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Emil C. Gotschlich, … , Rene Triau, Kenneth J. Sparks

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Research Article

Radioactive antigen binding tests have been developed to measure quantitatively the antibody response of 167 adults, 84 children, and 51 infants to several different preparations of group A and group C meningococcal polysaccharides. Almost all the adults injected responded and the geometric mean responses were approximately 15 μg/ml of antibody protein in individuals vaccinated subcutaneously with two preparations of group A vaccine. The geometric mean antibody concentration after immunization with two preparations of group C vaccine was approximately 35 μg/ml. Most children aged 7 yr responded to immunization with two group A vaccines, and their mean response was only slightly less than that seen in adults. There was no difference between the subcutaneous and the intradermal route if both were given with jet gun. The majority of infants aged 6-13 months responded to a preparation of group A vaccine and the geometric mean titer was approximately 1.2 μg/ml. Adults, children, and infants responded significantly less to a preparation of group A polysaccharide which was of low molceular weight.

Quantitative Determination of the Human Immune Response to Immunization with Meningococcal Vaccines

EMIL C. GOTSCHLICH, MICHEL REY, RENE TRIAU, and KENNETH J. SPARKS

From The Rockefeller University, New York 10021, Faculte Mixte de Medicine et de Pharmacie, Dakar, Senegal, and Institut Merieux, Lyon, France

ABSTRACT Radioactive antigen binding tests have been developed to measure quantitatively the antibody response of 167 adults, 84 children, and 51 infants to several different preparations of group A and group C meningococcal polysaccharides. Almost all the adults injected responded and the geometric mean responses were approximately 15 μg/ml of antibody protein in individuals vaccinated subcutaneously with two preparations of group A vaccine. The geometric mean antibody concentration after immunization with two preparations of group C vaccine was approximately 35 μg/ml. Most children aged 7 yr responded to immunization with two group A vaccines, and their mean response was only slightly less than that seen in adults. There was no difference between the subcutaneous and the intradermal route if both were given with jet gun. The majority of infants aged 6–13 months responded to a preparation of group A vaccine and the geometric mean titer was approximately 1.2 μg/ml. Adults, children, and infants responded significantly less to a preparation of group A polysaccharide which was of low molceular weight.

INTRODUCTION

Currently there are under development two meningococcal vaccines based upon the use of high molecular weight group-specific polysaccharides. Immunization of military recruits with the group C polysaccharide has been shown to be effective in preventing meningococcal disease (1), and in lowering the rate of acquisition of the nasopharyngeal carrier stage by group C meningococci (2). Such information has not yet been obtained for the group A vaccine, in part because these strains are exceedingly rare in the U. S. military population. The efficacy of this vaccine will probably need to be tested in the African "Meningitis belt" where disease is caused primarily by group A organisms (3).

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Administration of either the group A or the group C vaccine to adults induces the formation of antibodies belonging to the three major immunoglobulin classes which have both bactericidal (4) and opsonic properties (5). To date quantitative data is available on the response of children.[1] There is no published data on the immune response of infants to these vaccines. Inasmuch as this age group stands to benefit most from meningococcal immunoprophylaxis (6), quantitative data of their serological response is vital.

The present report will describe the immune response of adults, children, and infants to immunization with several preparations of meningococcal polysaccharides measured by means of quantitative radioactive antigen binding tests (7).

METHODS

Antigens. Group A meningococcal vaccines lots A-5, A-7, V-1, and group C meningococcal vaccines lots C-6 and C-7 were prepared as described by Gotschlich, Liu, and Artenstein (8). The group A polysaccharide contained in lots V-4 and V-5 was prepared by a modified procedure, employing cold phenol extraction to remove protein contamination.[2] A-5 and C-6 were prepared at the Walter Reed Army Institute of Research; lots A-7 and C-7 by E. R. Squibb & Sons, New York; and lots V-1, V-4, and V-5 by Institut Merieux in Lyons, France. The molecular size of the different preparations was assayed by gel filtration on Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). The void volume of the column was determined with Blue Dextran (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and the total volume with tritiated water (New England Nuclear Corp., Boston, Mass.). The distribution coefficient (Kd[3]) was calculated from the peak elution volume of the major peak of the polysaccharide (9). The column was monitored either by determination of phosphorus by the method of Chen, Toribara, and Warner (10) in the case of the group A polysaccharide, or by the resorcinol method of Svennerholm (11) in the case of the

[1] Manuscript in preparation.

[2] Manuscript in preparation.

[3] *Abbreviation used in this paper:* Kd, distribution coefficient.

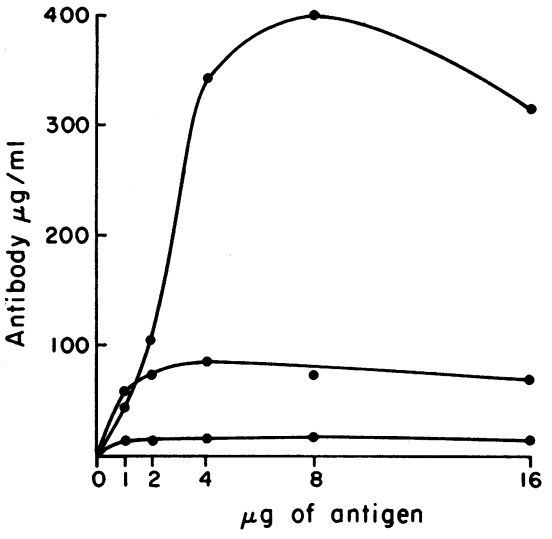

FIGURE 1 Quantitative precipitin curves obtained with human sera drawn 2 wk after immunization with meningococcal group-specific polysaccharide. The upper curve depicts reactions with group C antigen; the lower two curves with group A antigen.

group C antigen. The column was calibrated by determining the Kd of two preparations of group A polysaccharide. The molecular weights of these two preparations had been determined by ultracentrifugal methods and were respectively 170,000 and 25,000.[4] The two distribution coefficients were plotted against the logarithm of their molecular weight and the resulting line was used to estimate the average molecular weight of the vaccine preparations (12). This same procedure was used to obtain an estimate of the molecular size of group C vaccine preparations.

Quantitative precipitation analyses. Five sera obtained 2 or 3 wk after immunization with group A antigen and six sera obtained 2 wk after immunization with group C antigen were centrifuged at 70,000 *g* for 60 min to float the lipids, and centrifuged at 35,000 *g* for 30 min to remove all insoluble protein. A precipitin curve was constructed by adding to 1 ml portions of serum 100 μl of antigen solution dissolved in 0.2 M sodium EDTA pH 7.0 and containing 1,000 U penicillin and 1 mg of streptomycin. The quantity of antigen added consisted of 1, 2, 4, 8, and 16 μg. The test was carried out in duplicate in 3-ml conical centrifuge tubes which were sealed with parafilm and stored for 1 wk at 4°C. The tubes were gently shaken daily. The precipitates were sedimented by centrifugation for 30 min at 2,000 *g* at 4°C and washed twice with 2-ml volumes of cold saline. The precipitates were dissolved in 500 μl of 0.1 N NaOH and the protein concentration determined by the Folin reaction employing an autoanalyzer (13) (Technicon Co., Inc., Tarrytown, N. Y.). The test was quantitated by reference to a standard curve determined simultaneously using chromatographically purified human gamma globulin obtained from Pentex Biochemical (Kankakee, Ill.).

Radioactive antigen binding test with intrinsically labeled antigens. Radioactive group A polysaccharide was prepared by incubating group A meningococci, strain A-1, in Frantz medium (14) supplemented with 1 mc of ^{14}C-labeled sodium acetate (New England Nuclear Corp., Boston, Mass.). Group C meningococci of strain C-11 were grown in the same medium supplemented with 25 mc of tritium labeled sodium acetate (New England Nuclear Corp., Boston, Mass.). The polysaccharides were purified by the usual method (8). The recovered group A polysaccharide had a specific activity of approximately 2,800 cpm/μg and the group C had approximately 10,000 cpm/μg.

The radioactive antigen binding test was performed by adding 100 μl of radioactive antigen (0.17 μg of group A polysaccharide or 0.5 μg of group C polysaccharide) to 100 μl of serum, and after overnight refrigeration the globulins were precipitated by the addition of 200 μl of 80% saturated (room temperature) ammonium sulfate. After the precipitate had been sedimented by centrifugation for 60 min at 2,000 *g*, 100 μl of the supernate was dissolved in 10 ml of a liquid scintillation fluor consisting of Liquifluor (Pilot Chemicals, Inc., Watertown, Mass.) : Soluene (Packard Instrument Co., Inc., Downers Grove, Ill.) : toluene (42 : 100 : 858). The radioactivity was measured in a Beckman liquid scintillation counter, Model LS 133 (Beckman Instruments, Inc., Fullerton, Calif.).

Radioactive antigen binding test with group A polysaccharide labeled with ^{125}I. In order to be able to iodinate group A polysaccharide, phenolic groups were inserted by activating the polysaccharide with cyanogen bromide (15) and letting it react with tyramine.[5] Such polysaccharide could be readily iodinated by the method of Hunter, Greenwood, and Glover (16) and polysaccharide with a specific activity of approximately 1,000 cpm/ng was obtained. A double label technique was used in order to be able to do the binding tests on very small quantities of sera. The principle of this method was to add 1 μc of ^{22}Na per milliliter of antigen solution as a volume marker. To 10 μl of serum was added 10 μl of antigen solution and after overnight equilibration, 20 μl of 80% saturated (room temperature) ammonium sulfate was added and the precipitate sedimented by centrifugation. An arbitrary portion of the supernate was drawn off and discarded, taking care not to disturb the precipitates. This precipitate with a variable volume of supernate overlying it was counted in a Model 4233, two channel gamma spectrometer (Nuclear Chicago, Des Plaines, Ill.) with one channel set to count the sodium and the other channel the iodine. The iodine count represented the total amount of antigen left in the tube, both that bound and that present in the remaining supernate; the sodium count indicated the volume of supernate left. With these data it is possible to calculate the per cent of added antigen bound to antibody (17).

Three antigen concentrations containing approximately 4.0, 0.4, and 0.04 μg of radioactive antigen per milliliter were routinely used. All sera were heat inactivated for 30 min at 56°C and, when called for, were diluted in heat inactivated fetal calf serum. All volumetric measurements were done with Eppendorf pipets (Brinkmann Instruments, Inc., Westbury, N. Y.) and the test was carried out in disposable Microfuge tubes, and centrifuged in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.).

Statistical methods. Geometric mean antibody concentrations were calculated by taking an arithmetic average of the per cent binding. Inasmuch as the degree of binding is linearly related to the logarithm of the antibody concentration, this yields a geometric average of the antibody concentrations. The significance of differences in mean antibody concentration were tested by the Student *t* test.

[4] Manuscript in preparation.

[5] Manuscript in preparation.

Human sera. Dr. M. S. Artenstein kindly provided the sera of military recruits vaccinated with preparation A-7, C-6, and C-7. The source of the sera on which quantitative precipitation studies were performed, as well as the sera obtained from recruits injected with A-5, has been described before (2, 4). All other sera were obtained by personnel of the Department of Infectious Diseases of the School of Medicine of Dakar, Senegal. All subjects were volunteers, or informed consent was obtained from their legal guardians. They were observed clinically for 48 hr and rectal temperatures were obtained before, and at 6, 24, and 48 hr after vaccination on all individuals except the military recruits. No significant pyrexic responses were seen and only minor local reactions lasting for 24 hour were noted. A Ped-o-jet gun (Vernitron Corporation, Farmingdale, N. Y.) was used in some of the studies and was adjusted to inject a volume of 0.5 ml subcutaneously, or 0.25 ml intradermally. Serum was obtained from bleedings performed 2 wk after immunization. Both the infants and the children were in good health. The ages of the infants ranged from 6–13 months with more than half of them aged 9 months or less.

RESULTS

Quantitative determination of antibody by precipitation and by radioactive antigen binding capacity. One of the simplest methods for the quantitative determination of antibodies is the radioactive antigen binding test developed by Farr (7). Inasmuch as it measures the primary interaction of antibody with antigen, it is affected only by the concentration of antibody and by the average affinity of this antibody. The results of the radioactive antigen binding test have usually been expressed as the dilution of a serum which will bind a certain amount of antigen (antigen binding capacity, ABC) (7). However, because of the data available in the literature on the human immune response to the pneumococcal polysaccharides (19) in terms of micrograms of precipitating antibody, it was thought desirable to express the results obtained with the radioactive antigen binding test in terms of antibody concentration. To relate antigen binding capacity to antibody concentration,

TABLE I

Precipitating Antibody in Sera from Volunteers Immunized with Meningococcal Polysaccharides

Serum	Immunized with	Precipitating antibody to meningococcal polysaccharide	
		Group A	Group C
		μg/ml*	
W. C. B.	C	Not done	199
I. G.	A and C	38	38
J. S.	A and C	16.5	43.5
M. S. A.	A and C	47	401
E. C. G.	A and C	140	127
J. W.	A and C	85	36

* μg/ml of antibody protein.

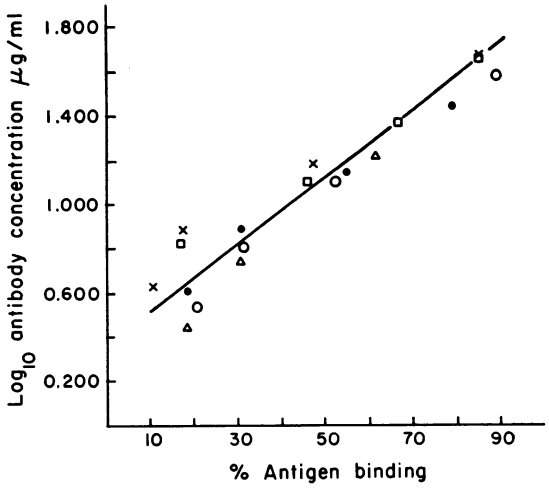

FIGURE 2 The antigen binding capacity of dilutions of five sera obtained from volunteers immunized with group A meningococcal polysaccharide. The per cent of added intrinsically labeled (^{14}C) group A polysaccharide bound by antibody is plotted against the logarithm of the antibody concentration as determined by quantitative precipitation. Serum: ○, I. G.; □, E. C. G.; ×, M. A. S.; ●, J. W.; △, J. S.

the radioactive antigen binding capacity of several sera with known content of precipitating antibody was determined. Sera were obtained 2 or 3 wk after intradermal immunization of six volunteers. One was immunized with group C vaccine only (W. C. B.) and the others with both group A and group C polysaccharide. Their immune response has been documented previously (4) by means of passive hemagglutination, bactericidal activity, and immunofluorscence. The concentration of antibody was determined by quantitative precipitation Three representative precipitin curves indicating the highest, the lowest, and an intermediate response are depicted in Fig. 1. The quantity of precipitating antibody found in each serum is summarized in Table I.

The capacity of these sera and dilutions thereof to bind intrinsically labeled group A and group C antigen was measured by the method of Farr as indicated in the Methods section and it was found that there was a linear relationship between the per cent of antigen bound and the logarithm of the antibody concentration. This linear relationship appeared to be valid between the limits of 10 and 90% antigen binding. The results are set forth in Figs. 2 and 3. The lines were drawn by the method of least squares, and the correlation coefficients were 0.94 in both instances.

The immune response of adults. The antigen binding capacity of sera obtained from 167 adults immunized with several different lots of meningococcal vaccines were measured and the quantity of antibody determined by reference to the calibration curves obtained with the

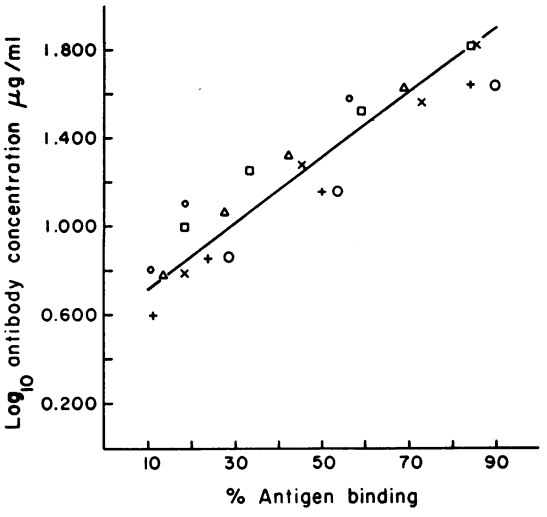

FIGURE 3 The antigen binding capacity of dilutions of six sera obtained from volunteers immunized with group C meningococcal polysaccharide. The per cent of added intrinsically labeled (^{3}H) group C polysaccharide bound by antibody is plotted against the logarithm of the antibody concentration as determined by quantitative precipitation. Serum: O, J. W.; □, W. C. B.; o, I. G.; △, E. C. G.; +, J. S.; ×, M. A. S.

standard antisera (Figs. 2 and 3). The results are set forth in the scattergrams (Fig. 4) and summarized in Table II.

Two groups of recruits were injected subcutaneously with 50 μg of two different preparations of group C vaccine. These two lots were physicochemically very similar as judged by their elution volume and profile on Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) The Kd of these preparations suggested that they both had average moleculer weights of approximately 300,000. The serological responses of the two groups were nearly identical, and all subjects had a rise in antibody concentration.

Four groups were injected with preparations of group A vaccine. The dose was 50 μg in every instance. The Kd of lot A-5 suggested that this preparation had an average molecular weight of approximately 84,000. It was administered intradermally by needle. The serological response of this group was significantly better than any other group injected with group A polysaccharide (vs. A-7 $P < 0.05$; vs. V-4 $P < 0.001$). Unfortunately, because lot A-5 and A-7 were administered differently, it is not possible to say whether the superior response observed with A-5 is due to the greater molecular size or the route of injection. Lot A-7 had a Kd suggesting a molecular weight of approximately 47,000 and was administered subcutaneously by needle. All of the subjects responded. The studies with V-1 and V-4 were performed in volunteer hospital personnel in Dakar, Senegal, and the results obtained are therefore not directly comparable to the ones obtained with American

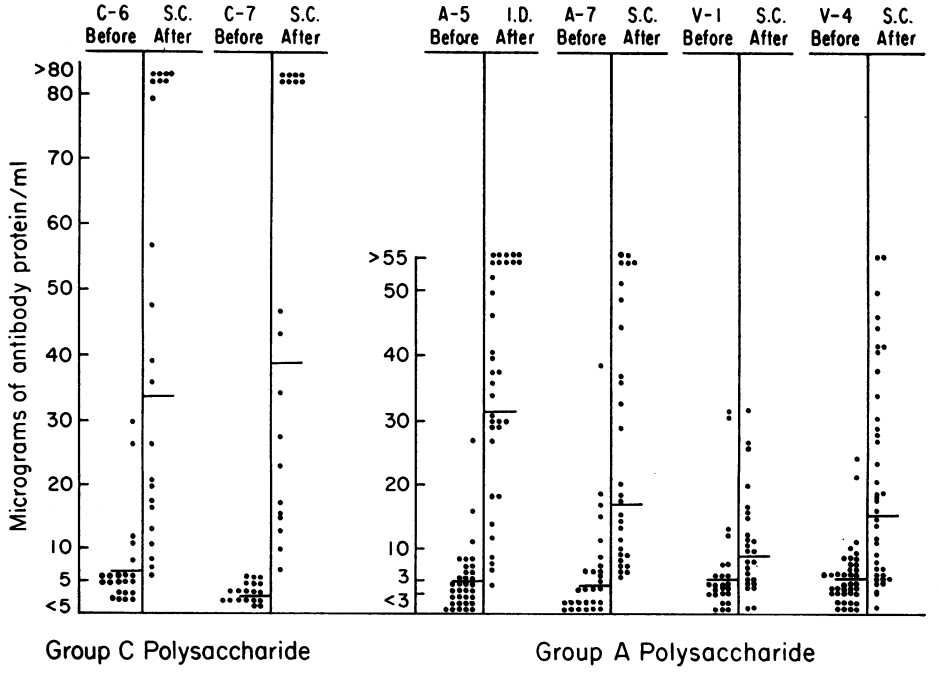

FIGURE 4 The antibody concentration found in sera of adults before and 2 wk after immunization with various preparations of meningococcal polysaccharide. The horizontal marks indicate the geometric mean antibody concentration.

TABLE II

The Antibody Responses of Adults Injected with Various Preparations of Group A and Group C Meningococcal Polysaccharide

Vaccine	Kd*	Route of administration	No., of subjects	No., of failures	Geometric mean antibody concentration	
					Pre-immune	Immune
					μg/ml	
C-6	0.30	s. c. (needle)	22	0	6.2	33.5
C-7	0.27	s. c. (needle)	19	0	<5.0	38.8
A-5	0.37	i. d. (needle)	34	0	4.7	31.4
A-7	0.50	s. c. (needle)	28	0	4.3	17.1
V-1	0.60	s. c. (needle)	25	5	5.3	9.0
V-4	0.27	s. c. (needle)	39	2	5.0	15.4

* Kd is the distribution coefficient as determined by Sepharose 4B gel filtration and provides an indication of the relative molecular size of the vaccine preparation (see text).

military recruits. V-1 and V-4 markedly different in molecular size. The former had a Kd of 0.60 suggesting an average molecular weight of approximately 30,000, the latter a Kd of 0.27 suggesting an average molecular weight of about 130,000. The serological responses to these vaccines were significantly different ($P < 0.001$) favoring the preparation with a higher molecular weight. Furthermore, five subjects (20% of the group) did not respond to lot V-1 (three had high antibody levels to start with and two had less than 3 μg/ml), whereas only two individuals (5.1% of the group) failed to respond to lot V-4 (one had a high initial titer and the other less than 3 μg/ml).

The immune response of children and infants immunized with group A polysaccharide. As a result of preliminary experiments with sera obtained from infants, it became obvious that the sensitivity which could be achieved with the Farr test using intrinsically labeled polysaccharide was inadequate. The group A polysaccharide was therefore labeled extrinsically with ^{125}I. To introduce covalently linked phenolic groups the polysaccharide was activated with cyanogen bromide and allowed to react with tyramine. Polysaccharide modified in this manner retains its antigenic integrity and is readily iodinated by the method of Greenwood et al. (17). The iodinated polysaccharide had a specific activity of approximately 1,000 cpm/ng.

The radioactive antigen binding test with this antigen was standardized by determining the antigen binding capacity of dilutions of a serum with known antibody content (E. C. G.), and the results are set forth in Fig. 5. A linear relationship between the per cent antigen bound and the logarithm of the antibody concentration was found between the limits of 10 and 80% antigen binding. Antibody concentration ranging from 0.1–44 μg/ml could be measured with three different antigen concentrations differing by tenfold.

This test was applied to the sera of 84 children and 51 infants immunized with various lots of group A polysaccharide and the results are summarized in the scattergrams in Fig. 6 and on Table III.

Children were immunized with 50 μg of three different lots of group A polysaccharide. Lot V-5 is a preparation whose physicochemical characteristics are very similar to lot V-4 which has been described previously. The experiment was designed to test the immunogenicity of these preparations in children and to compare subcutaneous with intradermal injection both performed by jet gun. The responses to V-4 and V-5 were similar and there was no significant difference between the intradermal and the subcutaneous route, although in both instances the subcutaneous route seemed slightly better. There was, however, a significant difference in the response to the subcutaneous administration of V-1 as compared to V-4 subcutaneously ($P < 0.001$) or V-5 subcutaneously ($P < 0.001$). All of the children who did not respond to the vaccine did have detectable antibody

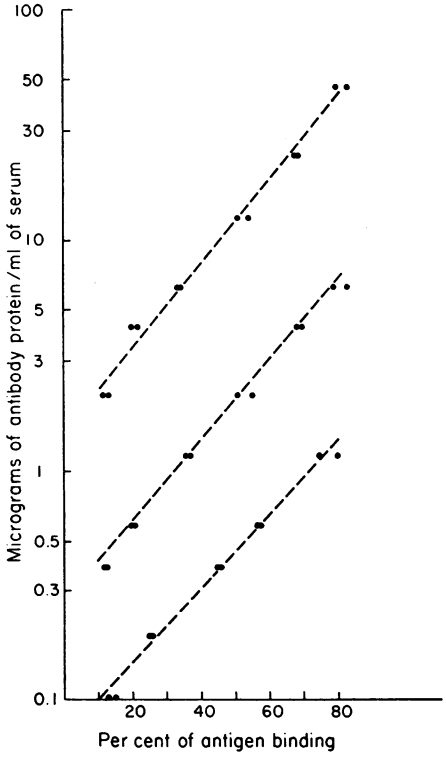

FIGURE 5 Antigen binding capacity of dilutions of a serum obtained 3 wk after immunization with group A polysaccharide. The per cent of added extrinsically ^{125}I-labeled group A polysaccharide bound is plotted against the antibody concentration. Three antigen concentrations differing by 10-fold were used.

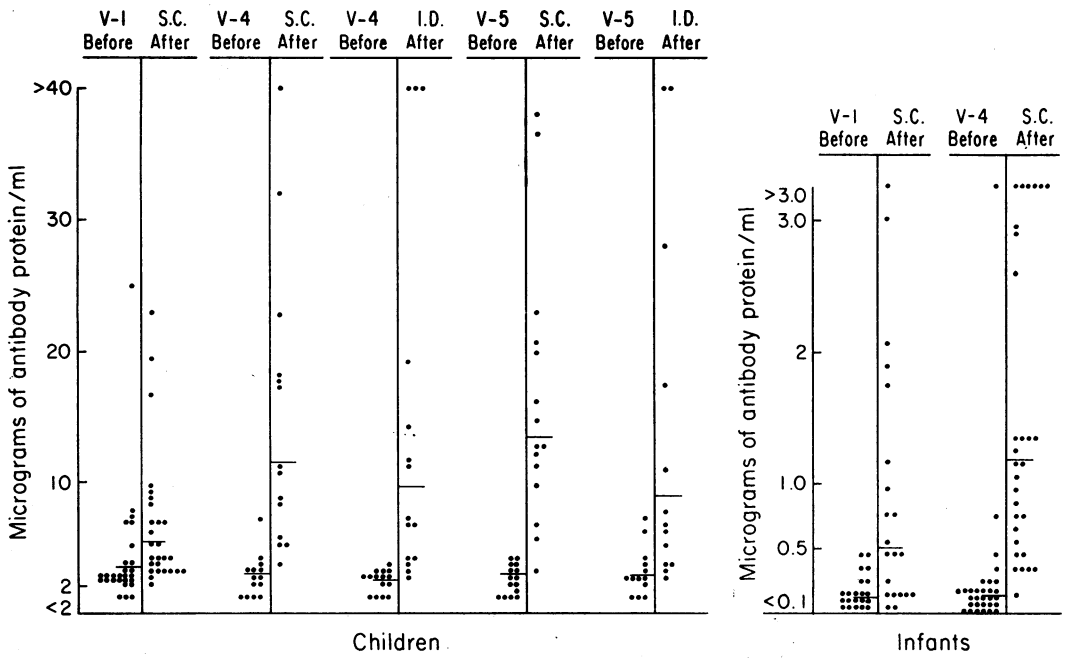

FIGURE 6 The antibody concentrations found in sera of children and infants before and 2 wk after immunization with various preparations of group A meningococcal polysaccharide. The horizontal marks indicate the geometric mean antibody concentration.

levels (greater than 2 μg/ml) in their preimmune sera.

Infants 6–13 months of age were injected subcutaneously by means of syringe and needle with 50 μg of lot V-1 or V-4 group A polysaccharide. The immune response of the infants was markedly lower than that seen among the children or adults with one exception. One 7 month old infant not included in the tabulation above to avoid skewing the distribution developed 40 μg of antibody per milliliter after immunization with vaccine V-4. His preimmune titer was less than 2 μg/ml. The other infants all had low responses but the majority did respond. Two individuals injected with V-1 did not develop a detectable level of antibody; that is to say, less than 0.1 μg/ml. One infant injected with V-4 had detectable antibody and did not have a rise following immunization. The response to V-1 was significantly ($P < 0.001$) inferior to the one obtained with V-4.

DISCUSSION

Of the methods presently available for the quantitative determination of low concentrations of antibody, the radioactive antigen binding test developed by Farr

TABLE III

The Antibody Responses of Children and Infants Injected with Various Preparations of Group A Meningococcal Polysaccharide

Vaccine	Route of administration	No. of subjects	Age	No. of failures	Geometric mean antibody concentration	
					Pre-immune	Immune
					μg/ml	
V-1	s. c. (needle)	27	6–9 yr	2	3.6	5.6
V-4	s. c. (gun)	14	7 yr	0	2.9	11.8
V-4	i. d. (gun)	15	7 yr	0	2.6	9.7
V-5	s. c. (gun)	15	7 yr	1	3.1	13.5
V-5	i. d. (gun)	13	7 yr	2	3.2	9.0
V-1	s. c. (needle)	21	6–13 months	2	0.14	0.51
V-4	s. c. (needle)	30	6–13 months	1	0.14	1.18

seemed the most suitable because of its simplicity and its versatility in terms of sensitivity. The quantity of antigen bound by a serum is a function of the concentration of antibody present and the average affinity of this antibody. In this study the radioactive antigen binding technique was standardized by reference to antisera with a known content of precipitating antibody to be able to convert the observed degree of antigen binding to antibody concentration. Strictly, this is correct only in the instance where the average association constant of the unknown serum and of the standard serum are identical. This criticism, of course, also applies to a greater or less extent to other immunological techniques such as quantitative precipitation. To explore the magnitude of the error introduced by ignoring differences in affinity of different antibodies, the antigen binding of dilutions of a number of sera with a known content of precipitating antibody was determined and the results are summarized in Figs. 2 and 3. All the points tend to fall rather closely on the line derived by least squares as is evidenced by the high correlation coefficients ($r = 0.94$) obtained in both cases indicating that for these sera the antigen binding capacity was closely correlated with the amount of antibody present as determined by quantitative precipitation. This suggests that little error is introduced by this treatment and that the association constant of these adult sera do not differ widely enough to be noticeable in this system.

The conversion of antigen binding capacity to antibody concentration, aside from the potential errors discussed above, has two advantages. It allows the results obtained in this study to be compared with the serological data obtained by quantitative precipitation on volunteers immunized with several pneumococcal polysaccharides. The other advantage is that the test is standardized by reference to antisera whose potency remains stable and which can readily be exchanged between different laboratories.

The response of 167 adults to vaccination with group A or group C meningococcal vaccines was measured. It can be concluded that the vast majority of adults injected with group C vaccine or with group A vaccine of high molecular weight had immune responses which quantitatively were comparable to those seen in individuals immunized with various pneumococcal polysaccharides (18). The responses to the group A vaccine apparently are lower than those obtained with group C vaccine. One preparation of group A vaccine, lot V-1, because of improper storage before packaging, was of considerably lower molecular weight than the other preparations tested. The immune response to this vaccine was significantly less, not only in adults but also in children and in infants. This is in accord with data obtained with various preparations of dextran of different molecular weight (19).

The response of children to two lots of group A vaccine of very high molecular weight, V-4 and V-5, were studied. The vaccine was administered by jet gun either intradermally or subcutaneously. In both instances the responses were comparable to those observed in adults injected subcutaneously by needle with vaccine lot V-4, and there was no advantage to intradermal injection. These data do not exclude the possibility that intradermal injection by needle may give a higher response than subcutaneous injection as suggested by the results obtained with adults, because there is considerable leakage from an intradermal injection site when performed with a jet gun.

Infants were injected with lot V-1 and V-4 and the response to the larger molecular weight vaccine was significantly higher. The mean response of these infants, who ranged in age from 6 to 13 months, was approximately tenfold lower than those observed in adults and children. The reasons for this lower response are at present unknown. The simplest possibility is that the infants are having a primary immune response, whereas the children are having an anamnestic response. It is true that the preimmune antibody levels of the infants are much lower than those seen in children and that almost half had no detectable antibodies (less than 0.1 μg/ml). This thesis can easily be tested in future studies by reimmunization of infants and observing whether they then produce a response akin to those seen in children or adults.

Controls consisting of infants not immunized a second time but studied over a longer period of time would resolve the question whether infants need more than 2 wk to mount their maximal immune response.

The crucial question is whether the immune response that the infants did mount is adequate to protect them from meningococcal disease. This will be answered definitively only by a large field trial. Nevertheless, it is a fact that agammaglobulinemic children are protected against meningococcal disease by passive immunoprophylaxis. It can be calculated that with the usual dose (0.7 ml/kg body weight) of concentrated gammaglobulin, and taking into account that the average concentration of antibody in adult sera is approximately 5 μg/ml, the concentration of antipolysaccharide antibody in the patient's blood stream would be less than 1.0 μg/ml. This consideration suggests that the low responses of the infants may be sufficient to be protective.

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