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THE ANTIBODY RESPONSE TO BACTERIOPHAGE φX 174 IN NEWBORN PREMATURE INFANTS *

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Quantitative studies in the past have indicated that the newborn human infant cannot be readily immunized against diphtheria or tetanus toxoids (1–3). It has been shown recently, however, that such newborns can usually be immunized to Salmonella antigens (4) and to certain viruses (5) and inconsistently rendered delayed-type hypersensitive to 2,4-dinitrofluorobenzene (6). These observations suggested that the incompetency of the newborn might be related to the type of antigen employed. It would be desirable, therefore, to study quantitatively the antibody response of newborns to an antigen likely to be an effective immunizing agent.

Bacteriophage φX 174 appeared to be an ideal antigen for this purpose, since minute amounts are highly antigenic for guinea pigs (7), and the assay for antibody is quantitative and extremely sensitive (8). When this bacteriophage was used for immunization, it was found that newborn premature infants produced antibodies as promptly and in as large amounts as did older children.

MATERIALS AND METHODS

Phage. Bacteriophage φX 174 was grown in *Escherichia coli* strain C in glycerol-casamino acid medium (9). The lysed culture was centrifuged at low speeds to remove cell debris. It was further purified by precipitation with ammonium sulfate, passage through a diethylaminoethyl (DEAE)-cellulose anion exchange column, and elution with 0.1 M ammonium acetate (10). This purified stock preparation was kept at 4° C in ammonium acetate buffer, pH 7.4, containing 0.01 per cent gelatin and 0.001 M calcium ion. Two phage stocks were used in this study: a preparation containing 2×10^{10} plaque-forming particles per ml in the first experiment and a preparation containing 4.5×10^{11} in the second. After purification, both preparations proved to be sterile upon bacteriologic culture. These bacteriophage preparations were extensively tested in guinea pigs, rabbits, and adult humans, and were found to be free of pyrogenicity, phlogogenicity, and any depressive effect on peripheral blood elements. In this paper, the number of phage particles will refer only to the plaque-formers.

Immunization. Newborn infants from the Premature Unit, and children, 2 to 10 years old, from the pediatric wards of Bellevue Hospital were studied. The older children were usually recovering from a viral upper respiratory infection. Dilutions of phage in saline were injected intramuscularly. Blood was obtained by heel or venipuncture before and at intervals after immunization.

Antibody titrations. The assay for antibody was carried out by the method described by Adams (8). The inactivation of phage by antibody follows first-order kinetics which is described by the relationship, $\ln(P_t/P_0) = K_t/D$. P_0 is the phage assay at zero time; P_t is the phage assay at time t minutes; D is the final dilution of antiserum; and K minutes^{-1} is the first order inactivation constant. The conditions necessary to obtain this exponential neutralization have previously been described (7). K, which is independent of dilution, is considered to be a measure of neutralizing antibody except, perhaps, for highly dissociable antibody.

One problem that was noted late in this study was a fall in the antibody titer of serum that had been stored at − 20° C for more than several months. For this reason, for one serum that had to be retitrated 1 year later to determine the physicochemical properties of the antibody, the K values so obtained were adjusted with regard to the original level to permit a valid comparison.

Double diffusion in agar was performed by the method of Preer (11). The tubes were kept at 4° C and observed for 2 weeks. The bacterial antigen preparation used for diffusion studies was prepared from a freshly grown *E. coli* C culture in nutrient broth which contained 5×10^9 organisms per ml. After sonication at 10 kv for 8 minutes, the supernatant was diluted 1:10 before use.

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Density gradient centrifugation. This was performed with 0.2 to 0.25 ml whole serum layered over a gradient formed from 21, 14, and 7 per cent saline in a Spinco model L ultracentrifuge with a SW 39 swinging-bucket rotor (12) at 22,000 rpm for 15 to 16 hours. The fractions were collected through a small perforation placed at the bottom of the centrifuge tube. Protein concentrations were determined by the Folin-Ciocalteu method (13). Samples were then pooled so as to obtain a rapidly sedimenting fraction that was rich in 19S γ-globulin and essentially free of 7S γ-globulin molecules, and a second fraction that contained the bulk of 7S γ-globulin and only small amounts of 19S γ-globulin. An intermediate fraction containing a mixture of the two types of molecules was not used. The purity of the three fractions was checked by capillary precipitin tests with antisera specific for 19S γ-globulin and antisera prepared to 7S γ-globulin (14). The fractions were exhaustively dialyzed against normal saline prior to assay for antibody activity.

Preparation of antibody against human 7S and 19S γ-globulin. Antiserum specific for 19S γ-globulin was prepared in rabbits by the subcutaneous injection in Freund's adjuvant of a pathological macroglobulin closely related antigenically to normal 19S γ-globulin. It was made specific for 19S γ-globulin by absorption with purified 7S γ-globulin, and gave only a single line of precipitation by immunoelectrophoresis against whole normal human serum (14). Antibody specific for 7S γ-globulin was prepared in a similar fashion by immunizing rabbits with fragment B, prepared from human 7S γ-globulin by papain digestion. It was made specific by absorption with fragment C and no longer reacted with 19S γ-globulin or γ-1-A-globulin (15).

RESULTS

Anti-φX formation in humans. In the first experiment, eight premature newborn infants and three older children were each injected with 10^9 φX. In the second experiment, in order to immunize on a weight basis, three premature and three older children received 10^9 φX per 1,500 g body weight. Serum was obtained at 1, 2, 3 to 4, and 6 to 8 weeks after immunization and the anti-φX content determined. One premature and one older infant were rechallenged with 10^9 φX 6 weeks after the first injection of phage, and serum was obtained 1 week later.

As can be seen in Table I, the antibody response to φX appeared to be generally similar in the newborn premature infants, even when they were immunized immediately after birth, and in the older children. All subjects in both experiments showed easily detectable serum antibody at

TABLE I

The anti-φX response in children and premature newborns

Expt.*	Group	Birth wt	K of sera†		
			1 wk	2 wk	3 or 4 wk
		g			
1	Children‡	nd§	4.3	37.6	44.0
		nd	4.0	37.0	28.6
		nd‖	2.6	36.8	nd
	Premature newborn‡	1,320‖¶	6.8	nd	nd
		1,600¶	4.7	36.8	53.2
		1,300¶	3.4	75.5	57.7
		1,550	1.4	64.8	88.0
		2,060	3.1	40.0	44.0
		1,900	4.8	165.0	nd
		1,880	7.6	51.0	86.0
		1,900	9.2	58.6	nd
2	Children	nd	0.3	6.3	nd
		nd	0.3	3.8	nd
		nd	0.4	1.9	nd
	Premature newborn	1,970	0.8	4.0	nd
		1,475	0.3	2.3	nd
		1,100	0.3	4.3	nd

* Experiments performed 1 year apart with different preparations of bacteriophage. Each individual in the first experiment received an i.m. injection of 0.1 ml containing 10^9 φX particles in saline regardless of weight. In experiment 2, each individual was immunized i.m. with 10^9 φX per 1,500 g body weight.

† Preimmunization sera contained no detectable antibody.

‡ The children were 2 to 10 years old; the premature newborns were less than 96 hours old unless otherwise indicated.

§ Not done.

‖ Reimmunized at 6 weeks with 10^9 φX and sera obtained 1 week later. *K* values were 977 and 830 for the older child and infant, respectively.

¶ Not immunized until 3 to 4 weeks of age.

1 week ($K = 0.3$ to 10), and antibody levels rose considerably for at least 2 to 3 weeks. Moreover, an efficient secondary antibody response was obtained in the one premature and the one older child tested.

Sera obtained from five newborns and three older children 1 or 2 weeks after immunization were tested for precipitating antibody by double Preer agar diffusion (11) with 4.5×10^{11} ϕX per ml. Of the 2-week sera, all the newborn and two of three from the older children gave one or more lines of precipitation. Two of three sera obtained 1 week after immunization of newborns also showed a precipitation line. No such precipitation occurred if a sonicated preparation of *E. coli* C was used as the antigen. Moreover, the time of appearance and intensity of the bands correlated with the K values. These findings led to the conclusion that the precipitating antibody was directed against the virus.

Physicochemical properties of antibodies to ϕX. During the primary antibody response to ϕX in guinea pigs, the anti-ϕX molecules are first of 19S and later of 7S type (7). The physicochemical characteristics of human antibody to ϕX were therefore investigated by separating the two major classes of antibodies by preparative zone ultracentrifugation, and by measuring the effect upon antibody activity of 2-mercaptoethanol and treatment with rabbit antihuman 7S or 19S γ-globulin. It is known that treatment with 2-mercaptoethanol causes dissociation of 19S γ-globulins to 7S units that lack antibody activity (16). Sera from four newborn premature infants and from four older children obtained at 1 and 4 to 8 weeks after immunization were treated with 0.8 per cent 2-mercaptoethanol and subjected to ultracentrifugation in a saline density gradient. Two sera from one premature infant were also treated with rabbit antibody that was specific for either human 7S or 19S γ-globulin. The anti-ϕX content was determined for all sera before and after these procedures.

Table II records the findings in one representative subject, a premature newborn immunized with ϕX 96 hours after birth. As can be seen, the initial anti-ϕX response was of the 19S type. The antibody activity was most concentrated in the rapidly sedimenting fraction after ultracentrifugation, and the antibody activity disappeared after

TABLE II

Physicochemical characterization of antibody to ϕX during the primary antibody response of one premature newborn *

Treatment of sera		K of sera 1 week	K of sera 7 weeks
None		3.5	116.0
2-mercaptoethanol†		0‡	115.0
Density gradient ultracentrifugation	Rapidly sedimenting	3.95	4.6
	Slowly sedimenting	0.66	48.1
Rabbit anti-19S§		0	94‖
Rabbit anti-7S¶		nd	0

* Birthweight 2,060 g; injected i.m. with 10^9 ϕX at 96 hours of age.
† Serum dilutions containing 0.8% 2-mercaptoethanol incubated for 30 minutes at 37° C.
‡ K less than 5×10^{-3}.
§ 0.2 ml incubated with 0.1 ml of a 1:10 dilution of anti-ϕX serum for 3 hours at 37° and 3 days at 4° C. Supernatant was then tested for antibody to ϕX.
‖ K adjusted with respect to original values. See Methods.
¶ Similar to the above except that 0.5 ml was added to 0.1 ml of a 1:100 dilution of anti-ϕX serum.

treatment with 2-mercaptoethanol or after the addition of rabbit antibody prepared specifically against human 19S γ-globulin. In 2 to 7 weeks these antibody molecules were replaced by antibody molecules of the 7S type. At 7 weeks the anti-ϕX activity was most concentrated in the slowly sedimenting fraction obtained by ultracentrifugation and was unaffected by treatment with 2-mercaptoethanol and rabbit antihuman 19S γ-globulin. Antibody activity disappeared, however, after treatment with rabbit antihuman 7S γ-globulin.

The other seven subjects in the experiment showed similar results; i.e. a change in the molecular species of anti-ϕX molecules from 19S to 7S type occurred between 2 and 6 weeks after primary immunization.

DISCUSSION

The studies reported here indicate that the antibody response to ϕX is remarkably similar in newborn premature infants and in children 2 to 10 years of age in the following respects. a) The amount of antibody produced by 1 week after immunization was 10^3 to 10^4 times the minimal detectable amount, and antibody levels continued to increase for at least the following 2 to 3 weeks. These results are similar to anti-ϕX formation in the guinea pig, in which antibody can be detected as early as 24 hours after immunization (7), which suggests that anti-ϕX production in the

human may also begin at this early time. b) Rechallenge with ϕX provoked a typical secondary response in the one infant and one older child tested. c) The physicochemical characteristics of anti-ϕX molecules formed during a primary response to 10^9 phage particles changed during 2 to 6 weeks after immunization from molecules belonging to the 19S class of γ-globulin to those of 7S sedimentation constant.

These results are in striking contrast to previous quantitative studies of the antibody response to diphtheria and tetanus toxoids in the newborn and older child (1–3). The latter studies suggest that the premature or full-term newborn cannot form detectable antitoxin during the first month of life. For example, after injection of 125 μg of diphtheria toxoid on alum, antitoxin cannot be detected for 4 to 10 weeks after birth in newborns, whether premature or full-term, while in older children, 0.05 to 0.1 U of antitoxin is usually present within 2 weeks after immunization. The marked difference in the immune responses of the premature newborn human to bacteriophage ϕX 174 compared with tetanus or diphtheria toxoid may be accounted for by immunization procedure, sensitivity of the assay system, or nature of the antigen. It would be unlikely for the immunization procedure to be the cause of the differences observed since the larger dose of toxoid (125 μg toxoid protein compared with approximately 10^{-2} μg phage protein) and the use of alum would be expected to enhance rather than suppress antibody formation. Although it has been shown that increasing the dose of antigen may result in a delay of detection of an immune response (7), such a mechanism would not account for an immunologic paralysis lasting 4 to 8 weeks. Difference in sensitivity of the antibody assays can be excluded because of the finding of precipitating antibody to ϕX within 1 week after immunization. This observation indicates that such sera contain at least 1 μg antibody nitrogen per ml (17); an equivalent amount of diphtheria antitoxin can be easily measured by the extremely sensitive technique of toxin neutralization in rabbit skin, which can detect as little as 0.0025 μg antitoxin nitrogen per ml (18). Thus, by exclusion, it appears that the nature of the antigen may be the most important factor responsible for the differences in the immunizing capacity of toxoid compared with ϕX. This possibility is further supported by other immunization experiments utilizing these antigens, which indicate the unusually good antigenicity of ϕX. For example, as little as 6×10^2 plaque-forming particles injected intravenously in saline may be sufficient to stimulate an immune response in a 300-g guinea pig (7). Moreover, the same early antibody response to 10^{10} ϕX is obtained in the rabbit, chicken, frog, and goldfish, although a similar immunization with diphtheria toxoid in two of these species does not produce detectable antitoxin (19). There is no information available at present concerning the qualities of either antigen that are responsible for these differences in antigenicity.

We have therefore tentatively interpreted our results to indicate that immunological maturation may not be represented by a single change from a nil-period to one of complete competence, but may involve at least one intermediary stage in which only certain types of antigens can induce a full antibody response.

In 1939, Kabat (20) first noted a change from 19S γ-globulins to nonhomogeneous, more slowly sedimenting components in horses immunized with pneumococcal polysaccharide. He suggested that this change might represent breakdown of the antibody into smaller active fragments. More recently, Stelos and Taliaferro (21) have described the change from a 19S to 7S type of antibody response during immunization with sheep red cells in the rabbit. A similar change in the immune response has been reported to other antigens in the rabbit (22, 23), to Salmonella in the human (24), and to ϕX in the guinea pig (7). The present studies indicate that a viral antigen in the human will produce a similar pattern of response, and that the premature newborn infant, although not mature immunologically, is capable of a 19S γ-globulin antibody response indistinguishable from that of an older child to one type of antigen. In this respect the results presented here differ from those of Smith (4), who found that the early 19S antibodies to Salmonella antigens were still active after depolymerization to the 7S state. The reason for this difference is not apparent at this time.

It has also been shown that a similarly early 19S type of antibody response to ϕX can occur in nonmammalian vertebrates (19). Thus, the early

19S antibody response is a general immune response of many species to some, if not to all, antigens and appears early in the development of the immune mechanism. It will be of particular interest, therefore, to examine more primitive species and other embryos or newborns for their capacity to form 19S and 7S types of antibody.

SUMMARY

Premature newborn infants and older children show a similar antibody response to injection of minute amounts of bacteriophage ϕX 174 as judged by the following criteria. *1*) Easily detectable neutralizing antibody is produced during the first week; *2*) the titer rises for an additional 2 to 3 weeks; *3*) an efficient secondary antibody response can be achieved by reimmunization 6 weeks later; *4*) a change occurs in the molecular species of antibody, from 19S to 7S sedimentation constant 2 to 6 weeks after immunization. These observations contrast with past studies demonstrating newborn incompetency toward other antigens.

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