influx into alveoli and ultrastructural differentiation. Pediat. Res., *11*: 197 (1977).
33. Zeligs, B. J., Nerurkar, L. S., and Bellanti, J. A.: Increase in fungicidal activity of rabbit alveolar macrophages during animal development. (In preparation.)
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Diphtheria
IgG subclasses
restricted antibodies
tetanus

Partially Restricted Antitoxins of Tetanus and Diphtheria in Man

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Summary

Antibodies of restricted specificity have been identified in the human in response to certain antigens. The present study analyzed tetanus and diphtheria antitoxins isolated from selected human sera and suggested a restricted reponse in antibody production to each of these antigens. Purified antibodies from eight serum specimens with elevated hemagglutination titers to tetanus and four to diphtheria yielded only IgG proteins in concentrations of 160–500 μg/ml. Although some of the tetanus specimens were derived from cord sera and tetanus immunoglobulin, none of the total group had antibodies of the IgA and IgM classes. Utilizing immunoelectrophoresis against heavy chain subclasses, genetic markers, and κ and λ quantitations, a predeliction for the κ IgG_1 subclass was established for both tetanus and diphtheria antibodies. The λ light chains were present in diminished quantities, IgG_2 heavy chains were absent, and the IgG_3 and IgG_4 chains were variably identified.

Speculation

The elucidation of those antigens which result in restricted antibody formation has apparent clinical significance, in view of the fact that some individuals possess selective defects in the production of certain IgG subclasses.

The light and heavy chain differences cause human immunoglobulins of each of the classes to exist in several forms, in accordance with variations in the way in which these chains combine. Since IgG heavy chains, as an example, occur in four subclasses (IgG_1, IgG_2, IgG_3, and IgG_4) and the light chains in two types (κ and λ), eight different IgG molecules are possible by varying combinations of light and heavy chains. Normally, the IgG_1 subclass constitutes about 66% of total IgG, 23% IgG_2, 7% IgG_3, and 4% IgG_4. When combined with the IgG_1 chain, the light chain κ:λ ratio is about 2:1.

Although most antibodies produced in the human appear to be heterogeneous, the response to certain antigens may result in antibody immunoglobulin which is relatively or even completely restricted. Rh antibody, as an example, is primarily an IgG_1 immunoglobulin with some IgG_3 and IgG_4 but no IgG_2 molecules (5, 10), whereas bacterial polysaccharide antigens elicit predominantly IgG_2 production (7). Antibodies against factor VIII are formed preferentially within the IgG_4 subclass (1). Levan provokes a highly restricted manufacture of κ IgG_2 globulins, approaching the homogeneity of a myeloma protein (17).

Utilizing available immunologic techniques, criteria have been proposed for establishing antibody specificity (3, 16). Employing several of these criteria, the results of this investigation suggest that the human responds with partial antibody restriction to the toxins of *Clostridium tetani* and *Corynebacterium diphtheria*.

MATERIALS AND METHODS

Eight specimens containing tetanus antibodies were derived from three sources: five were samples of newborn cord sera, two were taken from human antitetanus immunoglobulin pools, and one was serum of a 5-year-old burn patient taken several weeks after a tetanus toxoid booster immunization. Four sera containing diphtheria antibodies were from children, aged 7 months to 11 years, with recent clinical diphtheria. Specimens were obtained within a period not exceeding 6 months from the time of active disease. Antibody titers to tetanus and diphtheria in sera and in purified antibody preparations were measured by a hemagglutination technique using the toxoids of either antigen coupled to human type O Rh^+ red blood cells by the chromic chloride method (2).

Purified antibody preparations were prepared in the following manner. Tetanus or diphtheria toxoid (Wyeth, aluminum-precipitated toxoid containing 0.01 mg protein N/ml for tetanus and 0.004 mg protein N/ml for diphtheria) (3–4 ml) was incubated with 1 ml serum for 1 hr at 37°, then refrigerated overnight. The precipitate was washed 12 times. The supernatant and early washes were discarded after being tested for specific antibody activity. The precipitate was then incubated with acid-glycine buffer, pH 3.0 (1 ml), overnight at room temperature. The supernatant was dialyzed against normal saline for 1 hr and the residual material discarded. The dialysate (about 1 ml in quantity), representing a purified antibody preparation, was used for subsequent analyses.

The following methods were applied to sera or the purified preparations to establish the nature of the antibody isolated. (*1*) The major immunoglobulin classes were quantitated by double radial immunodiffusion using commercially available reagents (6). (*2*) Gm (a), Gm (b), and Gm (f) antigens (Gm numerical nomenclature: Gm (a) = 1; Gm (b) = 5; Gm (f) = 4) were typed by inhibition of hemagglutination using specific Gm antibodies and appropriate Rh coats of known Gm specificity, coupled to Rh positive red blood cells (13). The Gm (a) and Gm (f) markers were used to identify the IgG_1 heavy chain and the Gm (b) marker, the IgG_3 heavy chain. Gm (a) and (f) quantitations were done by an automated method, described by Litwin (4), which measures the percentage of inhibition of hemagglutination of each Gm type. (*3*) The κ and λ light chains were quantitated by double radial immunodiffusion on plates prepared in our laboratory using specific antisera derived from immunization of New Zealand white rabbits with isolated Bence-Jones proteins. (*4*) The heavy chain subclasses of IgG_1, IgG_2, IgG_3, and IgG_4 were identified by immunoelectrophoresis and double diffusion against specific heavy chain antisera obtained from immunization of New Zealand white rabbits with specific myeloma proteins having IgG_1, IgG_2, and IgG_4 chains. IgG_3 antisera was obtained by immunization of a baboon with an IgG_3 myeloma protein.

RESULTS

The major serum immunoglobulin classes in patients *Gr* (tetanus) and *39*, *40*, and *131* (diphtheria) were within normal limits, with mean values of IgG 1194 mg/dl, IgM 117 mg/dl, and IgA 239 mg/dl.

The titer of purified tetanus antibody, with one exception, was one to three 2-fold tube dilutions lower than that found in the sera. Of the major immunoglobulin classes, IgG was present, and IgM and IgA were absent in all separations (Table 1). The lowest limit of sensitivity for the detection of IgA and IgM was 5 μg/ml. The same information for the purified diphtheria antibody preparations is shown at the bottom of Table 1. Here, the antibody titers were all one 2-fold tube dilution lower than the levels in serum. As noted in the tetanus preparations, purified diphtheria antibody revealed the presence of IgG γ-globulin only. IgM and IgA were absent.

Table 2 shows the κ and λ light chain quantitations, and the corresponding IgG concentrations of the purified tetanus antibody preparations. Both κ and λ light chains were identified; the proportion of IgG molecules containing κ chains was 80–90% of the total, as compared to 10–20% for the λ. The lower limit of sensitivity for the detection of lambda molecules was about 25 μg/ml. The data for purified diphtheria antibody show similar proportions of κ- to λ-containing antibody.

The heavy chain analyses of tetanus purified antibody, as determined by the Gm systems which identify IgG_1 and IgG_3, and by immunoelectrophoresis with specific subclass antisera for all four types, are depicted in Table 3. For comparison, Gm phenotypes of the sera are also shown with each corresponding phenotype of purified antibody in the adjacent column. Each of the samples reveal Gm (a) and/or Gm (f) markers and, as expected, the IgG_1 heavy chain on which these antigens are located is present. Where Gm (b) is found, so also is the IgG_3 chain. The IgG_2 and IgG_4 heavy chain identification, shown by antisera reactions only, reveal the absence of IgG_2 chains in all of the specimens (with the exception of *TIG III*, which was not tested).

Data for diphtheria purified antibody are shown in Table 3. The Gm and heavy chain antisera analyses correspond. In each of the four specimens IgG_1 and IgG_4 heavy chains are present and IgG_2 and IgG_3 chains are lacking. The lower limit of sensitivity for the detection of heavy chains was ≈15 μg/ml.

The Gm (a) and Gm (f) allotypic quantitations, as a reflection of the amount of IgG_1 heavy chain present in the purified specimens, are shown in Table 4. Determinations were made on three of the tetanus and the four diphtheria samples. For the most part, the quantitations of these allotypic markers approximate the IgG concentrations measured, indicating a predominance of the IgG_1 chain in the purified preparations.

To determine what effect, if any, acid-glycine might have on immunoglobulin content, two specimens of normal sera, brought to a concentration of γ-globulin approximating that of the antibody preparations, were reacted with the buffer and then dialyzed against normal saline. For comparison, similar dilutions of sera were made with normal saline. The combined results of these studies are shown in Table 5. All immunoglobulin antigens tested were present after reaction with the buffer, although there was some decline in IgG concentration. The IgA and IgM globulins remained intact after being subjected to buffer, and both κ and λ light chains (in proper proportions) and all heavy chains were in evidence.

Because of the small quantities of purified material derived, and the number of procedures done with these specimens, antibody nitrogen determination was not attempted. To ensure that all specific antibody was removed from each of the original serum samples, the initial supernatant fluids, after precipitation from sera and the first washes of the precipitate, were tested for either tetanus or diphtheria antibodies. In none could antibody be detected. Further, the purified antibody of patient *Gr* was reprecipitated with tetanus antigen to determine whether any nonspecific γ-globulin was present in the supernatant of the purified preparation. Here, too, none was found. These results indicate that the purification process isolated all specific antibody and did not carry along nonspecific immunoglobulins.

Table 1. *Purified antibody preparations (immunoglobulin classes (micrograms per ml))*

Patient	IgG	IgM	IgA
Tetanus			
Be[1]	500	0	0
Fi[1]	300	0	0
Wi[1]	400	0	0
Pe[1]	200	0	0
Ho[1]	360	0	0
Gr	330	0	0
TIG II[2]	1440	0	0
TIG III[2]	960	0	0
Diphtheria			
39	250	0	0
40	405	0	0
21	560	0	0
131	400	0	0

[1] Cord blood specimens.
[2] Pooled human tetanus immunoglobulin.

Table 2. *Purified antibody preparations (light chain concentrations (micrograms per ml))*

Patient	κ	λ	IgG
Tetanus			
Be	380	78	500
Fi	295	65	300
Wi	370	30	400
Pe	170	25	200
Ho	335	40	360
Gr	325	60	330
TIG II	1350	NT[1]	1440
TIG III	1110	NT	960
Diphtheria			
39	225	45	250
40	345	90	405
21	470	NT	560
131	340	NT	400

[1] Not tested.

Table 3. *IgG allotypes*

Patient	Serum Gm phenotype	Purified antibody: Gm phenotype	IgG_1	IgG_2	IgG_3	IgG_4
Tetanus						
Be	a+ b+ f−	a+ b+ f−	+	−	+	+
Fi	a+ b+ f−	a+ b+ f−	+	−	+	+
Wi	a+ b+ f−	a+ b+ f−	+	−	+	+
Pe	a+ b+ f−	a+ b+ f−	+	−	−	−
Ho	a+ b+ f−	a+ b+ f−	+	−	+	+
Gr	a+ b+ f+	a+ b− f−	+	−	−	−
TIG II	a+ b+ f+	a+ b+ f+	+	−	+	−
TIG III	a+ b+ f+	a+ b+ f+	+	NT[1]	NT	NT
Diphtheria						
39	a− b+ f+	a− b− f+	+	−	−	+
40	a+ b+ f+	a+ b− f+	+	−	−	+
21	a+ b− f−	a+ b− f−	+	−	−	+
131	a+ b+ f+	a+ b− f+	+	−	−	+

[1] Not tested.

Table 4. *Purified antibody preparations (Gm (a) and Gm (f) Quantitations (micrograms per ml))*

	Gm phenotype	Gm (a)	Gm (f)	IgG
Tetanus				
TIG II	a+ f+	780	460	1440
TIG III	a+ f+	550	300	960
Gr	a+ f−	280	0	330
Diphtheria				
39	a− f+	0	250	250
40	a+ f+	100	90	405
21	a+ f−	700	0	560
131	a+ f+	250	100	400

Table 5. *Control sera at 1:20 dilution reacted with saline and acid-glycine buffer*

	IgA, μg/ml	IgM, μg/ml	IgG, μg/ml	κ, μg/ml	λ, μg/ml	IgG_1	IgG_2	IgG_3	IgG_4
BZ									
Saline	109	75	660	630	260				
Buffer	106	69	410	420	237	+	+	+	+
GN									
Saline	54	125	500	500	228				
Buffer	50	107	370	390	130	+	+	+	+

DISCUSSION

No significance, with regard to antibody selectivity, can be attributed to the expected absence of IgA and IgM classes of γ-globulins in the specimens derived from cord sera or from tetanus immune globulin pools. The failure to identify these immunoglobulins in the purified tetanus antibody preparation of the burn patient who received a tetanus toxoid booster is important. Of added significance, in terms of antibody restriction, was the absence of an IgA or IgM class of diphtheria antibody in the purified material derived from four patients who had recent clinical disease. These findings are at variance with those of Yount *et al.* (17), who describe IgA and IgM tetanus and diphtheria antibodies in one adult subject after the administration of tetanus and diphtheria toxoids. Conceivably, some IgA and IgM antibodies may have been present in our preparations, but in quantities too small to be detected by our test systems, *i.e.*, in amounts <5 μg/ml. Alternatively, since we were unable to determine precisely whether the times of blood sampling were still within the acute phases of disease, later sampling raises the possibility that IgA and IgM antibody production ceased at an earlier stage.

In order to be assured that exogenous factors were not responsible for the destruction of IgA and IgM molecules, two specimens of normal sera were incubated with the acid-glycine buffer, as in the separation procedure. No changes in IgA and IgM concentrations were observed. Some decline in IgG concentration was noted following the buffer treatment, but the normal κ:λ ratio remained unaltered and none of the heavy chain subclasses were eliminated. The fall in IgG content probably results from aggregation of these molecules (14) and may explain the somewhat lower antibody titers measured in the purified preparations when compared with the titers measured in serum.

Thus, using methods to quantitate immunoglobulin classes and light chains, to describe and quantitate Gm factors, and to identify heavy chain subclasses we have been able to demonstrate that there exists partial antibody restriction within the IgG class in response to the toxins of tetanus and diphtheria. Although uniform homogeneity of antibody could not be established, since some λ-containing molecules and IgG_3 and IgG_4 heavy chains were variably identified, the quantitative data strongly suggest a κ IgG_1 predeliction for these antitoxins.

The absence of the IgG_2 heavy chain in all of our specimens is clearly of significance. Although one might argue that IgG_2 in cord sera may be relatively scarce (15), others have shown cord sera to possess normal adult proportions of this heavy chain in relation to the other three subclasses (8, 9). Furthermore, our other preparations also lacked the IgG_2 chain. As IgG_2 antibody tends to be associated with bacterial capsular polysaccharides, the failure to find this molecule in any of our samples may relate to the protein nature of the tetanus and diphtheria antigens (14).

Several reports have described individuals with an inability to produce certain heavy chain molecules, while maintaining the capability to manufacture others (11, 12, 18). Such partial defects often result in an increased susceptibility to infections. The existence of heavy chain subclass deletions in the human adds clinical relevance to the recognition of those antigens which elicit restricted antibody responses.

REFERENCES AND NOTES

1. Anderson, B. R., and Terry, W. D.: Gamma G_4-globulin antibody causing inhibition of clotting factor VIII. Nature, *217*: 174 (1968).
2. Gold, E. R., and Fudenberg, H. H.: Chromic chloride: A coupling reagent for passive hemagglutination reactions. J. Immunol., *99*: 859 (1967).
3. Kunkel, H. G.: Individual antigenic specificity, cross specificity and diversity of human antibodies. Fed. Proc., *29*: 55 (1970).
4. Litwin, S. D., and Camp, F. R., Jr.: Automated detection of Gm factors of human γG globulin. Vox Sang., *17*: 194 (1969).
5. Litwin, S. D.: Allotype preference in human Rh antibodies. J. Immunol., *110*: 717 (1973).

6. Mancini, G., Carbonara, A. O., and Heremans, J. F.: Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry, *2*: 235 (1965).
7. McCracken, G. H., Jr., and Sarff, L. D.: Current status and therapy of neonatal *E. coli* meningitis. Hosp. Pract., *9*: 57 (1974).
8. Mellbye, O. I., Natvig, J. B., and Kvarstein, B.: Presence of IgG Subclasses and C1q in human cord sera. Protides Biol. Fluids Proc. Colloq. Bruges, *18*: 127 (1970).
9. Morell, A., Skvaril F., van Loghern, E., and Kleenola, M.: Human IgG subclasses in maternal and fetal serum. Vox Sang., *21*: 481 (1971).
10. Natvig, J. B., and Kunkel, H. G.: Human immunoglobulins: Classes, subclasses, genetic variants and idiotypes. Advan. Immunol., *16*: 1 (1973).
11. Rivat, L., Ropartz, C., Burtin, P., and Cruchaud, A.: Genetic control of deficiencies in γG subclasses observed among families with hypogammaglobulinemia. Nature, *225*: 1136 (1970).
12. Schur, P. H., Borel, H., Gelfand, E. W., Alper, C. A., and Rosen, F. S.: Selective γG globulin deficiencies in patients with recurrent pyogenic infections. N. Engl. J. Med., *283*: 631 (1970).
13. Steinberg, A. G.: Progress in the study of genetically determined human gamma globulin types (the Gm and Inv groups). Progr. Med. Genet., *2*: 1 (1962).
14. Turner, E. W., and Boyer, P. D.: Interaction of protein antigens and antibodies. III. Dissociation studies with diphtheria toxoid-antitoxin precipitates. J. Immunol., *69*: 265 (1952).
15. Wang, A. C., Faulk, W. P., Stukey, M. A., and Fudenberg, H. H.: Chemical differences of adult, fetal and hypogammaglobulinemia IgG immunoglobulins. Immunochemistry, *7*: 703 (1970).
16. Wilhamson, A. R.: Isoelectric focusing of immunoglobulins. In: Handbook of Experimental Immunology, Vol. 1, Chapt. 8, Ed. 2 (Blackwell Scientific Publications, Oxford, 1973).
17. Yount, W. J., Dorner, M. M., Kunkel, H. G., and Kabat, E. A.: Studies on human antibodies. VI. Selective variations in subgroup composition and genetic markers. J. Exp. Med., *127*: 633 (1968).
18. Yount, W. J., Hong, R., Seligmann, M., and Kunkel, H. G.: Structural gene defects and preponderance of γG_3 subgroup in patients with hypogammaglobulinemia. J. Clin. Invest., *48*: 92a (1969).
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