polysaccharides is, however, certainly no less effective than by the intracutaneous route, as indicated also in references 1 and 2. These groups averaged about the same antibody content as group 1, although several individuals showed much greater antibody formation. While slightly more polysaccharide was given groups 3 and 4, complaints due to reactions were fewer, perhaps because the bacterial proteins injected into group 1 were not present in the polysaccharide mixture. In these groups the antibody levels persisted almost unchanged for 8 months (Tables II and IV) and were not increased on reinjection. There was considerable individual variation in the rate of decrease after this period, but between one-tenth and one-half of the maximum antibody content was still circulating after 2 years.

Group 5: This group, the largest, was injected subcutaneously with 0.06 mg. each of S I, S II, and S V. Commercial products, made by the phenol method (13), were used.⁶ The data on the various bleedings are given in full in Table III. Beginning with bleeding 3, estimations of anti-C were carried out before the sera were analyzed for type-specific antibodies, and the trends of the antibody response to four different carbohydrate antigens can therefore be followed in detail. It will be noted that the anti-C content of most of the sera remained relatively constant. It is also evident that a vigorous response to one type-specific antigen by no means indicated a like response to one or both of the remaining two (*cf.* also 1, 2), and that all possible combinations

⁶ Manufactured by E. R. Squibb & Sons for use by the Pneumonia Commission.

of selective behavior were represented. It is apparent also, in conjunction with the figures in the other tables, that ability to form antibody to any one type was not correlated with ability to form antibody to the somatic C antigen of pneumococcus. While in groups 1 to 4 S II appeared to function as a better antigen in general than S I, the average antibody response to both was about the same in group 5. Except in a few students, antibody to S V was far lower in quantity than was anti-I and anti-II, but in one of the exceptions the value was the highest for any antibody in the group; consequently the average value for anti-V differs little from that for anti-I and anti-II. On account of the great individual variability shown, therefore, the average values given in Table IV are of service only in emphasizing the slow tapering off of the antibody levels and the failure of booster doses to produce the anticipated effect.

It is difficult to assign a reason for the failure of reinjections of the polysaccharides to produce a secondary rise in antibodies even when the antibody level had fallen, after 2 years, to a fraction of its original value. The specific polysaccharides of pneumococcus are, however, resistant to the usual carbohydrate-splitting enzymes (24). It is therefore possible that S I, S II, and S V enter into long lasting combination with some cellular constituent, and that this combination provides the observed antigenic stimulus over the comparatively long periods of observation to which the groups of students were subjected. If, at any period, the body should make its maximum response to the residual antigen available, injection of a further small quantity would not necessarily increase an already maximal effort at the production of antibody, even though the current level were below the original. One would not then expect reinjections of polysaccharide to exert much effect until all residues of previous injections had disappeared, and this state would be indicated by disappearance of the antibody. It is hoped to test this possibility, which would scarcely apply to protein antigens, as these are probably relatively rapidly degraded by the proteolytic enzymes of the body.

While it has long been known from studies with mice (25) and men (1, 2) that extremely minute quantities of the specific polysaccharides of pneumococcus are capable of eliciting an antibody response in the sera of these animals, the extreme variability of the data and their relative nature precluded any realization of the actual quantities of antibody circulating at the time of testing. The data now presented in the tables show that, in favorable instances, this response may be enormous when compared with the few hundredths of a milligram of polysaccharide injected. For instance, it can be calculated, with the use of an average human blood volume of 7 liters, that subject 53 produced within 3 weeks more than 25,000 times as much circulating anti-S II⁷ as the amount of polysaccharide injected. This result surpasses,

⁷ 217 mg. antibody N \times 6.3 = 1370 mg. antibody protein in 3.5 liters of serum.

relatively, the antitoxin-producing capacity of a horse⁸ calculated to have elaborated more than 10,000 times as much circulating antibody as the 60 mg. of diphtheria toxoid injected over a period of 1 month (26). When one considers that the average half-life of an antibody molecule (in the rabbit) has been calculated to be about 2 weeks (27),⁹ while the antibody level in subject 53 remained constant for 8 months, the relative enormity of the quantity liberated into the blood stream becomes even more striking. This is, of course, the extreme case, but there were several other subjects whose response to S I, S II, or S V was roughly equal in relative magnitude to that of the horse in question, while the majority of subjects fell far below this level. The data suffice to show, however, that in man the specific polysaccharides of pneumococcus are among the most powerful antigens known.

Although no evidence of a secondary rise in antibody levels was obtained following the reinjection of specific polysaccharides, the possibility was considered that subjects showing exceptionally high antibody production to one or another of the polysaccharides might have had previous experiences with pneumococci of the type in question. In several such instances questioning elicited no evidence of previous infections that might have been responsible. Nos. 53 and 54, both good producers of anti-S II, had had lobar pneumonia several years earlier, but correspondence with their physicians showed that the offending pneumococci had been of Types VII and I, respectively.

As noted in the tables and in references 1e and 6 many of the sera contained appreciable antibody to S VII, even after removal of the anti-C. This is the only one of the type-specific polysaccharides so far tested to give frequent reactions in the sera of normal individuals. Whether or not this is indicative of a cross-reaction with other antibodies is not yet clear.

It remains to consider the quality of the antibody formed and whether or not the quantities found in the serum would, in general, be sufficient to protect human beings against infection by pneumococci of the types of which the specific polysaccharides were injected. Evidence on the first of these points is presented in the accompanying paper (7) and in another yet to appear (23), from which it is apparent that antibodies elicited by small injections of pneumococci or their type-specific polysaccharides are not usually equal in protective power to antibodies from hyperimmunized animals.

⁸The experiment of Knorr (*Münch med. Woch.*, 1898, 45, 321, 362) frequently cited as the maximum recorded antibody rise (100,000 units per toxin unit injected), is not strictly comparable since it reflected an increase due to booster doses, not the primary injections. Even so, Knorr's result is of the same order of magnitude as those under discussion, as the figure should be 50,000 units per toxin unit injected, not 100,000, as given by Knorr, since the units were measured in the serum, not in whole blood, the volume of which was used for the calculation.

⁹"Half-life" is here understood to indicate the balance between formation and destruction. The rate of disappearance of serum globulin in man is similar (personal communication, Dr. David Rittenberg and Dr. David Shemin).

With regard to the protective value of the immunization procedure, the evidence that pneumonia patients may show circulating type-specific antibodies at the outset of the disease (28) would appear at first sight to argue against the protective power of such antibodies. However, it has been pointed out (28) that the pneumococcal test employed is 10 to 100 times as sensitive an indicator of antibody as the mouse protection test, which is usually negative under the same circumstances. The amounts of antibody circulating in the patients studied in reference 28 must therefore have been of a much lower order of magnitude than the precipitating and mouse-protecting (7) antibodies elaborated after injection of the polysaccharides. Evidence on the positive side was obtained from a study of serum therapy of pneumococcal pneumonia in rats (29). A sample of the rabbit serum concentrate used contained 7.13 mg. of anti-S I N per ml.,¹⁰ and 0.02 ml., injected intravenously into rats, regularly terminated 12 hour pneumonia due to Type I pneumococci, while 0.002 ml. did not. It was calculated that the 140 micrograms of antibody N (or less) having the curative effect would be diluted to about 10 micrograms (or less) per ml. of blood, or 80 micrograms (or less) per 4 ml. of serum in the average rat. Although this is only roughly double the average antibody response to the polysaccharide injections in man, the injected antibody sufficed to cure an already established infection, so that presumably less antibody would be required merely to protect. Also, infinitesimal amounts of antibody must have sufficed to protect mice in the tests employed in references 1 and 2, since values up to 10×10^6 protective units were obtained in sera which were obviously weak as compared with those from hyperimmunized animals. Since, in addition, active immunity should be more effective and lasting than passive immunity, it was concluded that further tests of the immunization of human beings against pneumonia were warranted. The results of a field test (16) appear to have justified this conclusion.

SUMMARY

1. A modification of the microanalytical quantitative precipitin method, five to ten times as sensitive as the older procedure, has been used to measure the type-specific antibody response in human beings. Injections of type-specific pneumococci or equivalent amounts of their type-specific polysaccharides led to comparable antibody production. In general, the few hundredths of a milligram of polysaccharides injected functioned as extraordinarily powerful antigens.

2. Subcutaneous injections of the polysaccharides were as effective as intracutaneous, and the resulting antibody levels, which were highly variable individually, remained relatively constant for 5 to 8 months, gradually taper-

¹⁰ A sample of the serum was kindly supplied by Dr. E. N. Irons and analyzed in this laboratory.

ing off during periods of observation that exceeded 2 years in some instances.

3. After the injection of several type-specific polysaccharides widely diverse combinations of selective and non-selective responses were observed.

4. Booster doses had little or no effect in increasing circulating antibody, even after 2 years. A tentative explanation is given of this unexpected result.

5. Possibly because they frequently harbor pneumococci, normal human beings appear to be in serological equilibrium with the C-antigen of pneumococcus, since in most instances the quite appreciable anti-C content of their sera remained relatively constant during periods of a year or longer. Many of the sera also contained antibody to S VII.

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