CLONAL NATURE OF THE IMMUNE RESPONSE

II. The Effect of Immunization on Clonal Commitment*

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Immunization of animals with carbohydrates or other antigens with a limited number of different antigenic determinants frequently elicits antibodies showing markedly restricted heterogeneity (1–4). Some antigenic determinants elicit very similar antibodies in nearly all syngeneic animals (5–7). An extreme case is the response to phosphocholine (PC) in BALB/c mice where 70–100% of the anti-PC antibody bears the TEPC-15 idioype (8, 9). Other antigenic determinants such as the alpha 1 → 3-dextran determinant of B1355 dextran or the β-N-acetyl-d-glucosaminide determinant of streptococcal group A carbohydrate (GAC) clearly elicit different responses in each animal (10, 11). The group A response is the most extreme case known, because although antibodies with at least several 100 different isoelectric focusing spectrotypes can be made by an individual strain, each mouse appears to express only one or two clones as the bulk of its antibody response (11).

The mechanisms that result in the restricted nature of these responses and in the different types of restricted responses to different antigens are not clear. In this report we have examined the time in the anti-GAC immunization sequence at which clonal commitment occurs. Somewhat to our surprise we have found that clonal commitment occurs very early in the immunization protocol, long before significant amounts of antibodies are found in the sera. In fact, some animals show commitment even before immunization. This finding reduces the possibility that the restriction results from affinity-dependent maturation, and indicates that the lymphocytes of nonimmune mice are idiosyncratic in their potential anti-GAC response capabilities. Such idiosyncrasies could reside either in regulatory circuits or in unequal representation of anti-GAC clonotypes in the B cells of individual mice.

Materials and Methods

Female Mice. SWR/J female mice were obtained from The Jackson Laboratories, Bar Harbor, Maine, at 6–8 wk of age.

Immunizations. Mice were immunized intravenously with group A streptococcal vaccine (300 μg of cell wall rhamnose/ml) (12). The immunization schedule consisted of 0.1 ml of one-tenth, * Supported by U. S. Public Health Service grants CA 16673, CA 13148, AI 15986, AI 15926, AI 15353, and AI 11635.

Abbreviations used in this paper: GAC-vaccine, streptococcal group A vaccine; GAC, group A streptococcal carbohydrate; PC, phosphocholine; pI, isoelectric point.

Briles, D. E., and R. J. Carroll. Manuscript submitted for publication.

J. Exp. Med. © The Rockefeller University Press - 0022-1007/80/07/0151/10 $1.00

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one-third, and undiluted vaccine on days 0, 7, and 14, respectively; mice were boosted on day 44 with undiluted vaccine and bled 10 d later.

Spleen Cell Transfers. Spleens were removed from nonimmunized donor mice or donors given one, two, three, or four injections of group A streptococcal vaccine (GA-vaccine). Immune spleens were removed in most cases 7 d (range 4–26) after the last immunization. The cells were prepared as described previously (13) and injected intravenously, along with vaccine, into six irradiated (400 R) recipients who were then given the remaining injections of the immunization sequence. Each recipient received one-sixth of the donor spleen. When a donor had already been given all four immunizations, the recipients were boosted with a single injection of undiluted vaccine before bleeding.

Isoelectric Focusing. Isoelectric focusing of anti-GAC antibodies and their detection with 125I-labeled GAC was performed as described previously (14, 15). Assignment of spectrotypes as either identical or different always relied on results of more than one isoelectric focusing gel. Before being dried down all gels were photographed with dark-field illumination to record the Na2SO4-precipitated immunoglobulin bands. These patterns were frequently useful in comparisons between sera because the precipitin bands are slightly more distinct than their autoradiographic images and could readily indicate distortions as a result of overloading.

Statistical Analysis. Mathematical analysis of the data is complicated by the fact that the groups of recipients and controls frequently differed in size. Thus, expressing the data simply as percentage of mice sharing clones would result in a bias. The larger groups would show higher percentages of sharing simply because each mouse would have more mice with which to be compared. To circumvent this problem the data have been expressed as the fraction of the possible comparisons between mice that show sharing of clones. The number of possible comparisons between mice is \( n(n - 1)/2 \), where \( n \) is the number of mice in the group being examined. The number of comparisons sharing a particular clone is \( a(a - 1)/2 \), where \( a \) is the number of mice with the clone in question. The total clonal sharing for a group of recipients (or control mice) is the sum of all comparisons for all clones shared within the group:

\[
\sum_{i=1}^{x} \frac{a_i(a_i - 1)}{2}
\]

where \( x \) equals the number of shared clones \( i \). Thus, the ratio of total shared comparisons per possible comparisons is \( \frac{\sum a_i(a_i - 1)/2}{n(n - 1)/2} \). Note that this ratio can be >1 because it is possible for a single mouse to have and share more than a single clone. To statistically determine if a donor showed evidence for clonal commitment, the Fisher exact test was used (16). For this test \( n(n - 1) \) was used as the number of possible clone pairs because for many mice two (predominant) clones were analyzed, thus doubling the number of possible pairs. The Fisher exact test compares the ratio \( [a(a - 1)/2]:n(n - 1) - [a(a - 1)/2] \) for the recipients of a donor with the same ratio for a control group of SWR/J mice with no donor. To compare pooled data for similarly treated donors with the same group of SWR/J control mice, a pooled chi-square analysis was used (16). To compare different donor groups, a Mann-Whitney rank test was used (16). In all of these tests the degree of significance is slightly underestimated because many recipients had only one rather than two predominant clones.

Results

Uniqueness of Anti-GAC Response of Individual SWR/J Mice. Although individual inbred mice immunized with GA-vaccine produce a pauciclonal response, they rarely produce anti-GAC antibodies with identical spectrotypes (11, 12, 17). This is seen for the SWR/J strain in Fig. 1 where the anti-GAC antibody of 15 different SWR/J mice is illustrated. Spectrotypic differences can confidently be interpreted as V-region differences because virtually all anti-GAC antibodies are of a single heavy isotype IgG3 (18). Although several of the spectrotypes in Fig. 1 appear to have very similar patterns, repeated side-by-side comparisons, using limiting dilutions of antisera to obtain maximum resolution, have indicated that no two sera contained identical spectrotypes. In making these comparisons between sera we considered only the major
clones or spectrotypes (sets of three to four bands) in each serum. This decision was made because detection of minor clones is not always reproducible, and, at loading concentrations high enough to insure detection of minor clones, the major bands of the sera frequently are so overloaded that minor bands are either obscured or displaced. Thus, in Fig. 1 and in the remainder of the paper we have arbitrarily limited our analysis to the major one or two spectrotypes of each serum. Within this limitation we can confidently state that sharing of major spectrotypes among individual mice is rare. Based on the frequency of spectrotypes sharing among several groups of SWR/J mice we have calculated that the clonal repertoire of anti-GAC antibody elicitable with GA-vaccine is on the order of 300 different spectrotypes.2

Time of Clonal Commitment. The immunization protocol used involves three weekly injections of GA-vaccine followed by a 1-mo rest and then a fourth injection. Although serum anti-GAC antibody levels can reach several milligrams per milliliter after the fourth injection, little if any antibody is detected until after the third immunization (12). By isoelectric focusing we have never observed SWR/J mice to produce 7S antibody before the third weekly injection of vaccine. To determine whether or not mice given zero, one, two, or three injections were committed to make particular anti-GAC clones, their spleen cells were transferred to six sublethally irradiated (400 R) syngeneic recipients (Fig. 2). The recipient mice were immunized with group A vaccine, and their sera were examined by isoelectric focusing to determine if any of them shared spectrotypes with sera from other recipients of the same donor.

53 transfers were performed. Examples of antibody patterns expressed are shown in
Fig. 2. Injection schedules for donor and recipient mice. Doses of vaccine are expressed as micrograms of cell wall rhamnose. Donor mice were bled on the day of spleen cell transfer. Recipient mice were bled 10 d after the last injection.

Fig. 3. Fig. 3 A depicts a transfer from an immune donor producing a single predominant clone at the time of transfer. Three of the four recipients made antibody identical by isoelectric focusing to that of the donor. The fourth recipient made no detectable antibody, a result found in <10% of the recipients for all transfers. Fig. 3 B shows the results obtained with a donor given a single injection of GA-vaccine before transfer. This donor, like all others given zero, one, or two primary doses of GA-vaccine, showed no detectable antibody even though the maximum loading vol of 15 µl of serum was applied to the gel. Radioimmunoassay showed these donor sera to contain <0.05 mg/ml of antibody. The recipients of this donor express three different spectrotypes; one (isoelectric point [pI] ~6.9) appears in four recipients, another (pI ~7.2) appears in five recipients, and the third spectrotyp, (pI ~7.7) is present only in a single recipient. Fig. 3 C shows the results obtained with two nonimmunized donors. Within each group of recipients there is considerable sharing of clones. The group at the left is particularly interesting. Each mouse has two predominant anti-GAC clones: one with a pI ~6.3 and the other with a pI slightly more basic, about pI ~6.5. The spectrotyp at pI ~6.3 appears to be identical in all recipients. The more basic clones, however, are not identical in each recipient, but show three slightly different spectrotypes (compare mice 4, 5, and 6 of donor 1). The existence of similar but not identical spectrotypes was observed in other transfers but was never as convincing as in this group of recipients. We are attempting to investigate this phenomenon further. More than one-half of the nonimmune donors failed to show any evidence for clonal commitment. The recipients of one such donor are seen in Fig. 3 D (right panel), where it can be seen that none of the recipients share anti-GAC spectrotypes. Whereas identity of spectrotypes among recipients is evidence for clonal commitment of the donor, a lack of spectrotypic identity in these experiments has little meaning because irradiated (400 R) SWR/J mice given a full 6-wk immunization schedule make anti-GAC antibody whether or not they receive donor spleen cells. Thus, the B cells producing antibody in the recipient could be either of recipient or donor origin.

To quantitatively evaluate the degree of spectrotyp sharing we have computed the ratio of the number of identical spectrotyp pairs:possible mouse pairs for each group of recipients. This ratio gives a much more accurate measure of the degree of similarity
Fig. 3. (A) Adoptive transfer of an anti-GAC clonotype from a hyperimmune donor to recipients, as evidenced by identity of isoelectric focusing spectrotypes. The donor was given four injections of group A vaccine before transfer. The recipients were given a single injection of vaccine at the time of transfer and bled 10 d later. Recipient mice were indicated by numbers 1-4. The donor serum is indicated by D. (B) Clonal commitment of a primed donor, as evidenced by anti-GAC spectrotypic sharing of recipient mice. The donor was given one injection of group A vaccine and made no anti-GAC antibody detectable by isoelectric focusing. The recipient sera were given three immunizations with group A vaccine and bled 10 d later. (C) Clonal commitment of two nonimmunized donors, as evidenced by anti-GAC spectrotypic sharing of recipient mice. The donor sera contained no anti-GAC antibody detectable by isoelectric focusing. The recipient mice were injected four times with group A vaccine and bled 10 d later. On the left, mice 1-6 received cells from donor 1, and on the right, mice 1-5 received cells from donor 2. At the position of serum 2-1 an artifact can be seen: the pattern with pi ~6.3 is spillover from sample 1-6. This pi ~6.3 band was not seen on other gels where sample 2-1 was focused. Sample 2-1 has a barely detectable spectrotypic that focuses slightly acidic of the major spectrotypic shared by mice 2-2, 2-3, 2-4, and 2-5. (D) Lack of detectable clonal commitment in a nonimmunized donor, as evidenced by a lack of spectrotypic sharing among recipient mice. Recipients, indicated by numbers 1-4, were immunized as in Fig. 3 C. The donor sera is indicated by D.
of recipients than simply the percentage of recipients sharing clones (Materials and Methods; Table I). Table I contains representative data from 8 of the 53 donors used in this study, and all of the data for the no-donor control mice. The table illustrates the method of calculating spectrotype pairs, mouse pairs, and the ratio of the two, and also reveals the sensitivity of this ratio for analyzing the degree of spectrotype sharing among recipients.

The spectrotype pairs/mouse pairs fractions for recipients of all 53 donors are plotted in Fig. 4. Solid symbols were used to represent those recipients whose donors were making antibody before transfer. Triangles represent those recipient groups that showed statistically ($P < 0.02$) more sharing than was observed in a control group of SWR/J mice with no donor. The arrow on the horizontal axis represents the spectrotype pair:mouse pair ratio for nonrecipient SWR/J mice. From this figure it

| Table 1 |
|---|
| **Evaluation of Clonal Commitment of Anti-GAC Antibody** |

| Group | Number of donor injections of GAC vaccine | Recipients | Percent respondents | Total mouse pairs | Total spectrotype pairs | Possible mouse pairs | Number of mice sharing specific spectrotype pairs | Actual spectrotype pairs Mouse pairs |
|---|---|---|---|---|---|---|---|---|
| 1 | 4 | 4 | 4 | 100 | 6 | 4, 3§ | 9 || 1.5 |
| 2 | 4 | 4 | 4 | 100 | 6 | 4, 4 | 12 | 2.0 |
| 3 | 4 | 3 | 3 | 100 | 3 | 3, 3 | 6 | 2.0 |
| 4 | 4 | 3 | 3 | 100 | 10 | 4, 5 | 16 | 1.6 |
| 5 | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 0 |
| 6 | 0 | 4 | 3 | 75 | 6 | 2, 3 | 4 | 0.7 |
| 7 | 0 | 4 (1)* | 2 | 50 | 6 | 2 | 1 | 0.2 |
| 8 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | 5 | 31 | 25 | 5 | 0.2 |

| Mice | No donor control groups | Total respondents | Total mouse pairs | Total sharing clones | Percent mice sharing clones |
|---|---|---|---|---|---|
| 9 | 400 R‡‡ | 15 | 2 | 13 | 0.0995 |
| 10§§ | No irradiation | 16 | 2 | 12.5 | 2 | 0.0083 |
| 11 | No irradiation | 15 | 0 | 10 | 0 | 0.0000 |
| 46 | 6 | 8.7 | 30 | 0.0060 |

* Number of recipients producing anti-GAC antibody detectable by isoelectric focusing. Numbers in parenthesis refer to the number of recipient mice failing to produce detectable antibody.

‡‡ Number of mice sharing at least one clone with at least one other mouse.

§§ Number of mice sharing particular clones. When two numbers are presented the first indicates mice sharing clone x, the second the number of mice sharing clone y.

¶ Number of clone pairs calculated from the number of mice sharing specific clones. For group 1, actual clone pairs = 3 + 3 = 6 + 3 = 9.

§ Values below single lines are totals.

** Values below double lines are calculated from totals.

‡‡‡ Mice given 400 R before the start of immunization.

§§ Data from (11); all other data from this paper.
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![Graph showing commitment of donor mice to anti-GAC clones. Solid symbols represent those recipient groups whose donor was making anti-GAC antibody detectable by isoelectric focusing. Triangles represent those recipient groups that showed significantly (P < 0.02) more spectrotype sharing than was observed in a control group of irradiated SWR/J mice with no donor (Tables I and II). The arrow on the vertical axis represents the spectrotype pair:mouse pair ratio for nonrecipient SWR/J mice.]

**TABLE II**

Summary of Clonal Commitment Data

| Number of donor injections | Number of donors | Total recipients | Percent donors committed* | Possible mouse pairs | Spectrotype pairs Mouse pairs | P vs. control | P vs. no injections |
|----------------------------|------------------|------------------|---------------------------|----------------------|-------------------------------|---------------|---------------------|
| 0                           | 10               | 49               | 20-80                     | 100                  | 30                           | 0.30          | <10<sup>-4</sup> |
| 1                           | 25               | 94               | 17-27                     | 163                  | 21                           | 0.13          | <10<sup>-4</sup> |
| 2                           | 3                | 15               | 100                       | 31                   | 18                           | 0.58          | <10<sup>-3</sup> |
| 3                           | 5                | 20               | 60                        | 31                   | 10                           | 0.32          | <10<sup>-3</sup> |
| 4                           | 12               | 47               | 92                        | 71                   | 102                          | 1.44          | <10<sup>-3</sup> |
|                              | 12               | 225              | 1                         | 0.005                |                               |               |                     |

No donor control groups

| Total mice                  |
|-----------------------------|
| 400 R                       |
| 31                          |

* Committed donors are those whose recipients share significantly (P < 0.02 by Fisher exact test) more clones than irradiated, no donor controls. Where a range is listed the second value includes all donors whose recipients shared more clones than expected, regardless of P value. Slightly more significant P values are obtained using the group of 31 nonirradiated controls.

I Total possible mouse pairs obtained by summing the possible mouse pairs for each donor's recipients.

Il Total spectrotype pairs obtained by summing the observed spectrotype pairs for each different donor's recipients.

¶ Donors with zero and four injections listed in Table I are a subset of those in this table.

NS, not significantly different; P > 0.05.

** No irradiation controls are the sum of groups 10 and 11 in Table I.

is apparent that donors need not have been making detectable anti-GAC antibody, or even been immunized for their recipients to extensively share clones. However, as the number of donor immunizations increased, so did the expression of donor antibody, as well as the degree of sharing of spectrotypes among the recipients. The relative degree of commitment for each class of donors is also shown in Table II. For each donor class the overall frequency of spectrotype pairs/mouse pairs was significantly different (P < 0.001) from that of nonrecipient mice by a pooled chi-square test.

**Discussion**

When SWR/J or other inbred strains of mice are immunized with streptococcal vaccine the bulk of the anti-GAC antibody they produce is composed of only one or two different spectrotypes per mouse (11, 14, 17, 19). From data in this and an earlier
paper it is apparent that different SWR/J mice rarely produce clones of anti-GAC antibody with identical spectrotypes. It can be calculated that the most probable number of different SWR/J spectrotypes is 310, with a 95% confidence interval of 90-2,600. Thus of the several 100 possible spectrotypes, each mouse appears to produce most of its antibody as one or two spectrotypes. The adoptive transfer studies in this paper make it clear that the commitment of mice to particular anti-GAC clones can occur in the absence of immunization and before the production of detectable antibody. These results make it unlikely that affinity maturation plays a role in restricting the heterogeneity of anti-GAC antibodies. This conclusion is supported by studies that indicate that the earliest detectable IgG (20) and IgM (12) anti-GAC antibodies are restricted in heterogeneity.

To understand the mechanisms that result in pauciclonal anti-GAC responses, two separate questions must be answered: (a) what maintains the initial clonal restriction during subsequent immunizations, and (b) why do only a few anti-GAC clones respond initially? Several potential answers to the first question exist. Clonal commitment could be maintained either by more efficient stimulation of memory than virgin B cells (21, 22), or by active suppression of the ability of virgin B cells to respond (23). Cramer and Braun (20) have used in vitro culture procedures to show that in antistreptococcal carbohydrate-immune rabbits most of the anticarbohydrate memory cells are specific for the predominant clones being produced in vivo. Thus, they feel that clonal commitment is merely an overgrowth of memory cells committed to the predominant clones. The origin of the initial clonal commitment is even more in doubt. It is possible that the anti-GAC responses of these mice in part reflect their earlier encounters with cross-reactive environmental antigens. Although previous exposure with cross-reactive antigens might explain why we are able to detect clonal commitment in some mice and not others, such cross-reactive stimulation does not readily explain why individual highly inbred SWR/J mice raised and housed under similar conditions would each make different predominant clones of anti-GAC antibody.

One mechanism that could result in the production of different anti-GAC clones by different syngeneic mice would be that naive anti-GAC B cells are very inefficiently stimulated by group A vaccine and that once a clone begins to expand, its more-easily stimulated memory cells have a selective advantage over virgin B cells expressing other anti-GAC spectrotypes. This type of chance activation hypothesis is not favored by the present data that indicate that clonal commitment can occur before immunization, unless such commitment results from environmental antigens. Adoptive transfer of spleen cells from germfree mice may resolve this issue.

Another mechanism that could account for clonal commitment of anti-GAC antibody would be the presence of regulatory anti-idiotype networks of T and/or B cells that allow synthesis of antibodies bearing only certain idiotypes. This hypothesis is consistent with data from other labs that show that anti-idiotype and anti-anti-idiotype can affect the expression of particular idiotypes (24-26). Although it is conceivable that regulation may be the source of anti-GAC clonal commitment it does not readily explain why different mice are committed to make different anti-GAC clones, but simply moves the problem to a different level.

A third possibility is that the initial clonal restriction is because B cell populations express only a portion of an animal’s genetic potential at any one time. Thus, the
mouse would only respond with those anti-GAC clones that were expressed at the time of immunization. This kind of chance expression of V regions during the generation of virgin B cells would be consistent with the observations that individual syngeneic mice differ both in the number of predominant anti-GAC spectrotypes produced (Fig. 2), and in the magnitude of their response to immunization with group A vaccine (12, 19, 27).

It is hoped that subsequent studies with the anti-GAC system will allow an assessment to be made of the relative contributions of regulation and differential B cell expression of clonotypes to the pauciclonal responsiveness of anti-GAC antibodies. Such studies may also help to explain the origin of other pauciclonal responses.

Summary

An inbred strain can produce several hundred different anti-group A carbohydrate (GAC) antibodies, as analyzed by isoelectric focusing. However, each individual mouse produces the bulk of its anti-GAC antibody as only one or two different spectrotypes, which appear to be randomly chosen. By using adoptive transfer techniques, we have observed that clonal commitment occurs very early in immunization, sometimes even before immunization, and thus does not result from competition among B cells for antigen.

We would like to acknowledge the excellent technical assistance of Elizabeth Nesbit, Bruce Linders, and Alex Schaffer; the statistical consultations of Doctors Seng-Jaw Soong, Alfred Bartolucci, and Raymond Carroll; and the preparation of the manuscript by Ann Brookshire. We are indebted to Dr. Max Cooper and Joyce Lehmeyer for their criticisms of the manuscript.

Received for publication 11 March 1980.

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