INFECTION AND IMMUNITY, Mar. 1981, p. 1132-1137
0019-9567/81/031132-06$02.00/0

Vol. 31, No. 3

Analysis of Human Anti-Diphtheria Antibodies by Isoelectric Focusing: Evidence for Restricted Clonal Heterogeneity of Anti-Fragment A Antibodies

CASEY D. MORROW,* ERIC M. MACY, AND RONALD H. STEVENS

Department of Microbiology and Immunology, School of Medicine, University of California, Los Angeles, Los Angeles, California 90024

The in vivo human humoral response to diphtheria toxoid-tetanus toxoid booster immunization was studied by isoelectric focusing analysis of sera obtained after immunization. The anti-diphtheria toxoid (immunoglobulin G [IgG]-Dip), anti-fragment A (IgG-Frag A), and anti-tetanus toxoid antibodies from 20 donors post-booster immunization were focused by using agarose isoelectric focusing and visualized by development with radiolabeled antigens. The quantities of the IgG-Dip and IgG-Frag A antibodies correlated with the number of bands seen on the isoelectric focusing pattern in that more bands were found in the spectrotypes of donors with high serum levels of antibody. No difference was apparent in the antibody spectrotypes obtained from sera of donors at successive times post-booster immunization. Individual heterogeneity of the different donors' spectrotypes was often found for IgG-Frag A antibodies, but a close comparison of several different donors revealed antibodies with the same spectrotype patterns. Thus, individual clones of antibody were revealed in humans after in vivo immunization, particularly when antibodies against antigens of restricted epitope size were analyzed. Additionally, the sharing of certain antibody spectrotypes among several individuals raised the possibility that certain antibody clones may be preferentially expressed in the human population.

Most of our knowledge of the regulation of the human humoral immune response has been derived from studies measuring the quantity, heterogeneity, and genetics of antibodies formed in vivo after immunization with appropriate antigens (2, 3, 7, 19-21, 23, 24). Studies on the human antibody response at a clonal level, however, have been limited because of the complexity of the antigens used for immunization and the clonal antibody heterogeneity of the resulting immune response. Such an analysis of human antibodies at a clonal level would seem necessary for a finer analysis of the genetics and regulation of human immune responses to naturally occurring pathogens.

In this study we have resolved the antibodies produced in response to immunization with tetanus toxoid (TT) and diphtheria (DT) toxoid (molecular weights, 160,000 and 62,000, respectively) by isoelectric focusing (IEF), an approach successfully used in murine studies (5, 14). Additionally, to reduce the complexity of the antigens and to simplify the resulting spectrotype pattern, we have used the smaller enzymatically active polypeptide of DT, fragment A (molecular weight, 21,145), as an agent for visualizing antibody clonal patterns (8). With this approach we have shown that, with decreasing antigen size, fewer antibody clones are detected in sera of immunized individuals and monoclonal responses can often be observed. Furthermore, it appears that certain spectrotypes may be preferentially expressed in the human population.

MATERIALS AND METHODS

Materials. DT-TT used for immunization was obtained from Lederle Laboratories, Pearl River, N.Y. All IEF equipment was obtained from LKB, Stockholm, Sweden. Diphtheria toxin was purchased from Connaught Laboratories, Willowdale, Ontario, and was further purified when necessary by chromatography on Sephadex G-150. TT was generously provided by Wyeth Laboratories, Marietta, Pa. Sodium sulfate was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. Dimethylsuberimidate was obtained from Sigma Chemical Co., St. Louis, Mo. X-ray film, developers, and fixers were obtained from Eastman Kodak, Rochester, N.Y. Rabbit anti-human immunoglobulin was obtained from Cappell Laboratories, Cochranville, Pa., and was purified by ammonium sulfate fractionation and diethylaminoethyl (DE52) chromatography. All other materials were obtained as previously described (20).

Methods: immunizations. Normal human donors were immunized with alum-precipitated DT-TT. Blood was drawn before immunization and at biweekly

intervals for 8 weeks.

Preparation of serum samples. Blood was drawn in heparinized syringes, diluted 1:1 with 0.9% saline, and separated from mononuclear cells (used for other studies) on Ficoll-Hypaque density gradients (4). The plasma was removed and stored at −20°C. An immunoglobulin-rich fraction for IEF analysis was prepared by mixing equal volumes of plasma and saturated ammonium sulfate for 1 h. The precipitate was reconstituted with 0.2 M glycine and dialyzed against 0.2 M glycine with three changes of the dialysate. The immunoglobulin fraction was then diluted to achieve the equivalent of a 2.5:1 concentration of immunoglobulin in the sera.

Radioimmunoassay. Plasma samples were assayed for anti-DT (immunoglobulin G [IgG]Dip), anti-fragment A (IgG-Frag A), and anti-TT (IgG-Tet) antibodies by a solid-phase radioimmunoassay as described previously (20). Briefly, DT, fragment A, or TT was used to coat flexible polyvinyl microtiter plates for at least 4 h at room temperature. The coating solutions were removed and stored for further use. The plates were then washed three times with 1% egg ovalbumin (OA), and the remaining binding sites were saturated with 5% OA for 1 h at room temperature. The plates were washed with 1% OA, the samples were added, and the final volume was adjusted to 0.3 ml with 5% OA. After 12 to 14 h, the samples were discarded and the plates were washed three times with 1% OA. Diethylaminoethyl-purified, isotype-specific rabbit anti-human antibody was iodinated (^{125}I) by the lactoperoxidase enzyme method. Iodinated antibody (specific activity, 2,000 to 4,000 cpm/ng) was added to the samples, and after 4 to 5 h, the plates were washed three times with 1% OA and eight times with water. Individual wells were cut out and counted on a Beckman gamma counter. Amounts of specific antibody were calculated relative to the specific activity of the ^{125}I-labeled rabbit anti-human antibody.

Preparation of antigens. Diphtheria toxin was converted to a toxoid by the method of Linggood et al. (16). Fragment A was obtained from the intact molecule after proteolytic nicking (12). A 20-ml amount of diphtheria toxin (10 to 12 mg/ml) was dialyzed for 5 h against 0.1 M tris(hydroxymethyl)aminomethane (Tris), pH 8. Proteolytic nicking of the toxin was accomplished by incubating the dialyzed toxin with 200 μl of 1 M ethylenediaminetetraacetic acid, 2.0 ml of dithiothreitol (1 M), and 200 μl of trypsin (1 mg/ml) for 10 min at 37°C. Soybean trypsin inhibitor was then added at twice the concentration of the trypsin; the solution was made up to 6 M urea, and 3.0 ml of 1 M iodoacetamide was added. The mixture was applied to a G-100 Sephadex column equilibrated with 6 M urea in 0.1 M Tris-hydrochloride (pH 8) buffer, and 10-ml fractions were recovered. The fractions corresponding to fragment A were pooled, and the purity was established by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When the pooled fragment A preparations showed >5% contamination with other proteins, the material was rechromatographed to remove those contaminants.

IEF. Agarose IEF was performed per LKB instructions and Rosen et al. (18). Briefly, the gel matrix was prepared by heating a 10% sorbitol–0.8% agarose (LKB agarose EF) solution to near boiling; LKB ampholines 3.5 to 9.5, specific for agarose IEF, were added to the solution at a final concentration of 0.5%, and the gel was poured onto a plastic sheet. After cooling, the gel was placed in a humidified box at 4°C overnight. The next day, the gel was blotted with Whatman filter paper for 10 min. Focusing was carried out on an LKB Multiphor apparatus. Immunoglobulin fractions (15 μl) were loaded onto the gel on small pieces of filter paper. In preliminary studies it was found that placing the samples nearer the cathode provided optimal focusing. Constant power (6.25 W/gel) was applied for 30 min, at which time the sample filter papers were removed. Power was maintained for an additional 35 min for complete focusing. At the conclusion of the run, the pH was measured by a flat-bed electrode. The gel was then immediately immersed in saturated sodium sulfate at 37°C for 1 h. Precipitated proteins were cross-linked with 0.43% dimethylsuberimidate in Tris-buffered (pH 8) saturated sodium sulfate for 45 min; non-cross-linked groups were blocked by 0.5% glycine in Tris buffer, pH 8, in water for 30 min at room temperature (17). The gel was then placed in 1% bovine serum albumin for 30 min. DT, fragment A, or TT was iodinated by the lactoperoxidase enzyme method. The iodinated proteins were diluted in 1% bovine serum albumin to a concentration of 30 to 60 μg/ml (specific activity, 2,000 to 3,000 cpm/ng). The gels were incubated with the iodinated proteins for 3 h and then washed overnight in 15 liters of phosphate-buffered saline, pH 7. The next day, the gel was fixed in 10% trichloroacetic acid, washed twice with 95% ethanol, and press dried. Routinely, the gels were stained with 0.35% Coomassie brilliant blue and then autoradiographed on Kodax X-R Omat film.

RESULTS

Concentrations of IgG-Tet, IgG-Dip, and IgG-Frag A in sera of donors. The amount of IgG-Dip antibody in the sera of donors increased 2.8- to 31-fold (median, 6.0-fold) after booster immunization. As expected, different individuals' antibody levels varied considerably after immunization. In general, however, the serum antibody levels in each of the immunized individuals were IgG-Tet > IgG-Dip > IgG-Frag A.

The percentage of IgG-Dip antibodies directed against fragment A varied with individuals, ranging from 71 to 6%. No obvious correlation existed between the levels of IgG-Dip and IgG-Frag A (Table 1).

Spectrotypes of IgG-Tet, IgG-Dip, and IgG-Frag A antibodies. In preliminary studies it was found that, in most individuals, no distinct clones of antibodies could be visualized by IEF before booster immunization, although a faint smear was observed in some individuals. After immunization, distinct antibody spectrotypes were observed in the sera.

Serum samples were ordered with respect to antibody levels, and the spectrotypes of IgG-Tet, IgG-Dip, and IgG-Frag A were determined by

TABLE 1. *Serum concentrations of IgG-Frag A, IgG-Dip, and IgG-Tet antibodies*

Donor	Concn (ng/μl of serum)[a]			IgG-Frag A/IgG-Dip (ng) × 100%[b]
	IgG-Frag A	IgG-Dip	IgG-Tet	
1	41.0	189	87	21
2	35.0	144	200	24
3	28.0	88	210	37
4	20.0	32	141	62
5	18.0	118	111	15
6	14.0	90	212	15
7	11.0	70	270	16
8	10.0	37	89	26
9	9.0	40	47	22
10	6.0	22	194	30
11	5.5	21	32	26
12	5.0	7	34	71
13	4.5	50	45	8
14	3.7	30	153	12
15	2.1	31	176	7
16	1.8	14	80	13
17	1.6	25	158	6
18	1.2	14	79	9
19	1.1	6	18	18
20	0.9	9	58	10

[a] At 2 to 4 weeks post-booster immunization. IgM-specific antibodies were less than 5% of total IgG as determined by radioimmunoassay.

[b] A plot of these values versus nanograms of IgG-Frag A or IgG-Dip per microliter of serum had r values of −0.29 and −0.10, respectively.

agarose IEF. Analysis of IgG-Tet antibodies by IEF routinely showed complex smears, and individual clones were rarely observed (Fig. 1). The spectrotype pattern of IgG-Dip showed that multiple bands could be visualized in the pH range of 7 to 9 (Fig. 2). A greater number of bands with a darker intensity was observed in those samples with higher serum concentrations of IgG-Dip.

The IgG-Frag A antibodies showed spectrotypes similar to, but more restricted than, the IgG-Dip spectrotypes. The striking feature of the IgG-Frag A response was the visualization of distinct clones and the variety of the IEF spectrotypes (Fig. 3). For example, donor 2 had a multibanded, complex spectrotype, whereas donor 8 showed a single dark band with two to three minor light bands. Another interesting donor was number 1, in which most of the IgG-Frag A antibodies were confined to a pH range of 7 to 9, but a major portion of the antibodies also focused at pH 5 to 5.5. The spectrotype patterns of donor IgG-Frag A antibodies were not altered when the serum was obtained at different times after immunization (data not shown).

Evidence for antibodies with the same specificities and pI's in different individuals. After screening sera from 20 individuals, it appeared that some of the fragment A spectrotypes were common among different donors. In particular, a pattern of four bands at a pI of approximately 7.5 to 8.5 (i.e., donors 16 and 9) and a pattern of two bands at pI 8.5 to 9.0 (i.e., donors 8 and 4) appeared to be similar. Sera from donors with similar clones were run individually or were mixed with one another and focused to determine whether there were common IgG-Frag A bands. Donors 16, 9, 17, and 15 had identical spectrotypes in the four-band (pH

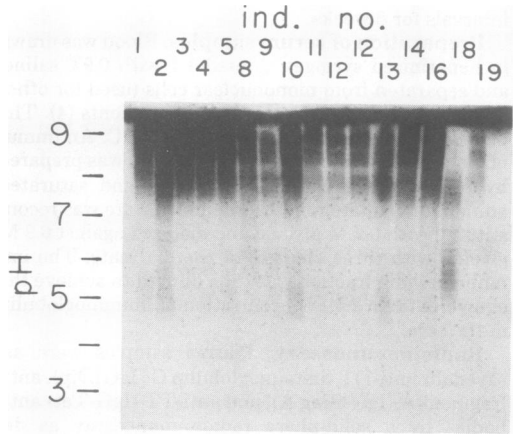

FIG. 1. *Spectrotype of IgG-Tet antibodies. Immunoglobulin fractions from 15 donors were focused as described in the text. Gels were incubated with iodinated TT (3,000 cpm/ng) for 3 h at room temperature, washed overnight, fixed, dried, and autoradiographed 24 h. The numbers refer to those individuals listed in Table 1.*

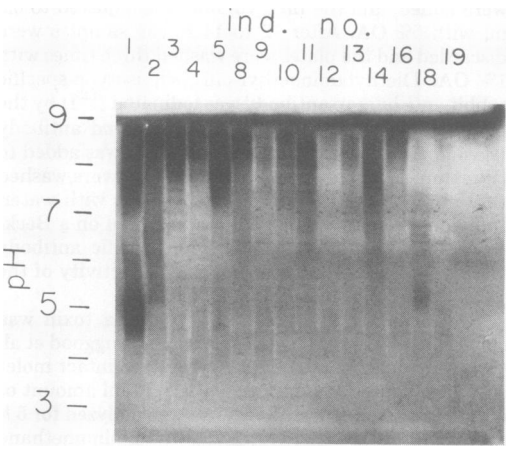

FIG. 2. *Spectrotype of IgG-Dip antibodies. Immunoglobulin fractions from 15 donors were focused as in Fig. 1. Gels were incubated with iodinated DT (2,500 cpm/ng) for 3 h. The gels were autoradiographed for 24 h. Numbers correspond to those individuals listed in Table 1.*

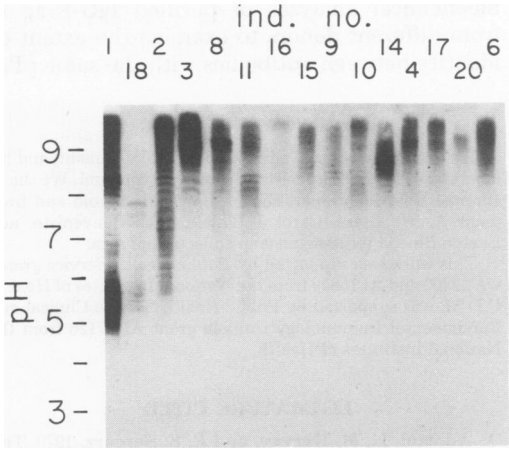

FIG. 3. *Spectrotype of IgG-Frag A antibodies from various donors. Plasma samples from 15 donors were focused and incubated with fragment A (3,000 cpm/ng) for 3 h. The gel was autoradiographed for 9 h. The numbers refer to those individuals listed in Table 1.*

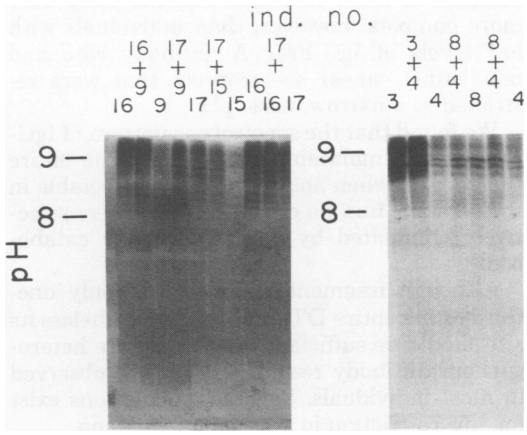

FIG. 4. *Repeat IgG-Frag A spectrotypes among different individuals. Sera from donors 16, 9, 17, 15, 3, 4, and 8 were focused and developed with fragment A (3,000 cpm/ng). Where single numbers appear, one serum was focused (15 μl); two numbers refer to an equal mixture (10 μl each) of sera. Gels were autoradiographed for 24 and 9 h, respectively.*

7.5 to 8.5) and two-band (8.5 to 9.0) regions (Fig. 4). Donors 3 and 4 do not share the same two clones at the basic 8.5 to 9.0 pI's, as is evident by some overlapping (i.e., broad, dark bands on the gel). We have further confirmed the dissimilarity of clones from individuals 3 and 4 by focusing the two sera in close proximity. The double-band pattern of donor 3 consistently focused at slightly lower pI's (data not shown). In contrast, donors 4 and 8 share the two bands at pI 8.5 to 9.0. In addition, these donors do not share the four-band pattern found in donors 16, 9, 17, and 15.

DISCUSSION

There have been numerous reports on the use of DT-TT for preventive immunization which have dealt with the development of effective immunization protocols (9, 10, 15, 22). There have been no studies, however, which have examined the degree of antibody clonal heterogeneity within an individual or shared among different individuals after such immunizations.

In murine systems, IEF has been successfully used for studying the clonal antibody repertoire of immune responses to a variety of antigens. The success of this analysis has resulted from the ability of IEF to distinguish among up to 10^4 different clonotypes based on the different multibanding patterns exhibited by clones of antibodies (14).

When highly heterogeneous immune responses are analyzed by IEF, however, the individual clonotypes cannot be adequately resolved and a smear results. We observed such a pattern for the IgG-Tet responses from the individuals tested. This most likely results from the size of the molecule (molecular weight, 160,000) and the large numbers of epitopes presumably exposed to the immune system.

In an attempt to reduce the epitope density of the immunogen, we next examined the humoral response to DT (molecular weight, 62,000). Different individuals had varying quantities of serum IgG-Dip, ranging from 200 (donor 1) to 6 (donor 19) ng/μl. When the sera were analyzed by IEF and developed with iodinated DT, individual clones of antibody were resolved. The greatest resolution of individual clonotypes, however, occurred when individuals were immunized with DT and the IEF antibodies were developed with fragment A, a proteolytically derived fragment (molecular weight, 21,145) of DT.

Previously, Barzaral et al. had shown that the percentage of serum DT antibodies directed against fragment A was variable in different individuals (3). We also found this variability, and it is clear that the percentage of DT antibodies directed to fragment A was not related to the quantity of antibody produced. In general, however, the complexity of the fragment A spectrotype correlated with the amount of IgG-Frag A. For instance, donors 1 and 2, who had the most anti-Frag A antibody, also had multibanded, complex spectrotypes with antibodies that had acid and basic pI's. Donors 5, 8, and 9, with intermediate levels of serum IgG-Frag A, had multibanded spectrotypes that were not as

complex as those of donors 1 and 2. They were more complex, however, than individuals with low levels of IgG-Frag A antibody who had nondistinct, smear spectrotypes that were restricted to a narrow, basic pH.

We found that the spectrotype pattern of IgG-Frag A was maintained throughout the entire time period when antibodies were detectable in the serum. Thus, no clonal products were selectively eliminated by normal antibody catabolism.

Although fragment A consists of only one-third of the entire DT molecule, nevertheless its size should be sufficient to elicit a more heterogeneous antibody response than that observed in most individuals. Several explanations exist for this restriction in antibody expression.

First, fragment A may express only a limited number of epitopes to the immune system. As we are immunizing with whole DT molecule, it is possible that a large portion of the fragment A molecule may be sequestered within the remainder of the molecule. If this is so, then we would expect that many of the antibodies would share binding capacity to only a limited portion of the molecule. Studies are in progress with purified clones of antibody to determine whether the IgG-Frag A response is indeed directed at limited numbers of epitopes. A second, and less likely, reason for the restricted antibody heterogeneity would include the possibility that only a limited repertoire of variable region genes exists for fragment A.

A third possibility would be that a form of immune regulation dictates the expression of a dominant clone, as has been shown for the control of idiotype expression in mice (1, 6).

Lastly, as diphtheria was a major source of infant mortality up until two generations ago (11), it is possible that selection for protective clones of antibody has occurred in the human population. Future in vivo and in vitro studies of the humoral immune system may help to resolve these alternatives.

An important finding was the identical IgG-Frag A spectrotypes found among several different donors.

If these spectrotypes are indeed identical antibodies, then it would suggest not only that restriction of antibody expression occurs in each individual, but also that the population as a whole is limited to a small number of IgG-Frag A antibody species.

Identity of spectrotypes, however, does not necessarily prove identity of antibodies. For instance, Hansburg et al. have shown in the murine system that antibodies to dextran B1355 can sometimes have the same spectrotype but different idiotypes (13). We are at present using biochemical analyses of purified IgG-Frag A from different donors to examine the extent of identity between antibodies with the same pI's.

ACKNOWLEDGMENTS

We gratefully acknowledge William H. Hildemann and his laboratory for use of isoelectric focusing equipment. We thank Birgitte Keld for preparation of diphtheria toxoid and fragment A. We thank Carol J. Thiele, Etty Benveniste, and Evelyn Pineda for assistance in collection of sera.

This work was supported by Public Health Service grants CA 12800 and AI 15859 from the National Institutes of Health. C.D.M. was supported by Public Health Service Clinical and Fundamental Immunology training grant AI 07126 from the National Institutes of Health.

LITERATURE CITED

1. **Adorini, L., M. Harvey, and E. E. Sercarz.** 1979. The fine specificity of regulatory T cells. IV. Idiotypic complementarity and antigen-bridging interactions in the anti-lysozyme response. Eur. J. Immunol. **9**:906–909.
2. **Bandilla, K. K., F. C. McDuffie, and G. J. Gleich.** 1969. Immunoglobulin classes of antibodies produced in the primary and secondary responses in man. Clin. Exp. Immunol. **5**:627–641.
3. **Bazaral, M., P. J. Goscienski, and R. N. Hamburger.** 1973. Characteristics of human antibody to diphtheria toxin. Infect. Immun. **7**:130–136.
4. **Boyum, A.** 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. Suppl. **97**:77–108.
5. **Briles, D. E., and J. M. Davie.** 1980. Clonal nature of the immune response. II. The effect of immunization on clonal commitment. J. Exp. Med. **152**:151–160.
6. **Cancro, M. P., N. H. Sigal, and N. R. Klinman.** 1978. Differential expression of an equivalent clonotype among Balb/C and C57BL/6 mice. J. Exp. Med. **147**: 1–12.
7. **Curtis, J. E., E. M. Hersh, J. E. Harris, C. McBride, and E. J. Freireich.** 1970. The human primary immune response to keyhole limpet haemocyanin: interrelationships of delayed hypersensitivity, antibody response and *in vitro* blast transformation. Clin. Exp. Immunol. **6**:473–491.
8. **DeLange, R. J., R. E. Drazin, and R. J. Collier.** 1976. Amino acid sequence of Fragment A, an enzymically active fragment from diphtheria toxin. Proc. Natl. Acad. Sci. U.S.A. **79**:69–72.
9. **Edsall, G., J. S. Altman, and A. J. Gaspar.** 1954. Combined tetanus-diphtheria immunization of adults: use of small doses of diphtheria toxoid. Am. J. Public Health **44**:1537–1545.
10. **Edsall, G., H. J. Banton, and R. E. Wheeler.** 1951. The antigenicity of single graded doses of purified diphtheria toxoid in man. Am. J. Hyg. **53**:283–295.
11. **Frost, W. H.** 1928. Infection, immunity and disease in the epidemiology of diphtheria. With special reference to some studies in Baltimore. J. Prev. Med. **5**:325–343.
12. **Gill, D. M., and L. L. Dinius.** 1971. Observations on the structure of diphtheria toxin. J. Biol. Chem. **246**:1485–1491.
13. **Hansburg, D., R. M. Perlmutter, D. E. Briles, and J. M. Davie.** 1978. Analysis of the diversity of murine antibodies to dextran B1355. III. Idiotypic and spectrotypic correlations. Eur. J. Immunol. **8**:352–359.
14. **Kreth, H. W., and A. R. Williamson.** 1973. The extent of diversity of anti-hapten antibodies in inbred mice: anti-NIP (4-hydroxy-5-iodo-3 nitrophenacetyl) antibodies in CBA/H mice. Eur. J. Immunol. **3**:141–147.
15. **Levine, L., J. Ipsen, and J. A. McComb.** 1961. Adult

immunization, preparation and evaluation of combined fluid tetanus and diphtheria toxoids for adult use. Am. J. Hyg. **73**:20–35.

16. **Linggood, F. V., M. F. Stevens, A. J. Fulthorpe, A. J. Woiwood, and C. G. Pope.** 1962. The toxoiding of purified diphtheria toxin. Br. J. Exp. Pathol. **44**:177–187.
17. **Nicolotti, R. A., D. E. Briles, J. A. Schroer, and J. M. Davie.** 1980. Isoelectric focusing of immunoglobulins: improved methodology. J. Immunol. Methods **33**:101–115.
18. **Rosen, A., K. Ek, and P. Aman.** 1979. Agarose isoelectric focusing of native human immunoglobulin M and α_2-macroglobulin. J. Immunol. Methods **28**:1–11.
19. **Rowley, M. J., and I. R. Mackay.** 1969. Measurement of antibody-producing capacity in man. I. The normal response to flagellin from *Salmonella adelaide*. Clin. Exp. Immunol. **5**:407–418.
20. **Stevens, R. H., and A. Saxon.** 1978. Immunoregulation in humans. Control of antitetanus toxoid antibody production after booster immunization. J. Clin. Invest. **62**: 1154–1160.
21. **Stevens, R. H., and A. Saxon.** 1979. Differential synthesis of IgM and IgG antitetanus toxoid antibody following *in vivo* booster immunization of humans. Cell. Immunol. **45**:142–150.
22. **Volk, V. K., R. Y. Gottshall, H. D. Anderson, F. H. Top, W. E. Bunney, and R. E. Serfling.** 1962. Antigenic response to booster dose of diphtheria and tetanus toxoids. Seven to thirteen years after primary inoculation of noninstitutionalized children. Public Health Rep. **77**:185–194.
23. **Wells, J. V., H. H. Fudenberg, and I. R. Mackay.** 1971. Relation of the human antibody response to flagellin to Gm genotype. J. Immunol. **107**:1505–1511.
24. **Whittingham, S., J. D. Mathews, M. S. Schanfield, J. V. Matthews, B. D. Tait, P. J. Morris, and I. R. Mackay.** 1980. Interactive effect of Gm allotypes and HLA-B locus antigens on the human antibody response to bacterial antigen. Clin. Exp. Immunol.**40**:3–15.