Staphylococcal nuclease is a chemically well-defined protein antigen the immune response to which is under \(H-2\)-linked \(Ir\) gene control (1). Antibodies directed against nuclease have been shown to contain idiotypic determinants related to the antibody combining site (2). In a recent study, the genetic linkage of such determinants in the A/J mouse strain was investigated (3). No influence of the \(H-2\) locus on idiotype expression was demonstrated. The A/J idiotype was present in immune sera from \(H-2\) congenic high (A/J, \(H-2^a\)) and low (A.BY, \(H-2^b\)) responder strains sharing the same background, including heavy chain (\(C_H^1\)) allotype group, \(Ig-1^e\). The idiotype was absent from a high responder strain (B10.A, \(H-2^a\)) with the same \(H-2\) haplotype as A/J, but a different background and the heavy chain allotype group, \(Ig-1^b\). An analysis of the segregation of the A/J idiotype and \(Ig-1^e\) heavy chain allotype marker in progeny of the backcross (B10.A x A/J) × B10.A showed linkage of the idiotype to the \(Ig-1^e\) locus. However, two animals in a series of 19 were found to have an apparent recombinant phenotype, being \(Ig-1^b/Ig-1^b\) homozygotes but expressing the A/J idiotype. This implied a recombination frequency of 10.5%, although obviously this was based on a very small number of animals. As this frequency was considerably higher than that observed for other idiotype markers (4–7), it was deemed important to examine a much larger number of animals to see if it could be confirmed. In this communication, we report on the results obtained from 101 backcross animals as well as progeny tests on some of the putative recombinants.

Materials and Methods

**Mice.** B10.A mice were purchased from The Jackson Laboratory, Bar Harbor, Maine or raised in our own breeding colonies. Offspring of the cross (B10.A × A/J) × B10.A as well as backcrosses of putative recombinants to B10.A were produced in our own colonies. All animals were immunized at 8–12 wk of age.

**Antigen.** Staphylococcal nuclease was purified from the extracellular broth of cultures of *Staphylococcus aureus* Foggi strain by published methods (8) with final purification by affinity chromatography by the method of Cuatrecasas et al. (9).

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1. *Abbreviations used in this paper: C_H, heavy chain; V_H, heavy chain variable region; Nase, nuclease; SRBC, sheep erythrocyte.*
Immunizations. Mice were immunized with 100 µg of nuclease in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and bled from the retro-orbital plexus 3 wk later. Some animals received booster immunizations of 25 µg nuclease in saline at weekly intervals to produce sufficient antibody for assay.

Antibody Assay. Anti-nuclease antibodies were assayed by testing their ability to inhibit the degradation of DNA by nuclease, as previously described (1).

Anti-Idiotype Antisera. Anti-idiotype antisera were prepared in Lewis rats by hyperimmunization with A/J anti-nuclease antibodies purified by affinity chromatography on Sepharose columns containing covalently bound nuclease, also as previously described (2).

Determination of the Presence of the A/J Idiotype. The presence of the A/J anti-nuclease idiotype was determined by the ability of the anti-idiotype antisera to inhibit the antibody-mediated inactivation of nuclease. The idiotype was considered present if the activity of the antibody in the presence of the anti-idiotype reagent was significantly greater than the activity in the absence of the anti-idiotype reagent (P < 0.05). Data are reported in terms of the percent of antibody activity inhibited by the anti-idiotype antisera. A maximum inhibition of 60-70% was achieved with the preparations used in these experiments.

Determination of Allotype. The allotype of individual sera was determined by using an Ouchterlony double diffusion system with specific anti-allotype sera prepared by immunizing allotypically dissimilar animals with pertussis-anti-pertussis complexes according to the methods of Herzenberg and Herzenberg (10). All sera were tested for the presence of both the Ig-I* and Ig-I' allotypes. For more sensitive analysis, an inhibition of passive hemagglutination assay was performed. Briefly, an anti-allotypic antiserum with specificity for Ig-I* markers was titered to determine the highest dilution causing hemagglutination of sheep erythrocytes coated with purified Ig-I* γ-G globulins by the glutaraldehyde method (11). A dilution of 1:4,000 was used in these experiments. The ability of various dilutions of both pre-immune and immune sera of selected backcross animals to inhibit this hemagglutination was then determined. Controls in all experiments were sera from normal A/J and B10.A animals.

Results

Segregation of the Ig-I' Allotype Marker and A/J Idiotype. The segregation of the Ig-I' Cλ allotype marker and the A/J idiotype was studied in the progeny of the backcross of (B10.A x A/J) x B10.A. These strains were chosen for this study for three reasons: (a) the A/J idiotype has a very high degree of penetrance. As yet, there has been no A/J or (A/J x B10.A)F1 animal examined that has failed to express the A/J idiotype; (b) there is no crossreactivity between the A/J and B10.A anti-nuclease idiotypes, allowing the unambiguous assignment of an idiotype to a given animal (c) H-2a is a high responder H-2 type, so that in this backcross all of the progeny should make sufficient anti-nuclease antibodies to be informative.

101 animals from the backcross (B10.A x A/J) x B10.A were immunized with nuclease and bled 3 wk later. The allotype and idiotype of individual sera was assessed as described in Materials and Methods.

The results of this analysis are represented graphically in Fig. 1. Of 36 animals identified as Ig-I'/Ig-I' heterozygotes, all had detectable idiotype as demonstrated by significant inhibition of their anti-nuclease antibodies by the anti-idiotype antisera; the amount of inhibition ranged from 20 to 70% (Fig. 1, bottom). For the 65 animals identified as Ig-I'/Ig-I' homozygotes, two patterns of reactivity with the anti-idiotype antisera could be distinguished (Fig. 1, top). Antibodies from 58 of these animals showed less than 10% inhibition by the anti-idiotype antisera (range -5.9% + 7.1% and none significantly different from zero). These animals were considered idiotype negative. Seven of the
DAVID S. PISETSKY AND DAVID H. SACHS

Fig. 1. Idiotype analysis of (B10.A × A/J) × B10.A backcross. The bar graphs illustrate the distribution of inhibition values obtained for the antinuclease antibodies from 101 backcross animals. The number of animals showing inhibition in each 5% range of values is plotted. Top, 65 Ig-1b/Ig-1b homozygous backcross animals; bottom, 36 Ig-1b/Ig-1e heterozygous animals.

homozygotes, however, had antibodies which demonstrated inhibition. The inhibition values for these animals (Table I) were comparable to those obtained for the heterozygotes on the backcross population as well as (B6 × A/J)F1 animals (3) and were all significantly different from zero. Animals in this group were considered to be positive for the idiotype. Table II presents a summary of the results obtained with the backcross population.

These results indicated linkage of the A/J anti-nuclease idiotype marker to the Ig-1e C\textsubscript{H} allotype marker, in accordance with our previous results on fewer animals (3). There was again an unusually high apparent frequency of recombination of 7% (7/101). The distribution of recombinants among Ig-1b/Ig-1b homozygotes and Ig-1b/Ig-1e heterozygotes was also unusual in that there were no heterozygotes which bore a recombinant phenotype. This difference in recombination frequency between homozygotes and heterozygotes (7/65 vs. 0/36) was statistically significant ($P < 0.05$ from normal distribution). It is also to be noted that there was an unequal distribution of animals with respect to the C\textsubscript{H} allotype with a preponderance of animals being Ig-1b/Ig-1b homozygotes. The origin of the difference, which is also statistically significant, is unclear, although similar distributions have also been reported by others (12).

While the results of this analysis suggested a high recombination frequency in this backcross at least two mechanisms other than recombination could have led to the observed phenotype: (a) suppression of the Ig-1e allotype in Ig-1b/Ig-1e heterozygotes without concomitant suppression of the A/J idiotype; and (b) the operation of regulatory genes which allowed the occasional expression of the
1606 RECOMBINATION OF GENES DETERMINING ANTI-NUCLEASE IDIOTYPES

**Table I**
*Idiotype Analysis of Putative Recombinants*

| Animal number | Inhibition of inactivation % |
|---------------|-----------------------------|
| 622           | 42.3                        |
| 790           | 27.1                        |
| 944           | 46.3                        |
| 59            | 38.5                        |
| 217           | 57.1                        |
| 802           | 59.3                        |
| 817           | 56.0                        |

Results of the inhibition of inactivation assays for Ig-1b/Ig-1b animals identified as idiotype positive. All inhibition values were statistically significant (P < 0.05).

**Table II**
*Segregation of the A/J Idiotype and Allotype in (B10.A × A/J) × B10.A Backcross*

|                          | Ig-1b/Ig-1b | Ig-1b/Ig-1e |
|--------------------------|-------------|-------------|
| A/J Idiotype (positive)  | 7           | 36          |
| A/J Idiotype (negative)  | 58          | 0           |

Progeny of the backcross of (B10.A × A/J) × B10.A were immunized with 100 µg nuclease in CFA and bled 3 wk later. The presence of the A/J idiotype was assessed by inhibition of inactivation method as described in Materials and Methods. Allotype was determined by Ouchterlony diffusion analysis.

A/J anti-nuclease idiotype in animals homozygous for the Ig-1b allotype. The following experiments were designed to consider these alternate explanations for the high apparent recombination frequency.

**Attempts to Detect Hidden Allotype.** Several investigators have reported detection of an allotypic marker in animals that were not expected to have that marker on the basis of standard allotyping tests and family studies (12-15). This phenomenon has been termed hidden allotype and has suggested the existence of regulatory genes which may control the expression of structural genes for immunoglobulins. In the context of the present work, one could suggest that the putative recombinants were in fact Ig-1e/Ig-1b heterozygotes in which regulator genes suppressed or blocked the expression of genes coding for the Ig-1e allotype without altering the expression of genes coding for the A/J idiotype. Animals demonstrating this type of regulatory control would be falsely considered to be Ig-1b/Ig-1b homozygotes and identified as recombinants. To test for this possibility, a more sensitive system for the detection of the Ig-1e allotype was devised, measuring the ability of sera from backcross animals to inhibit the hemagglutination by anti-allotypic sera of sheep erythrocytes (SRBC’s) coated with purified γ-G globulin from A strain animals. By use of this test, amounts of Ig-1e allotype equivalent to 0.2% of that found in normal A/J animals could be detected. Table III shows the results of tests on putative recombinants as well as animals designated as homozygotes and heterozygotes.
These data show the results of an allotype detection assay testing ability of various sera to inhibit hemagglutination of SRBC's bearing purified A strain gamma G globulin by 1:4,000 dilution of anti-allotype sera. In addition to sera from normal B10.A and A/J animals, sera from putative recombinants and other randomly selected backcross animals were tested. Included are four animals identified by Ouchterlony as Ig-1\textsuperscript{a}/Ig-1\textsuperscript{e} heterozygotes (25, 58, 210, 803), two identified as Ig-1\textsuperscript{b}/Ig-1\textsuperscript{e} homozygotes (207, 801), as well as seven putative recombinants. Results presented show the allotype as determined by Ouchterlony analysis, as well as the reciprocal of log\textsubscript{2} of the dilution of sera which caused inhibition of hemagglutination.

These data show the results of an allotype detection assay testing ability of various sera to inhibit hemagglutination of SRBC's bearing purified A strain gamma G globulin by 1:4,000 dilution of anti-allotype sera. In addition to sera from normal B10.A and A/J animals, sera from putative recombinants and other randomly selected backcross animals were tested. Included are four animals identified by Ouchterlony as Ig-1\textsuperscript{b}/Ig-1\textsuperscript{e} heterozygotes (25, 58, 210, 803), two identified as Ig-1\textsuperscript{a}/Ig-1\textsuperscript{b} homozygotes (207, 801), as well as seven putative recombinants. Results presented show the allotype as determined by Ouchterlony analysis, as well as the reciprocal of log\textsubscript{2} of the dilution of sera which caused inhibition of hemagglutination.

Progeny Testing of Putative Recombinants. Progeny testing of the putative recombinants should determine whether the phenotype of such animals was the result of the recombination of structural genes for heavy chain variable region (V\textsubscript{H}) markers or was the result of regulatory phenomena allowing the occasional expression of an idiotype marker in a Ig-1\textsuperscript{b}/Ig-1\textsuperscript{b} homozygote. In the case of a true recombinant, one would expect one-half the offspring of another backcross to B10.A to demonstrate the recombinant phenotype. On the other hand, if regulatory phenomena were occurring one would expect progeny of the putative recombinants to demonstrate the recombinant phenotype at a frequency similar to that observed in the backcross itself. Table IV shows the results of idiotype analysis of immune sera of such progeny. Five of the
animals progeny tested in this manner were identified as recombinants in the initial backcross while one animal, number 993, was the offspring of one of these initial recombinants. Of the 46 animals examined for the presence of the A/J idiotype, 21 were positive for this marker. This value is consistent with the value of 50% expected for the segregation of a single locus, and substantiates the hypothesis that the animals tested were true recombinants. Furthermore, the observation that the A/J idiotype has never been demonstrated in a large number of animals of strains CB.20, B10, B10.A, and a variety of other B10 congenics, (3 and data not shown) makes it unlikely that the structural genes for this idiotype are present in these strains but only sporadically expressed.

Discussion

In this report, we have shown that in the backcross of (B10.A × A/J) × B10.A there was a high frequency of animals that bore a recombinant phenotype, being Ig/1b/Ig-1b homozygotes at the CH allotype locus but bearing the A/J anti-nuclease idiotype. Since the frequency of such animals was so high, it was considered important to show that the observed phenotype was in fact the result of a recombination event and did not arise as a result of some other mechanism.

The possibility of regulatory control leading to allotype suppression was tested for by use of a more sensitive allotype detection assay. By use of this technique it was shown that sera from putative recombinants contained less than 0.2% the amount Ig-1e bearing γ-G globulin as did normal animals or Ig-1b/Ig-1e heterozygotes from the same backcross. Thus, suppression of allotype expression without concomitant suppression of idiotype expression was considered an unlikely explanation for the observed recombinant phenotype. Additionally, this type of anomalous control of Vh and Ch gene expression would be unlikely on theoretical grounds as it would necessitate either (a) suppression of all Ig-1d allotype bearing immunoglobulin except that involved with anti-nuclease antibody or (b) the trans transcription of variable and constant region genes producing hybrid molecules with the variable region sequences derived
from genes on one chromosome and constant region sequences from the other chromosome. While the presence of such molecules was suggested in work on variable and constant region allotypes in rabbits (16, 17), they involved on the order of 1% of antibody molecules or antibody-producing cells. Our results would demand a much higher level of hybrid molecule formation to account for the levels of A/J idiotype seen in the putative recombinants if they were instead Ig-1b/lg-le heterozygotes with suppressed levels of Ig-1e allotype.

The best test for recombinants is assessment of whether or not they breed true. In progeny tests so far, almost 50% of the progeny of our putative recombinants have demonstrated the recombinant phenotype. This number is much greater than that expected if the operation of an unlinked regulatory gene were allowing the infrequent emergence of the A/J idiotype in B10.A animals. If the latter process were occurring, the frequency of the recombinant phenotype in the progeny of recombinants should have been similar to that observed for recombination in the backcross, or about 7%. Thus, our results are most consistent with the segregation of linked variable region genes that arose from true recombination events.

One facet of this study that requires additional explanation is the unequal frequency of recombinants among Ig-1b/Ig-1b homozygous and Ig-1b/lg-le heterozygotes. The explanation which we consider most likely, is that there is more than one idiotype recognized by our anti-idiotypic antisera and that the genes coding for these different idiotypes are widely separated on the chromosome region containing V\_H region gene sequences. Fig. 2 a illustrates the possible arrangement of genes coding for anti-nuclease idiotypes in an F\_1 animal. For the sake of simplicity, only two such genes are shown linked to each allotype locus, although the number of such genes could, of course, be higher. In Fig. 2 b, a possible recombinant event is shown with the cross-over point occurring between the genes coding for the two different idiotypes present on each chromosome. In Fig. 2 c, the resulting haploid chromosomes, A and B, that will be transmitted to the next generation are shown. In this case, animals that inherit chromosome A are scored as recombinants since they express the A/J anti-nuclease idiotype 1 as well as the Ig-1b allotype. Animals that inherit chromosome B, however, are not scored as recombinants since they can still express A/J anti-nuclease idiotype 2 although also having an anti-nuclease idiotype from B10.A. They could however, be shown to be recombinants by backcrossing them to strain A/J and testing the offspring for the presence of the B10.A idiotype. An antiserum specific for the B10.A idiotype has been prepared and could be used for this experiment. However, since there is no way to distinguish recombinant from nonrecombinant heterozygotes, this approach would necessitate backcrossing and progeny testing of all heterozygotes to identify the recombinants. An alternate approach would be the development of antiseras of more restricted specificity which could discriminate between the various hypothetical A/J anti-nuclease idiotypes; these antiseras could be used to directly test the available backcross animals and identify heterozygotes missing one or more A/J idiotypes. In fact, as our experiments were performed, the only recombinants with an Ig-1e allotype that could be readily identified would have resulted from a recombination event between the genes coding for
RECOMBINATION OF GENES DETERMINING ANTI-NUCLEASE IDIOTYPES

Fig. 2. Hypothetical map of V\textsubscript{H} region genes for antinuclease antibodies. In the map shown, A/J α-Nase 1 and 2 and B10.A α-Nase 1 and 2 represent hypothetical anti-nuclease idiotypes and are used for illustrative purposes only; they are not to be confused with other Nase markers that have been serologically defined and mapped. The relative positions of genes coding for these markers are arbitrary. The figure illustrates the following: a) position of different idiotypic and allotypic markers on the two chromosomes of a (B10.A × A/J)F\textsubscript{1}; b) a recombinational event involving those chromosomes; and c) the resultant chromosomes from a crossover as shown in b) which separates genes coding for different idiotypic markers.

The anti-nuclease idotype 2 and allotype; if the map distance between these loci were small, such recombinants would be rare as was observed. Preliminary results in fact have demonstrated that there are at least two idiotypes that can be recognized by our antisera.\textsuperscript{2} If these multiple genes coding for A/J antinuclease idotype account for the unequal recombination frequency, then the figure of 7\% is an underestimate of the recombination frequency, which is more accurately 7/65 or 11\%.

The meaning of the high recombination frequency for the A/J idotype

\textsuperscript{2} D. S. Pisetsky and D. H. Sachs. Manuscript in preparation.
marker(s) is not yet clear. It is possible that the chromosomal region involved with \( V_H \) genes is extremely large. The genes in this area could represent structural and/or regulatory genes for \( V_H \) region sequences; the size of the region would thus be a reflection of the large number of \( V_H \) sequences that are encoded in the germ line. The region could also be very large without necessitating the presence of a large number of \( V_H \) sequences in the germ line if \( V_H \) region genes were interspersed among genes coding for other proteins, perhaps unrelated to antibodies, although this possibility is less attractive.

Another possible explanation for the high recombination frequency involves the operation of unusual genetic mechanisms affecting \( V_H \) region genes. If genes for \( V_H \) sequences are somatically derived, there could be unusual features of the DNA that make it conducive to the multiple rearrangements necessary for the creation of a large number of different antibodies (i.e. generation of diversity). Recent experiments by Hozumi and Tonegawa (18) suggest that there is a rearrangement of genes coding for the constant and variable region of antibodies with these genes separated in embryonic tissue but close together in antibody producing cells. It is possible that the structural features that allow for such somatic rearrangements favor recombination. It is hoped that further studies with these recombinant animals will provide insight into some of the genetic processes that may be operating in this important chromosomal region.

Summary

Rat antisera detecting binding site-specific idiotypic determinants on anti-nuclease antibodies from A/J mice have been used to define the A/J anti-nuclease idiotype and to investigate its genetic linkage as a variable region marker. Analysis of the segregation of the A/J idiotype in progeny of the backcross (B10.A × A/J) × B10.A showed linkage of the idiotype to the Ig-1\( ^e \) heavy chain allotype locus. There was, however, a very high apparent frequency of recombination, with 7 of 101 backcross animals having a recombinant phenotype. All of these putative recombinants were accounted for by \( Ig-1^b/Ig-1^b \) homozygotes which bore the A/J idiotype, and none by \( Ig-1^b/Ig-1^c \) heterozygotes lacking the idiotype. On progeny testing of these animals in another backcross to B10.A the recombinant trait bred true. If this idiotype is indeed a marker for variable region structural genes, then the germ line gene pool must be very large or there must be special genetic mechanisms to account for the increased recombinational frequency observed.

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