MOUSE-PROTECTIVE TITERS OF SERA OF VOLUNTEERS FOLLOWING INJECTION OF PNEUMOCOCCI OR THEIR TYPE-SPECIFIC POLYSACCHARIDES*

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(Received for publication, December 13, 1945)

The antipneumococcic mouse-protective activity of sera from human subjects before and after inoculation with pneumococci or type-specific polysaccharides was measured in this study and compared with the amounts of precipitable antibody nitrogen in the same sera. The measurements of the precipitable antibody nitrogen have been reported in the preceding paper (1). The mouse protection tests add knowledge of the *in vivo* potency of the antibodies to the evidence of their activity *in vitro*, and they indicate that a comparison with measurements of mouse-protective antibodies should be feasible in other similar studies.

Methods and Materials

Technic.—Mouse protection tests were carried out by the method used by the New York City Health Department, Bureau of Laboratories, for standardization of therapeutic antipneumococcic sera with the exception that the small volumes of sera available permitted few duplicate tests, each with ten mice per serum dilution.

The Type I and Type II cultures were passed through mice from one to five times weekly and stored between passages at 10°C. in beef heart phosphate broth containing 3 per cent horse blood. For the test an 18 hour blood broth culture of the heart blood of a mouse injected 5 hours previously was adjusted turbidimetrically so that 10^{-8} ml. in a volume of 0.5 ml. contained about 2 microorganisms (1 M.L.D.). All culture dilutions were made with beef heart phosphate broth. The test dose of culture for each type was 10^{-3} ml. contained in 0.5 ml. of broth, approximately 100,000 M.L.D. This dose of culture is protected against by 1/10 unit of serum (2). As controls of the virulence of the culture poured blood agar plates were made of the 10^{-6}, 10^{-7}, 5×10^{-8}, 2×10^{-8}, 10^{-8}, and 5×10^{-9} ml. culture dilutions in 0.5 ml. volumes and one mouse was injected intraperitoneally with 0.5 ml. of broth and 0.5 ml. of each of these culture dilutions at the beginning and at the end of each test. Control mice which received two or more pneumococci as determined by plate counts died within 72 hours. The sera were diluted in 0.85 per cent sodium chloride solution so that the desired amounts were contained in 0.5 ml.

As a control serum the National Institute of Health bivalent Type I and Type II horse serum P-11 was included in each test. Monovalent Type I or Type II hyperimmune rabbit

* This investigation was supported in part through the Commission on Pneumonia, Board for the Investigation and Control of Influenza and other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

serums were also included in a number of the tests. Mice were injected intraperitoneally with 0.5 ml. of the test culture dilution and 0.5 ml. of the serum dilution drawn into a 1 ml. insulin type syringe. Up to ten mice were injected with each serum dilution, the number of mice being determined by the amount of serum available. All injections were completed within 60 minutes of the time dilution of the culture was begun. The mice were inspected twice daily for 120 hours. Sickness and deaths were recorded and cultures on blood agar plates were made from the heart blood of sufficient mice to determine that deaths were due to uncomplicated pneumococcus septicemia. Although the test was considered complete at 96 hours, the occurrence of a large number of deaths after this time was taken into account in planning second tests of sera. The majority of the mice used were the CFW strain. Their weights ranged from 15 to 19 gm. At the conclusion of each test the unit value of each serum in comparison with the control serum was calculated from the deaths and survivals at 96 hours by the 50 per cent endpoint method of Reed and Muench as adapted by Goodner and Horsfall (3). The established values of control serum P-11 are Type I, 300 units per ml.; Type II, 150 units per ml.

From most of the subjects less than 5 ml. of serum was received from each bleeding. As 0.5 ml. of a serum containing 0.2 unit per ml. or 0.1 ml. of a serum containing 1 unit per ml. is necessary for protection against the test dose of culture, it can be seen that duplicate tests using ten mice for each serum dilution were not possible with those sera which were in this range of unit potencies. Only the results of tests in which 50 per cent endpoints could be calculated are reported below.

The results of repeated tests of National Institute of Health control serum, hyperimmune rabbit sera, and those human sera in which two tests were carried out with ten mice per serum dilution showed satisfactory agreement. In thirteen Type I tests, the 50 per cent survival endpoint of control serum P-11 averaged 440 units per ml. with a range of from 320 to 650 units per ml.; in thirteen Type II tests with the same serum, the average was 260 units per ml. with a range of from 115 to 380 units per ml. Type I hyperimmune rabbit serum, lot 3-745, contained 1.44 mg. specifically precipitable nitrogen per ml. In seven Type I mouse protection tests of this serum in comparison with P-11 the average was 2,180 units per ml., with a range of from 1,650 to 2,480 units per ml. The ratio, mouse protection units to milligrams of antibody nitrogen, averaged 1,500 with a range of from 1,145 to 1,720 units per mg. Type II hyperimmune rabbit serum, lot 579RS D39, contained 0.94 mg. specifically precipitable nitrogen per ml. In six Type II mouse protection tests of this serum in comparison with P-11, the average value was 540 units per ml. with a range of from 276 to 1,030 units per ml. The ratio, mouse protection units to milligrams of antibody nitrogen, averaged 580 with a range of from 294 to 1,096 units per mg. Duplicate mouse protection tests with ten mice per serum dilution were not completed for any of the human sera tested against Type I. Duplicate tests with ten mice per dilution were completed for eight human sera tested against Type II organisms. The greatest variation in results for any one of these eight sera occurred with serum 53-6 which titered 19 and 14 units per ml. respectively in two tests.

Subjects.—The sera studied were representative samples from the immunized subjects described in the preceding paper, and were selected chiefly because the volumes of the serum samples were larger.

Group 1 received two intravenous injections, 3 days apart, of a total of 10 billion each of the Type I and Type II pneumococci containing 0.03 mg. and 0.04 mg. respectively of Type I and Type II specific polysaccharides (S I and S II). Group 2 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 received two intracutaneous injections 6 days apart of polysaccharide solution, 0.05 mg. each of S I and S II. Group 4 received the same dose as group III but by two subcutaneous injections 8 days apart. Group 5 received 0.06 mg. each of S I, S II, and S V subcutaneously in two doses 3 days apart.

TABLE I

Type I Antibody Nitrogen, Mouse-Protective Titer, and the Ratio, Mouse Protection: Antibody Nitrogen in the Sera of Immunized Subjects

Group	Subject	Bleeding No.	Antibody nitrogen Micrograms per 4 ml. (1)		Mouse-protective titer		
			Anti-C	Anti-I	Units per ml.	Ratio, Units per ml.: antibody N mg. per ml.	No. of mice per dilution
1	10	2		15	0.17	45	1 to 3
	15	1		82	4.0	195	5 to 10
		3		64	4.3	269	10
		5		60	3.8	253	9 to 10
		7	28	44	3.1	282	5
	16	2		25	0.8	128	3
		5		25	2.1	336	1 to 3
	17	7	13	11	0.5	182	3 to 5
2	29	2		40	4.7	470	10
		3		31	3.3	426	10
		4		30	1.5	200	10
		5	25	27	0.6	89	10
		6	24	14	0.8	228	5
	31	2		19	0.49	103	1 to 2
		3		7	0.14	80	3
		4		12	0.2	67	1 to 3
3	39	1		20	0.35	70	1 to 2
		2		22	0.15	27	3
	43	1		37	0.44	48	1 to 3
4	54	1		16	2.2	550	3
		2		28	1.25	178	3
		3	41	23	1.2	209	5
	56	1		86	6.2	288	8
		3		149	2.3	62	10
	59	1		45	0.53	47	2 to 3
5	78	4	18	46	0.8	70	5
		5	37	41	0.4	39	10

RESULTS

The results of tests of postinoculation sera for Type I protective antibody are shown in Table I, and for Type II antibody are shown in Table II. Twenty-seven samples from twelve subjects were tested for Type I protection, and thirty-one samples from fourteen subjects were tested for Type II protection. Only two samples were tested for both Types I and II and they

TABLE II

Type II Antibody Nitrogen, Mouse-Protective Titer, and the Ratio, Mouse Protection: Antibody Nitrogen in the Sera of Immunized Subjects

Group	Subject	Bleeding No.	Antibody nitrogen (1) micrograms per 4 ml.		Mouse protective titer		
			Anti-C	Anti-II	Units per ml.	Ratio, Units per ml.: antibody N mg. per ml.	No. of mice per dilution
1	9	3		102	4.9	192	10
		4		99	4.5	182	10
	13	4		15	1.5	400	3 to 10
	17	2		152	8.2	216	10
		3		136	8.9	262	10
		7	13	55	3.65	265	5
					2.9	211*	5 (repeated)
	19	1		24	26.1	4350	7 to 10
		2		144	11.5	319	10
		7	18	52	2.7	208	8 to 10
		8	29	43	3.1	288	7 to 10
2	24	4		31	0.42	54	3
	30	2		7	0.6	343	3
		3		9	0.24	107	3
		6	32	4	0.6	600	5
3	38	1		121	6.2	205	8 to 10
		2		142	14.4	406	10
					19.0	535*	10 (repeated)
		4	67	137	27.0	788	5
	48	1		42	1.3	124	10
		4	40	39	2.9	297	5
4	52	1		58	1.3	90	10
		4	7	48	0.7	58 Av.	5
					0.9	75 67*	5 (repeated)
	53	1		247	38.5	623	10
		3		251	26.0	414 Av.	10
					32.0	510 462*	10 (repeated)
		4	18	231	23.0	398 Av.	10
					27.0	468 433*	10 (repeated)
		5	12	140	23.1	660 Av.	10
					18.8	537 599*	10 (repeated)
		6	37	155	19.0	490 Av.	10
					14.0	361 426*	10 (repeated)
5	62	4	9	45	0.5	44	5 to 10
		5	7	25	0.3	48	5 to 10
	74	5	70	48	3.4	283	10
	78	4	18	25	0.5	80	5
					0.5	80	5 (repeated)
	82	4	41	44	0.6	54	10

* Figures chosen from duplicates to be used in Table III.

appear in both tabulations, namely bleeding 7 of subject 17 and bleeding 4 of subject 78.

All the subjects tested had, with one exception, protective antibodies in at least one postinoculation sample sufficient to protect against the 0.001 ml. test dose of culture containing 100,000 M.L.D. In most cases the serum could be diluted and still protect against the test dose. In general, Type I antibodies were lower in titer than Type II, corresponding to the lesser amounts of precipitable antibody nitrogen found for Type I as compared with Type II. There was a tendency for the mouse-protective titer to be lower in the later bleedings from the same subjects, just as the quantities of antibody nitrogen tended to be lower.

The number of subjects in the groups receiving different antigens was too small to justify comparisons of antigenic potency with respect to the mouse-protective titer produced by individual antigens. Variations in precipitable anti-C antibody or repeated inoculations with antigens had no pronounced effect on mouse-protective titer or the ratio of mouse-protective titer to antibody nitrogen.

Samples of sera taken before inoculation from eleven subjects were tested for mouse-protective activity using amounts of culture smaller than 0.001 ml. Eight, from subjects 10, 15, 16, 31, 43, 54, 56, and 59, were tested for Type I antibodies, and three, from subjects 9, 48, and 53, were tested for Type II antibodies. Amounts of 0.1 ml. of serum were given to each of four mice with small doses of culture: 10^{-3}, 10^{-5}, 10^{-6}, 10^{-7} ml. or 100,000; 1,000; 100; and 10 M.L.D. respectively. The sera of two subjects showed mouse-protective activity, No. 56 against Type I, No. 9 against Type II, both protecting against a maximum of 10^{-5} ml. containing 10,000 M.L.D. Neither was potent enough to protect against 0.001 ml. containing 100,000 M.L.D., the test dose of culture used for the determination of units. Type I precipitable antibody nitrogen, 11 micrograms per 4 ml., was found in the serum from subject 56. Neither Type I nor Type II precipitable antibody nitrogen was found in the sera of five other subjects that showed no mouse protection. Pre-immunization sera from the remaining five subjects were not examined by the method, used later, which was sufficiently accurate to measure such small amounts of precipitable antibody nitrogen (1).

In order to find out whether a regular relationship existed in these low titer sera between mouse protection units and precipitable antibody nitrogen, the ratios of the two values were calculated. The ratio varied widely from 27 to 550 for Type I, and from 44 to 4,350 for Type II. When the results were listed in the order of the number of mouse protection units, there was a marked tendency for lower ratios to be associated with lower mouse-protective values. For both Type I and Type II the average ratio (Table III) was the lowest for sera with from 0 to 1 unit of protection per ml. and higher for sera with from

TABLE III

Relationship of Mouse Protection to the Ratio, Mouse Protection: Antibody Nitrogen

Kind of serum	Mouse-protective titer		Antibody nitrogen micrograms per 4 ml.		Ratio	
	Range of observations Units per ml.	No. of sera	Range of observations	Average	Mouse protection units per ml.: antibody nitrogen mg. per ml.	
					Range of observations	Average
Type 1 human	0 to 1.0	14	7 to 46	24	27 to 228	87
	1.1 to 5.0	12	16 to 149	49	62 to 550	286
	More than 5.0	1	86	86	288	288
Type 1 rabbit hyperimmune lot 3-745	1650 to 2480	1 Examined 7 times	5760		1145 to 1720	1514
Type 2 human	0 to 1.0	9	4 to 48	26	44 to 600	155
	1.1 to 5.0	10	15 to 102	55	90 to 400	228
	5.1 to 39	12	24 to 251	157	205 to 4350	768
Type 2 rabbit hyperimmune lot 579	276 to 1030	1 Examined 6 times	3760		294 to 1096	579

TABLE IV

Effect of Normal Serum on the Mouse-Protective Titer of Hyperimmune Rabbit Antipneumococcic Serum

Antiserum	Diluent	Units of mouse protection per ml.	Ratio: Mouse-protective units per ml. / Antibody nitrogen mg. per ml.
Type 1	0.85 per cent NaCl	2570	1785
Lot 3-745	Normal human AA 232	805	559
First test	Normal rabbit 1	2180	1514
Type 1	0.85 per cent NaCl	930	646
Lot 3-745	Normal human AA 233	620	431
Second test	Normal human AA 233*	670	465
	Normal rabbit 2	905	628
	Normal rabbit 2*	1010	701

* Duplicate test carried out at same time.

1 to 5 units of protection per ml. Only one serum was tested for Type I which had more than 5 units per ml. More than 5 units of Type II mouse-

protective activity per ml. were found in twelve sera ranging from 6.2 to 38.5 units per ml. and the ratio was 770, even higher than that found in the hyperimmune rabbit serum.

The possibility that the relatively large amounts of serum components other than antibody contained in serum of low potency interfered with the protective action of the antibody was explored by measuring the mouse-protective titer of hyperimmune rabbit antipneumococcic serum with normal human sera as a diluent, and comparing the titer with that of the same hyperimmune rabbit sera diluted with normal rabbit serum and with saline (Table IV). Normal human and normal rabbit sera were chosen that had no protective action in the amounts used in the test. Relatively large amounts of normal human serum as diluent caused a consistent lowering of the values resulting from the titration, but these ratios were not as low as the ratios of mouse protection to antibody nitrogen described above for sera titering less than one unit per ml.

DISCUSSION

Because of insufficient amounts of serum only part of the mouse protection tests were carried out with the full number of mice and with repetitions of the tests as prescribed for the titration of therapeutic sera. Agreement was good in the findings with hyperimmune sera examined repeatedly and in the human sera in which determinations were repeated using 10 mice per dilution. The results can therefore be considered reliable. All of the subjects tested except one were shown to have substantial amounts of mouse-protective activity after immunization and the results for eleven subjects showed the development or increase in titer of mouse-protective antibody after immunization.

The low ratio of units of mouse protection per milliliter to milligrams of antibody nitrogen per milliliter in human sera of the lowest titer suggested that the precipitable antibody nitrogen when present in small amounts was of low potency. The possibility that technical factors in the protection or precipitation tests could have led to the same findings was also considered. Interference with the protective activity of small amounts of antibody by the relatively large amounts of other serum components in weak human sera was studied by titering hyperimmune rabbit sera diluted in human sera as compared with saline dilution (Table IV). Dilution with either of two different human sera reduced the mouse-protective activity of rabbit hyperimmune sera, but the difference was not great enough to provide an explanation of the lowest ratios observed. A potential error, due to the presence of non-specific precipitable substances tending to make the analyses too high, was also considered. Further studies of animal sera of low and high potency have been undertaken to clarify this question but they are not completed.

Comparsion of the mouse-protective titers after immunization with the titers observed by others (4–6) can be made by calculating the proportion of sub-

jects whose serum contained 1 unit of protection or more; *i.e.*, 0.1 ml. amounts protected mice against 100,000 M.L.D. of the same pneumococcic type. In this study the proportion was 5 out of 12 for Type I and 9 out of 14 for Type II. Felton *et al.* studied mouse-protective titers of human sera from large groups of subjects following inoculations with pneumococcic polysaccharides and found a somewhat smaller number containing one unit or more as defined above. In one study (4) of 533 subjects who received varying amounts of antigen, the sera of 152 or 29 per cent protected against 100,000 M.L.D. of Type I, and the sera of 270 or 51 per cent protected similarly against Type II. In another study (5), in which relatively large amounts of antigen were used to inoculate 1,099 subjects, the sera of 260 or 24 per cent of the injected individuals protected against 100,000 M.L.D. of Type I culture and the serum of 440 or 40 per cent protected against a similar amount of Type II culture. Other authors (6) administered smaller amounts of antigens and had even smaller numbers of subjects whose sera developed protection against 100,000 M.L.D. or more of culture. The data summarized do not show striking differences between postinoculation antibodies in the present studies, and those reported by others.

CONCLUSIONS

1. Sera of human subjects immunized with pneumococci or their type-specific polysaccharides showed type-specific antibody by mouse protection tests as well as on determinations of the specifically precipitable nitrogen.

2. The ratio, mouse-protective units to antibody nitrogen, was less in human sera of low potency than in more potent human or animal sera.

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