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Antibody Studies in Hypogammaglobulinemia *

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Serum immunoglobulins are detectable in the majority of individuals with even extreme hypogammaglobulinemia, and evidence of antibody formation has been found in some (1–5). It has been suggested that antibody production might be detectable in all hypogammaglobulinemic patients if sufficiently sensitive assay methods were employed (2). This report describes the results of a study of antibody formation in eight hypogammaglobulinemic children using a potent antigen, bacteriophage ØX 174, and two sensitive methods for evaluating antibody formation, *in vivo* phage clearance rates and *in vitro* phage neutralization. The magnitude and nature of the antibody responses observed after primary and secondary immunizations will be discussed in comparison with a group of immunologically sound individuals.

Methods

Patients and controls. Patients were eight children with abnormal serum immunoglobulin levels and previously established objective evidence of defective immunologic responsiveness (Table I). These children included three sets of siblings.

Patients 1 and 2 are identical twin girls who have had recurrent severe bacterial infections since infancy. Patient 3 is a boy with repeated bacterial pneumonias and otitis media since the age of 4 years. Patients 4 and 5 are brothers with a history of only one severe infection each. Patient 6 has had numerous episodes of pneumonia since infancy. She has a palpable spleen and lymph nodes. Patients 7 and 8 are brothers with ataxia telangiectasia who have had recurrent pneumonias, otitis media, and β-hemolytic streptococcal pharyngitis since infancy. None of these patients has a family history of recurrent infections, malignancies, or collagen disease, other than siblings as noted.

Lymph node biopsies of Patients 2, 3, 4, and 5 showed features previously described as typical in hypogammaglobulinemia (3). The nodes were hypocellular, germinal centers were absent, and mature plasma cells were absent or only very rarely seen. A lymph node was not examined from Patient 1. Patient 6 had very different lymph node morphology, with many large germinal centers with mitotically active cells, but no mature plasma cells. Nodes from Patients 7 and 8 showed small, ill-defined germinal centers with prominent histocytes and rare, scattered plasma cells.

Patients 4 and 5 have remained well without therapy. The others have had marked reductions in numbers of infections since the institution of gamma globulin therapy.

Controls were five children and one adult from a school for the retarded, with normal serum immunoglobulin levels and histories indicating no unusual susceptibility to infections.[1]

Phage preparation. Bacteriophage ØX 174 was grown and purified as described by Uhr, Finkelstein, and Baumann (6).[2] Purified phage was sterilized by passage through a 0.3-μ Millipore filter.[3] Lack of pyrogenicity or effect on peripheral leukocyte count was established by animal testing. The final preparation was stored in single dose vials at − 60° C until thawed for use.

Phage clearance studies. After a blood sample from each subject was obtained to determine the original level of serum phage neutralizing activity, 1.5×10^9 plaque-forming units (PFU) of phage per kilogram of body weight was given intravenously. It has been stated that ØX remains largely in the vascular space after such administration (6). This quantity of phage produced a circulating level of approximately 10^8 PFU per ml of serum. Blood samples were obtained 15 minutes after phage administration and two to three times daily thereafter until phage was no longer detectable. Each sample

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[2] Bacteriophage was obtained through the kindness of Dr. Neal Groman of the Microbiology Department, University of Washington, Seattle, Wash.

[3] Millipore Filter Corp., Bedford, Mass.

TABLE I

Clinical and immunologic information on patients studied

Patient	Age	Sex	Immunoglobulins IgG	IgA	IgM	Blood type	Isoagglutinin* titer	Antigenic stimuli: typhoid, diphtheria toxoid, polio, influenza vaccines†
	years		*mg/100 ml*					
1	10	F	375	23	35	A+	Anti-B 1:16	No evidence of induced antibody
2	10	F	206	<4.5	15	A+	Anti-B 1:8	No evidence of induced antibody
3	14	M	170‡	<4.5	9	O+	Anti-A 0 Anti-B 0	No evidence of induced antibody
4	4	M	170	4.5	15	O+	Anti-A 0 Anti-B 0	No evidence of induced antibody
5	9	M	120	120	15	O+	Anti-A 0 Anti-B 0	No evidence of induced antibody
6	3	F	12	<4.5	6	O+	Present: too weak to titer	No evidence of induced antibody
7	4	M	200	14	460	A+	Anti-B 1:4	Typhoid O agglutinin 1:5; H agglutinin 1:80; no other antibodies.
8	6	M	80	14	400	A+	Anti-B 1:16	Typhoid O agglutinin 1:5; H agglutinin 1:20. Schick test became negative. No other antibodies.
Adult (normal)			1,200	280	120			

* Isohemagglutinins can occur in hypogammaglobulinemic patients with severe impairment of antibody response (3).
† Three or more injections with each antigen.
‡ While on exogenous γ-globulin. Native level estimated to be less than 100 mg per 100 ml.

was serially diluted, and the number of PFU per milliliter of serum was determined by using the agar overlay pour plate method (7).

Neutralizing antibody. After circulating phage had disappeared, blood was obtained at intervals, and the level of specific serum ØX-neutralizing activity was determined. Phage neutralization occurs in an exponential fashion, and the rate of inactivation (K) is described by the relationship: $K = 2.3 \times (D/T) \times \log (Po/Pt)$. D = the reciprocal of the serum dilution; T = the time in minutes during which the reaction occurred; Po = the phage titer at the start; Pt = the phage titer at time T (7). K then may be used as a measure of antibody activity.

Heterologous coliphages, λ and f_2, were used to establish the specificity of acquired anti-ØX activity.[4]

Characterization of antibody. Antibody to phage was characterized in each serum sample by exposure to 2-mercaptoethanol (2-ME) as described by Grubb and Swahn (8), and by sucrose density gradient ultracentrifugation (9). In some instances chromatography on DEAE cellulose by the method of Kochwa, Rosenfield, Tallal, and Wasserman (10) was also done. For ultracentrifugation, 0.1 ml of serum in an equal volume of normal saline was placed onto a continuous gradient of 5 to 25% sucrose in normal saline. Normal human serum free of anti-ØX activity was also layered on as a marker when hypogammaglobulinemic serum was being studied. The tubes were centrifuged for 15 hours at 25,000 rpm on a SW 39 rotor in the Spinco model L refrigerated ultracentrifuge at 30° F. Tube contents were collected in ten equal fractions by puncturing the tube bottom. IgG and IgM were identified in the fractions by the use of specific rabbit antisera in agar double diffusion plates. Other amounts of the fractions were incubated with ØX to locate phage-neutralizing activity in relation to the immunoglobulins.

The various methods correlated exactly in that antibody activity that was 2-ME sensitive also sedimented rapidly on ultracentrifugation and eluted with the 1 M NaCl fraction from DEAE. That some of the 2-ME sensitive activity may have been related to IgA antibody cannot be ruled out except that no discrepancies between 2-ME and ultracentrifugation results were seen (11). In the text, therefore, antibody that is 2-ME sensitive and rapidly sedimenting will be called IgM, and that which is 2-ME resistant and slowly sedimenting will be called IgG.

Quantitations of immunoglobulins. Immunoglobulin levels in patient sera were quantitated by radial diffusion of serum in antibody-agar plates (12).[5]

Results

Clearance of circulating phage. In all controls and in four of eight patients it was possible to follow the disappearance of phage from the circulation. Uhr established that ØX is eliminated from the circulation of animals in two exponential phases. The first slower phase is said to reflect nonimmune mechanisms, and the second more rapid phase results from the effect of specific antibody (6) In Figure 1, the shaded area represents the range of clearance of the six control subjects, and the individual lines represent patients. Three patients cleared in the control range, with two

[4] These were also obtained from Dr. Neal Groman.

[5] Through the courtesy of Dr. John Fahey.

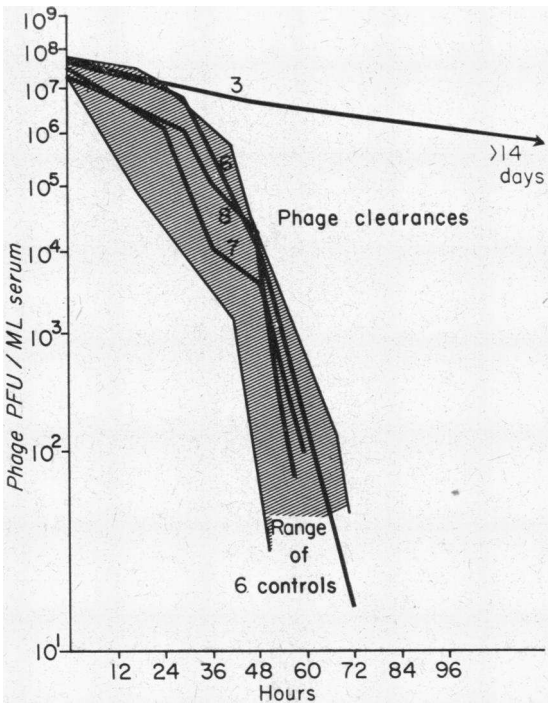

FIG. 1. CLEARANCE RATES OF CIRCULATING PHAGE. Clearances in Patients 3, 6, 7, and 8 and in six immunologically normal individuals. PFU = plaque-forming units.

phase curves characteristic of antibody formation. The fourth patient maintained high levels of circulating phage for the 14 days we were able to observe him. No phage remained by 1 month. The rate of clearance in this last patient approximated the rate of inactivation of ØX when it was incubated *in vitro* at 37° C either alone or with nonimmune serum. He was the only patient who did not produce neutralizing antibody at any time.

Although phage clearance could not be measured in the remaining four patients, no phage was detectable in sera obtained from them at 1 week after inoculation.

Although not included in the present data, we had the opportunity to study one full-term newborn at 6 hours of age who also demonstrated a two-phase clearance curve with phage disappearance by age 3 days and neutralizing antibody by age 4 days.

The primary antibody response. Serum was obtained 1 week and 1 month after primary inoculation and assayed for phage-neutralizing activity. The results are summarized in Figure 2 and Table II.

At 1 week, controls had developed 10^3- to 10^4-fold increases in serum neutralizing activity over preimmunization levels, with K values comparable to those found by Uhr and associates in a group of healthy newborns and children (13). All patients except No. 3, who had persistent circulating phage and developed no increase in serum antiphage activity at any time, showed approximately tenfold increases in activity. In both controls and patients all antibody appeared to be IgM, as it was 2-mercaptoethanol sensitive, was limited to the bottom fractions of sucrose density gradients after centrifugation, and did not pass through DEAE columns in the manner of IgG.

At 1 month all but one of the controls showed further increases in antibody activity, and about 10% of this activity was now associated with IgG. Among the patients, only No. 7 and 8 developed higher levels of activity than at 1 week and had a

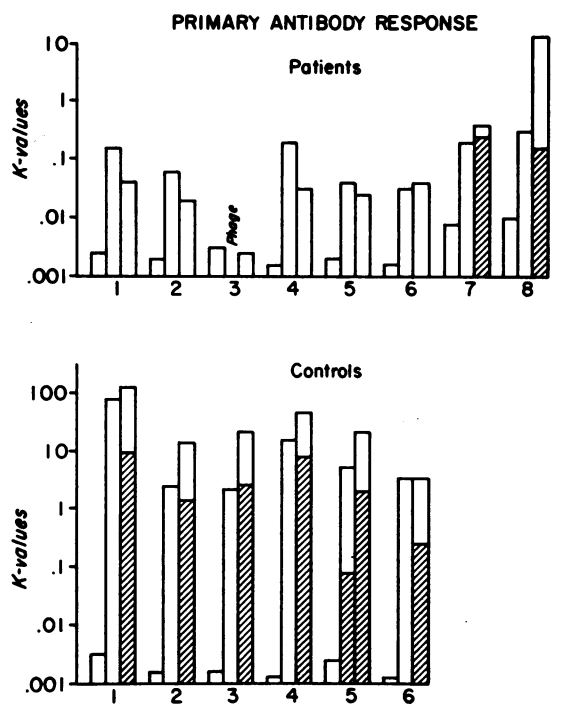

FIG. 2. PRIMARY ANTIBODY RESPONSE TO ØX. Bars represent serum phage inactivation rate (K values) at a given time. From the left, the first bar represents preimmunization serum; the second bar represents serum at 1 week; and the third bar represents serum at 1 month after primary immunization (third bar represents serum at 4 months for Patients 7 and 8). Each entire bar represents total serum antiphage activity; the shaded portion represents 2-mercaptoethanol (2-ME) resistant activity.

TABLE II

Neutralizing antibody responses of patients and control subjects

	Primary response				Secondary response			
	K values*				K values*			
	Pre.	1 Wk.	1 Mo.	4 Mo.	Pre.	1 Wk.	1 Mo.	8 Mo.
Patients								
1	0.004†	0.2/0‡	0.06/0	§	0.004	0.7/0		0.3/0.03
2	0.003	0.08/0	0.03/0		0.006	20/0		0.3/0.2
3	0.005	Phage‖	0.004		0.004	Phage	0.004	
4	0.002	0.3/0	0.05/0		0.009	1.4/0.03	4.8/	6.5/5.6
5	0.003	0.06/0	0.04/0		0.03/0	0.6/0.06	7.8/1.7	1.5/ .9
6	0.002	0.05/0	0.06/0		0.001		0.4/0	
7	0.009	0.3/0		0.6/0.4	0.11/0	25.6/2.8	1.6/0.2	0.2/0
8	0.010	0.5/0		13.3/0.2	2.9/0	850/36	107/	53/1.5
Controls								
1 (Hea)	0.005	90/0	126/9.7		20/17	310/310	280/214	54/64¶
2 (Kr)	0.002	4/0	14/1.4		9.6/9.6	372/246	117/115	2/2
3 (Mart)	0.002	3.5/0	33.6/4.1		9/7	750/440	252/123	48/34
4 (PQ)	0.001	19.6/0	68/8.9		16/16	210/170	72/61	60/75
5 (Bou)	0.004	7.3/0.09	36.8/3					
6 (Gag)	0.001	5.4/0	5.3/0.4					

* K = serum phage inactivation rate.
† Approximates K of phage incubated alone at 37° C.
‡ Numerator = total serum neutralizing activity; denominator = 2-mercaptoethanol (2-ME) resistant activity.
§ Not done.
‖ Circulating phage still present.
¶ This apparent increase in activity has been noted by Uhr on occasion with 2-ME (23).

mixture of IgG and IgM antibody. The others had generally lower levels of activity, which still consisted of only IgM antibody.

Thus, although primary responses to this antigen could be elicited in seven of eight hypogammaglobulinemic children, such responses were of reduced magnitude and less well sustained than in controls. In most cases they did not show the progression of response from early IgM to later IgG antibody normally seen with this antigen (13).

The secondary response. All patients and four control subjects were given intravenous boosters of phage 4 to 8 months after primary immunizations (Figure 3, Table II). The amount of phage was the same as was used for primary stimulation. Serum was obtained before re-immunization and at 1 week, 1 month, and 8 months thereafter for antibody determinations.

Prebooster sera of both controls and patients showed a marked reduction in activity from sera obtained at 1 month after the primary stimulation. Antibody in sera from controls was almost entirely IgG. Antiphage activity in patient sera was no higher than preprimary stimulation levels in four (Patients 1, 2, 3, and 6) and somewhat above this level in three (Patients 4, 5, and 7). Patient 8 maintained a relatively high level of activity. All antibody in patient sera at this time was IgM.

One week after restimulation, antibody levels in control subjects were 3 to 25 times greater than the highest recorded during the primary response, and most of the activity was associated with IgG. Antibody activity then gradually declined so that by 8 months levels less than 30% of those at 1 week remained. IgG antibody still predominated.

Secondary responses in the patient group were quite varied. Seven of eight patients challenged responded with antibody levels at 1 week several- to 1,000-fold greater than the highest responses observed after primary stimulation, but in contrast to the control responses antibody activity was still predominantly in the IgM fraction. Antibody was still present in the sera of six patients who were observed to 8 months. In some, IgG antibody predominated, whereas in others IgM did.

Antibody specificity. In each patient the antibody formed was determined to be specific for ØX; inactivation of control phages λ and f_2 did not occur.

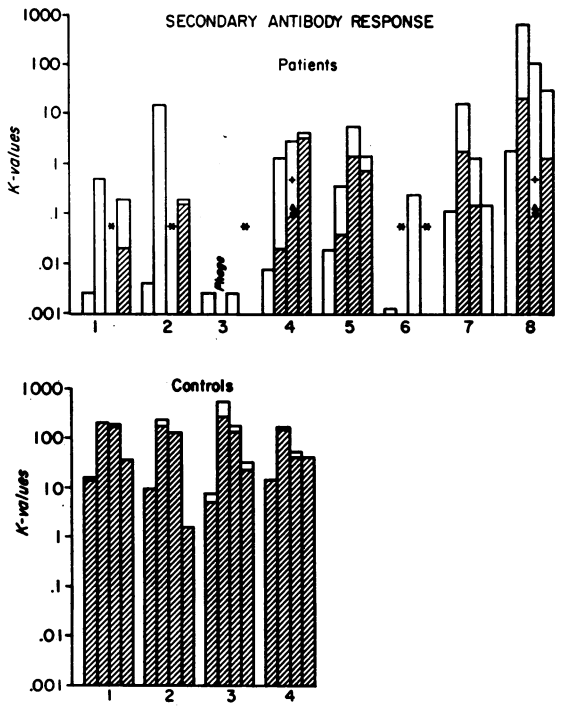

FIG. 3. SECONDARY ANTIBODY RESPONSE TO ØX. From the left, the first bar represents serum before ØX booster; the second bar represents serum 1 week after the booster; the third bar represents serum 1 month after the booster; and the fourth bar represents serum 8 months after the booster. An asterisk indicates that the determination was not done; a plus sign indicates that 7 S and 19 S antibodies are present and that 2-ME sensitivity has not been determined. Each entire bar represents total serum antiphage activity, and the shaded portion represents 2-ME resistant activity.

Discussion

Primary and secondary antibody responses to bacteriophage ØX 174 were elicited in seven of eight hypogammaglobulinemic children. This supports previous reports of limited antibody production in many patients with hypogammaglobulinemia. However, patient antibody responses clearly differed from those of the control group whose responses were similar to those obtained by Uhr and associates in normal infants (13) using the same antigen. ØX has also been used as an antigen in lower species, including mammals (6), chickens (14, 15), amphibians, and fish (15). In normal humans and animals, rapidly sedimenting antibodies appeared first and were replaced later by slowly sedimenting antibodies. Although the chicken exhibited the coexistence of both types of antibodies after immunization, rapidly sedimenting antibody appeared first (14). Since the hypogammaglobulinemic patients produced mostly IgM antibody after primary and secondary stimulation, they showed not only a quantitative reduction of the normal response, but also a distortion of the usual immunoglobulin sequence of response for this antigen.

Patients also differed among themselves. One (Patient 3) cleared phage slowly and produced no neutralizing antibody after either primary or secondary stimulation and hence truly represented a nonresponder. After primary stimulation, Patients 1, 2, 4, 5, and 6 developed poorly sustained IgM responses of considerably lower magnitude than control values. Patients 7 and 8 with the primary diagnosis of ataxia telangiectasia developed antibody levels that were higher and more persistent than those of the other patients and included some IgG as well as IgM antibody. After challenge, all patients except for 3 showed anamnestic responses consisting solely or largely of IgM antibody. Patients 4 and 5, whose primary responses had been similar to those of Patients 1, 2, and 6, did develop some IgG as well as IgM antibody during the early secondary response, unlike the others.

These differences in antibody response furnish further evidence of the nonhomogeneity of the patient group. As noted earlier, clear differences among patients in terms of severity of infections, lymphoid morphology, immunoglobulin levels, and homograft reactions exist (16). Because of this heterogeneity, it is difficult to fit the first six patients into the usual classifications of the hypogammaglobulinemias (4). None of the males can be proven to have a sex-linked disease since no other relatives are affected. These six patients could be called congenital sporadic cases. Certainly the presence of two pairs of affected siblings among the six suggests that at least for these four the disease is of a congenital nature. Patients 1 and 2, who by blood groups and dermatoglyphics are identical twins (concordance of $> 99\%$), show a greater than tenfold difference in antibody response 1 week after secondary stimulation. Their serum IgA levels also differ to as great a degree (Table I). This suggests that factors other than purely genetic ones can influence both serum immunoglobulin levels and degree of antibody re-

sponse and indicates that immunoglobulin levels may be of only limited value in classifying types of immunologic defects. Patients 4 and 5, brothers, are unusual in that they have had only one severe infection each and continue well without therapy. Yet, their immunoglobulin levels and the degree of impairment of their antibody responses are not greatly different from other patients studied (Table I). Patient 6 is similar to other reported cases of girls with hypogammaglobulinemia and splenomegaly (17, 18). Patients 7 and 8, brothers, are typical cases of ataxia telangiectasia with hypogammaglobulinemia (19, 20). With the exception of the last two patients, who have a defined primary diagnosis, this diversity could represent either variations in the expression of a single basic defect or a number of separate defects with superficially similar clinical presentations.

The observation of secondary responses involving IgM antibody requires further comment. Many antigens, including ØX, have been said to induce a primary response in which the first antibody to appear is IgM. IgG antibody subsequently replaces the IgM, and after secondary stimulation IgG antibody predominates, although this scheme may require re-examination in view of the studies of Freeman and Stavitsky, who showed that protein antigens resulted in primary responses with the simultaneous appearance of IgM and IgG antibodies (21). Furthermore, this pattern of response is not true for all antigens, since, for example, typhoid O (9) and brucella (22) evoke persisting IgM antibody responses. With systems in which this sequence does occur, Uhr and Finkelstein, using ØX (23), and Svehag and Mandel, using poliovirus (24, 25), determined that for these antigens the IgM system does not possess immunologic memory. They showed that successive low doses of antigen elicited only brief, nonsustained IgM responses that did not increase in magnitude with repetition, whereas larger doses of antigen resulted in the above described sequential response. The cessation of IgM synthesis after small doses of antigen was thought to result from antigen depletion. That occurring with larger doses of antigen was felt to result from the suppressive effect of IgG antibody on IgM synthesis, perhaps by rendering antigen unavailable to the IgM system. Secondary stimulation produced no IgM response greater than that obtained with the primary stimulation. Evidence supporting this negative effect of IgG antibody on IgM antibody synthesis has accumulated (26–29).

Certain conditions have been defined under which IgM antibody synthesis can be prolonged and IgG synthesis suppressed. These include irradiation (23, 25), the administration of drugs such as 6-mercaptopurine (6-MP) (29), or the use of certain germ-free animals (30).

Furthermore, reports of macroglobulin anamnestic responses to a variety of antigens have recently appeared. Borel, Fauconnet, and Miescher, using erythrocytes as antigen, produced IgM secondary responses in rabbits (31) and mice (32) by varying antigen dosage or site of administration or by using 6-MP. Nossal, Austin, and Ada, with *Salmonella flagella* antigen, were able to obtain either predominately IgG or IgM secondary responses by employing different dose combinations for primary and secondary stimulation (33). Porter demonstrated IgM memory in rabbits, using bovine or human albumin, by waiting until primary antibody was no longer detectable before restimulating the animals (34). Rosenquist and Cambell found increases in both rapidly and slowly sedimenting antibody after secondary stimulation using ØX in the chicken (14).

These studies illustrate some of the diverse factors that influence the expression of the primary or secondary antibody response. Most of these experimental models that result in prolonged IgM synthesis or IgM secondary responses seem to have in common either an immature, damaged, or perhaps phylogenetically more primitive lymphoreticular system exposed to a "normal" dose of antigen, or a normal lymphoreticular system exposed to a "reduced" amount of antigen. In the patient studies here presented, the persistence of IgM antibody is in keeping with the concept of an impaired system of IgG synthesis, which allows prolonged IgM synthesis. The nature of such impairment is entirely speculative.

Summary

After administration of a bacteriophage antigen, primary and secondary antibody responses were demonstrable in seven of eight hypogammaglobulinemic children as determined by measurement

of circulating phage clearance rates and the acquisition of specific serum phage-neutralizing activity. However, patient antibody responses differed both quantitatively and qualitatively from those of control subjects. Primary and secondary responses in patients were marked by a predominance of IgM antibody with minimal development of the IgG response seen normally with this antigen. Differences in antibody responses support the concept that the congenital sporadic hypogammaglobulinemias are an etiologically heterogeneous group. The demonstration of macroglobulin secondary responses provides further evidence that the IgM immunoglobulin system can express immunologic memory under certain circumstances.

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