GENETIC CONTROL OF THE IMMUNE RESPONSE TO STAPHYLOCOCCAL NUCLEASE

VIII. Mapping of Genes for Antibodies to Different Antigenic Regions of Nuclease

By DAVID S. PISETSKY AND DAVID H. SACHS

(From the Transplantation Biology Section, Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014)

Staphylococcal nuclease is a well-characterized protein antigen that has been used in our laboratory for a variety of studies on the genetic control of immune responses (1). Rat antisera detecting binding site-specific idiotypic determinants of anti-nuclease antibodies have been used to study the genetics of variable region genes (2-4). Thus, genes determining the predominant idiotype produced in A/J mice in response to nuclease were found to be linked to the heavy chain allotype locus, were subject to recombination with respect to this locus at a high frequency, and were independent of H-2-linked Ir genes in their expression (3, 4). In a preliminary study (3), it was shown that there existed at least two distinct noncross-reacting idiotypes determined by genes linked to heavy chain allotype linkage group (IgCH)[1] characteristic of strain A/J (IgCH^e) or SJL (IgCH^b). These two idiotypes were both present in immune sera from strain BALB/c (IgCH^a), but they were absent from sera of strain CB.20 (IgCH^b). The strain BAB.14 (IgCH^b), a recombinant strain which was produced during the transfer of the IgCH^b allotype locus of B6 to the BALB/c background, bore only the A/J idiotype. This result suggested that the recombinational event in the BAB.14 occurred within the gene segment which codes for variable regions of the immunoglobulin heavy chain (V_H) rather than between constant region (C_H) and V_H genes as had been inferred from other studies (5-7).

A genetic map of V_H region genes in the BALB/c strain was proposed as:

$$\text{A/J}\alpha\text{Nase} - \text{SJL}\alpha\text{Nase} - \text{IgCH}^a$$

where A/JαNase and SJLαNase represent loci for genes determining A/J and SJL anti-nuclease idiotypes, respectively, and IgCH^a represents the heavy chain allotype linkage group of BALB/c (8). From these studies, however, it was not possible to determine whether the A/J and SJL idiotypes were directed against the same or different antigenic determinants, or whether in fact they were on the same or different molecules. To answer some of these questions,

---

[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; CFA, complete Freund's adjuvant; C_H, constant region of the immunoglobulin heavy chain; CNBr, cyanogen bromide; IgCH, heavy chain allotype linkage group; V_H, variable region of the immunoglobulin heavy chain.

anti-nuclease antibodies from various strains have been fractionated into two populations on the basis of their ability to bind to a fragment of nuclease comprising the 99th–149th amino acids. By analysis of the idiotypes in these two fractions, the map order for strain BALB/c has been confirmed, and at least three new idiotypic markers have been identified, allowing additional map positions to be assigned. These results and analyses are the subject of this report.

Materials and Methods

Mice. A/J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine; SJL mice from Texas Inbred Mice Co., Houston, Texas; and BALB/c from National Institutes of Health Animal Production. CB.20 mice were a gift of Dr. M. Potter of the National Cancer Institute. BAB.14 mice were a gift of Dr. M. Weigert, Institute for Cancer Research, Philadelphia, Pennsylvania. All mice were males except those of strain SJL.

Rats. Adult male Lewis rats were purchased from Microbiological Associates, Walkersville, Md.

Preparation of Anti-Nuclease Antibodies. Groups of 5–25 mice were immunized with 100 μg of purified nuclease in complete Freund's adjuvant (CFA) and boosted weekly with 10 μg of nuclease in saline beginning on the 3rd wk after immunization. For the experiments reported here, pooled hyperimmune sera obtained after the 4th and 5th boost were used.

Preparation of Anti-Idiotypic Antisera. Lewis rats were immunized every 2 wk with 500 μg of affinity column-purified anti-nuclease antibodies in CFA. Sera from individual animals showing the highest activity were used in these experiments.

Assay of Anti-Nuclease Antibodies. The assay of anti-nuclease antibodies has been previously described (9). Briefly, the ability of anti-nuclease antibodies to inactivate the enzymatic activity of nuclease was determined at increasing dilutions of serum, and the plateau value for units inactivated per milliliter of antiserum was calculated.

Assay for the Presence of Idiotypes in Anti-Nuclease Antibodies. The details of this assay have been previously reported (2). Briefly, the ability of anti-idiotypic antisera to inhibit the inactivation of nuclease by antibodies was determined. To perform the assay, an anti-nuclease preparation was preincubated with anti-idiotypic antiserum for 5 min, and then incubated with nuclease. The units inactivated were determined and then compared to an identical amount of anti-nuclease antibody preincubated with 0.1% bovine serum albumin (BSA) in saline. The inhibitory activity of the anti-idiotypic antisera was calculated using the formula

$$\% \text{ inhibition} = \frac{A(\text{Nase} + \alpha\text{Nase} + \alpha\text{ID}) - A(\text{Nase} + \alpha\text{Nase})}{A(\text{Nase}) - A(\text{Nase} + \alpha\text{Nase})}$$

in which A(Nase + αNase + αID) indicates the activity measured in the presence of nuclease (Nase), antibody (αNase), and anti-idiotype (αID), etc., as previously described. In all cases, the final dilutions of the antibody preparations used had comparable activities in terms of units of nuclease inactivated. Results presented were obtained from at least duplicate determinations on each antibody preparation. An antibody preparation was considered to contain a given idiotype if the anti-idiotype produced statistically significant ($P < 0.05$) inhibition of inactivation.

Preparation of Nuclease Fragment (99-149). The nuclease fragment comprising the carboxy terminal 99th-149th amino acids was prepared according to published methods (10). Briefly, nuclease was degraded with cyanogen bromide (CNBr) and the resulting cleavage products were separated by gel filtration chromatography on Sephadex G-50. The major peak with absorbance at 280 nm was then chromatographed twice on phosphocellulose, developing the column with a linear elution gradient of ammonium acetate 0.1 M, pH 3, to 1.0 M, pH 8. Purity of the resulting peptide was assessed by use of a complementation assay in which the ability of nuclease fragment (99-149) to combine with nuclease fragment (1-126) to form an enzymatically active structure was determined (11). By comparison with a preparation of fragment (99-149) of known purity, a purity of ≅90% was determined for the preparation used in these experiments.

Fractionation of Anti-Nuclease Antibodies. To separate antibodies capable of binding to the fragment (99-149), affinity chromatography was employed. Fragment (99-149) was coupled to

TABLE I

Strains Used in the Present Study

Strain	H-2 Haplotype	Allotype	Anti-nuclease idiotypes
A/J	a	e	A/J
SJL	s	b	SJL
B10.A	a	b	B10
BALB/c	d	a	A/J, SJL
CB.20	d	b	B10
BAB.14	d	b	A/J

CNBr-activated Sepharose 4B as previously described (12). Hyperimmune sera were passed over this column. The antibodies not binding were considered to be devoid of specificities for determinants represented on the fragment and were termed anti-nuclease $(1-99)_N$, in which the subscript N indicates that the antibodies were prepared against intact, native nuclease, according to previously established conventions (13). To remove bound antibodies, the column was eluted with 0.5 M guanidine, 0.01 M KPO_4, pH 7, containing 0.1% BSA. The material obtained by this elution method was termed anti-nuclease $(99-149)_N$. Fractionated antibody preparations could be stored at $-20°F$ without loss of activity for at least 2 mo.

Results

Fractionation of Antibodies. The fractionation procedure used in these experiments is based on the ability of antibodies prepared against the native form of a protein antigen to bind to a peptide fragment of the protein. This approach has been previously used in fractionation of goat anti-nuclease antibodies to yield preparations of restricted heterogeneity (12), and a theoretical basis for this fractionation has been described in terms of the conformational equilibrium of the peptide in solution (14). The peptide used in these studies, nuclease (99-149), comprises the carboxy terminal 99th to 149th amino acids of nuclease.

Table I lists the strains used in these studies along with their *H-2* haplotype, C_H allotype, and known anti-nuclease idiotypes. Antibodies from these strains were fractionated into anti-nuclease $(99-149)_N$ and anti-nuclease $(1-99)_N$ fractions as described in Materials and Methods. Various eluting conditions were tested for their ability to remove bound antibodies from the column; the most stable and active preparations were obtained using 0.5 M guanidine containing 0.1% BSA. Higher concentrations of guanidine or other chaotropic agents were able to remove slightly more bound protein, but the resulting preparations were less active. With the elution conditions described, the anti-nuclease $(99-149)_N$ fraction contained from 2 to 6% of the inactivating capacity of the unfractionated sera for the strains examined. In contrast, anti-nuclease $(99-149)_N$ preparations from goat anti-nuclease antisera comprised 11.5% of the total antibodies (12). It has not yet been determined whether in mice anti-nuclease $(99-149)_N$ antibodies represent a smaller percentage of the total anti-nuclease response than in the goat, or whether the mouse antibodies have become denatured during the fractionation procedures yielding preparations of lower specific activity.

Idiotype Analysis of Antibody Preparations. The idiotypes present in fractionated and unfractionated antibodies of the five strains tested were assayed by use of antisera prepared in Lewis rats against purified anti-nuclease
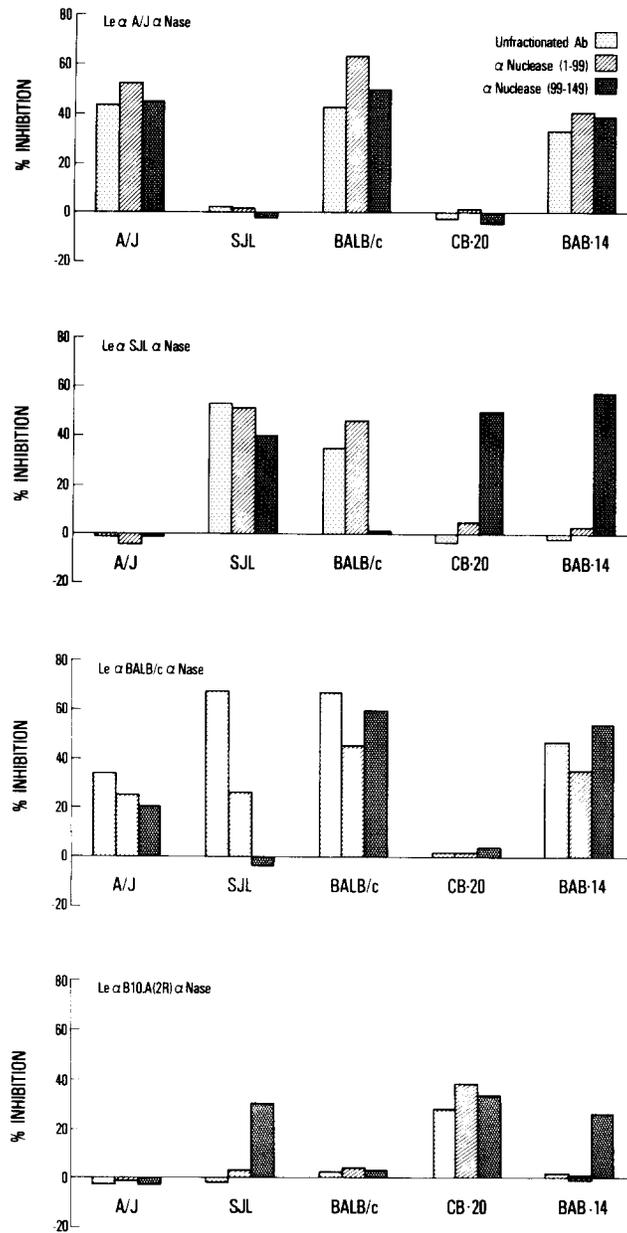
Fig. 1.   Determination of anti-nuclease idiotypes by inhibition of antibody inactivation. Anti-nuclease antibodies from strains A/J, SJL, BALB/c, CB.20, and BAB.14 were fractionated into anti-nuclease $(1-99)_N$ and anti-nuclease $(99-149)_N$ as described in Materials and Methods. Fractionated antibody preparations as well as unfractionated antisera were tested with anti-idiotypic antisera specific for A/J, SJL, BALB/c, or B10.A(2R) anti-nuclease antibodies. Results are plotted in terms of the percentage of antibody-mediated inactivation that is inhibited by the anti-idiotypic antisera.

antibodies from strains A/J, SJL, BALB/c, and B10.A(2R). B10.A(2R) is a high responder strain with a recombinant *H-2* haplotype on the B10 background. Since *H-2* type has been found not to influence idiotype expression (3), the markers recognized by this sera will be referred to (for simplicity) as B10 idiotypes.

Fig. 1 presents in graphic form the idiotype analysis of the unfractionated antibodies as well as the anti-nuclease $(1-99)_N$ and anti-nuclease $(99-149)_N$ preparations from the strains analyzed. The percent inhibition of inactivation produced by the different idiotypes is shown. It is apparent from this figure that each anti-idiotypic antiserum was capable of recognizing at least two sets of idiotypic determinants, one detected in the anti-nuclease $(1-99)_N$ population and the other in the anti-nuclease $(99-149)_N$ populations. Thus, additional anti-nuclease idiotypes can be defined and distinguished in terms of the specificity of the antibodies, i.e. either anti-nuclease $(1-99)_N$ or anti-nuclease $(99-149)_N$.

Also apparent in Fig. 1 is the fact that in all cases, the pattern of reactivity of the anti-nuclease $(1-99)_N$ fraction was the same as that of unfractionated antibodies. This result was interpreted to indicate that the inhibition of nuclease inactivation assay for idiotypes detects only those idiotypes present in highest concentration. Studies by Berzofsky et al. (15) showed that within the anti-nuclease sera of strains A/J, SJL, and B10.A, antibodies with specificity for fragment (99-149) were less abundant than antibodies with specificity for fragment (1-126). Since anti-nuclease $(1-99)_N$ should be of similar composition to anti-nuclease $(1-126)_N$, this result is consistent with our observation that idiotypes of anti-nuclease $(1-99)_N$ were more readily detected in unfractionated antisera than idiotypes of anti-nuclease $(99-149)_N$.

The most surprising and, perhaps, important result seen in Fig. 1 was that the pattern of reactivity with the anti-idiotypic antisera of the anti-nuclease $(99-149)_N$ and anti-nuclease $(1-99)_N$ antibody populations differed for several strains. The following differences were noted. (*a*) Anti-nuclease $(1-99)_N$ antibodies from strains SJL and BALB/c expressed cross-reacting idiotypes, whereas anti-nuclease $(99-149)_N$ antibodies from the same strains did not. This result was obtained using anti-idiotypic antisera prepared against either SJL or BALB/c antibodies. (*b*) Anti-nuclease $(99-149)_N$ from CB.20 and SJL shared a cross-reacting idiotype, whereas the anti-nuclease $(1-99)_N$ antibodies did not. Again, an identical result was obtained using the anti-idiotypic antisera prepared against SJL or B10.A(2R) antibodies. (*c*) Unlike BALB/c, strain BAB.14 expressed an anti-nuclease $(99-149)_N$ idiotype that cross-reacted with anti-nuclease $(99-149)_N$ from CB.20 and SJL. On the basis of these results, five distinct patterns of reactivity of antibody populations with the anti-idiotypic antisera could be distinguished. The idiotypes thus identified were termed NASE idiotypes and were identified by sequential letters A through E (Table II).

Discussion

The results presented in this communication confirm and extend our previous finding on the strain distribution of anti-nuclease idiotypes. By fractionation of antibodies to individual antigenic regions of the nuclease molecules, we have

Table II
Strain Distribution of NASE Idiotype Markers

Antigenic determinant	Idiotype markers	Positive strains
Nuclease (1-99)$_N$	NASE-A	A/J, BALB/c, BAB.14
	NASE-B	SJL, BALB/c
	NASE-C	CB.20, B10
Nuclease (99-149)$_N$	NASE-D	A/J, BALB/c, BAB.14
	NASE-E	SJL, CB.20, B10, BAB.14

Various anti-nuclease idiotypes, designated NASE markers, are listed along with their strain distribution and the antigenic region to which they are directed.
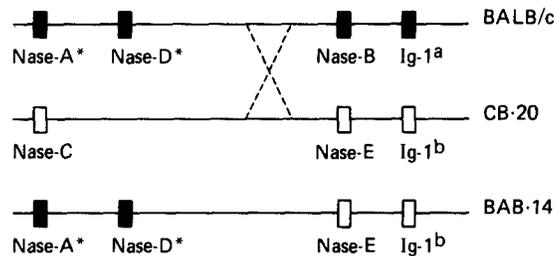

Fig. 2. Proposed map of genes determining anti-nuclease idiotypes. Map positions of genes for various NASE idiotype markers were assigned to account for results obtained on idiotype determinations of anti-nuclease antisera from strains BALB/c, CB.20, and the recombinant strain BAB.14. Distances with respect to the IgCH allotype locus as well as the relative positions of the NASE-A and NASE-D markers are arbitrary. The recombinational event that resulted in the BAB.14 strain is shown as involving the strain CB.20, as this strain was the source of antibodies used for the idiotype determinations. * indicates that position of these markers relative to each other is arbitrary.

been able to identify five distinct anti-nuclease idiotypes. These idiotypes can be distinguished by the antigenic region to which they are directed (either nuclease [1-99] or nuclease [99-149]) and by their strain distribution. This number of idiotypes represents the minimum number needed to account for the observed results, and it is likely an underestimate of the true number of anti-nuclease idiotypes present in immune sera. For each of five strains examined, a unique pattern of expression of these idiotypes has been identified.

For three of these strains, BALB/c, CB.20, and BAB.14, it is possible to map the genes that determine the different anti-nuclease idiotypes. To construct such a map, it is necessary that the genes determining these idiotypes all be linked to the C_H allotype locus. Since these three strains are congenic, with known genetic differences only at the C_H allotype locus and closely linked genes, the differences in their idiotype expression can be accounted for entirely on the basis of the linkage of idiotype genes to the C_H allotype locus. This linkage, however, does not imply that the structures recognized by the anti-idiotypic antisera are present exclusively on the heavy chain, nor that the gene linked to the C_H allotype locus is a structural rather than a regulatory gene.

Fig. 2 shows maps of idiotype genes for strains BALB/c, CB.20, and BAB.14

using the NASE idiotype markers defined in Table II. In these maps, the recombination event that occurred during the development of the BAB.14 strain is illustrated as involving the CB.20 strain, since the CB.20 strain was the source of antibodies of the B6 allotype used in the idiotype determination. A precise designation of the actual strain involved in this event cannot be given, since the time of occurrence of the recombination is unknown. These maps indicate that the crossover point between the BALB/c and B6 (CB.20) chromosomes occurred among the V_H genes rather than between V_H and C_H genes.

Two lines of evidence support this placement: (a) the BAB.14 strain has acquired the NASE-A and NASE-D markers from BALB/c, but not the NASE-B marker; (b) the BAB.14 strain expressed the NASE-E marker, which indicates that V_H region genes from B6 were acquired along with the IgCH[b] allotype locus. Since the BAB.14 strain has V_H genes from BALB/c and B6, the recombinational event must have occurred between different V_H region genes. At present, the position of the NASE markers can be made only relative to the crossover point and the C_H allotype locus. The map distances between the genes, as well as the ordering of NASE-A and NASE-D as shown is arbitrary. Nevertheless, such mapping is consistent with a linear array of V_H region genes.

These data also indicate that genes determining antibodies directed against the same antigenic regions of the nuclease molecule may have different map positions in different strains. Several examples are evident from our maps: (a) the genes determining anti-nuclease $(99\text{-}149)_N$ antibodies in strain CB.20 (NASE-E idiotype) are close to the allotype locus, whereas genes determining anti-nuclease $(99\text{-}149)_N$ antibodies in BALB/c (NASE-D idiotype) are distant, being localized to the other side of crossover point; (b) genes determining anti-nuclease $(1\text{-}99)_N$ in BALB/c (NASE-B idiotype) are close to the allotype locus, whereas anti-nuclease $(1\text{-}99)_N$ idiotype in strain CB.20 (NASE-C) and strain BALB/c (NASE-A) are both more distant, distal to the postulated crossover point; (c) in the BAB.14 strain, genes for idiotypically distinct anti-nuclease $(99\text{-}149)_N$ idiotypes occur at different positions, on either side of the crossover point. These results are similar to those obtained by Berek et al. (16) for mapping anti-streptococcal idiotypes, and they support the notion that genes for antibodies to the same antigenic determinants may map at nonhomologous positions in different strains. This suggests that different anti-nuclease idiotypes are not necessarily allelic.

In a previous study (3), two interesting features of the strain distribution of anti-nuclease idiotypes were noted: first, strain BALB/c (IgCH[a]) shared idiotypes with strain A/J (IgCH[e]) and SJL (IgCH[b]); and second, strain B10 (IgCH[b]) did not share idiotypes with strain SJL (IgCH[b]). From these data we speculated on possible mechanisms of gene evolution that could have led to this distribution. It is clear from the studies presented here that the situation is more complex. Thus, whereas BALB/c mice have anti-nuclease $(1\text{-}99)_N$ idiotypes in common with both A/J and SJL, the anti-nuclease $(99\text{-}149)_N$ idiotypes are shared only with strain A/J. Similarly, although strains SJL and CB.20 do not share idiotypes for anti-nuclease $(1\text{-}99)_N$ antibodies, they do share an idiotype for anti-nuclease $(99\text{-}149)_N$. These results suggest that genes coding for different antibodies to the same antigen may have evolved independently. For the B10 and SJL

strains, two models to account for such independent evolutionary development can be suggested. In the first model, one can postulate that both strains derived from a common ancestor. As the strains evolved, however, there was divergence of only some of the V_H region genes that had been inherited in common in such a manner that genes for the anti-nuclease $(1-99)_N$ idiotypes in the two strains no longer coded for cross-reacting idiotypes. This sequence of gene evolution would be consistent with allelism of genes determining antibodies to the same antigenic region if the genes for anti-nuclease $(1-99)_N$ idiotypes in both strains derived from the same ancestral genes. It is equally plausible, however, that the genes for the two idiotypes evolved from different ancestral genes. Alternatively, in a second model, one can consider both strains to have evolved from different ancestors with different V-gene repertoires. One can then propose that by convergent evolution, genes coding for cross-reactive anti-nuclease $(99-149)_N$ idiotypes emerged in both strains, although not necessarily at the same chromosomal location. At the moment, the constraints placed upon this evolutionary development by C_H allotype and other genes, e.g. immune response genes or genes coding for light chains, are unknown. It is hoped that analysis of more idiotypic markers will give further insight into this problem.

Summary

Antibodies to staphylococcal nuclease have been fractionated into two populations on the basis of their ability to bind to the cyanogen bromide cleavage product of nuclease comprising the C-terminal portion of the molecule from the 99th to the 149th amino acid. The two populations of antibodies, anti-nuclease $(1-99)_N$ and anti-nuclease $(99-149)_N$, have been prepared from a variety of strains, and analyzed using anti-idiotypic antisera raised against whole anti-nuclease antibodies from strains A/J, SJL, BALB/c, and B10.A(2R). Anti-nuclease $(1-99)_N$ antibodies had the same pattern of reactivity with the anti-idiotypic antisera as did unfractionated antibodies, whereas a different pattern was found for anti-nuclease $(99-149)_N$ preparations. On the basis of these studies, five anti-nuclease idiotypes, designated NASE markers, have been identified and defined on the basis of their antigenic specificity and strain distribution. With these additional markers, it has been possible to provide more detailed maps of variable (V) region genes in the strains BALB/c, CB.20, and the recombinant BAB.14. A recombinational event between V region genes during the development of the BAB.14 strain is suggested by the positioning of these NASE markers.

References

1. Sachs, D. H., J. A. Berzofsky, C. G. Fathman, D. S. Pisetsky, A. N. Schechter, and R. N. Schwartz. 1977. The immune response to staphylococcal nuclease: a probe of cellular and humoral antigen-specific receptors. *Cold Spring Harbor Symp. Quant. Biol.* 41:295.

2. Fathman, C. G., and D. H. Sachs. 1976. Genetic control of the immune response to nuclease. II. Detection of idiotypic determinants by the inhibition of antibody-mediated nuclease inactivation. *J. Immunol.* 116:959.

3. Fathman, C. G., D. S. Pisetsky, and D. H. Sachs. 1976. Genetic control of the immune response to nuclease. V. Genetic linkage and strain distribution of anti-nuclease idiotypes. *J. Exp. Med.* 145:569.

4. Pisetsky, D. S., and D. H. Sachs. 1977. The genetic control of the immune response to staphylococcal nuclease. VI. Recombination between genes determining the A/J anti-nuclease idiotypes and the heavy chain allotype locus. *J. Exp. Med.* 146:1603.

5. Blomberg, B., W. R. Geckeler, and M. Weigert. 1972. Genetics of the antibody response to dextran in mice. *Science (Wash. D. C.).* 177:178.

6. Riblet, R., M. Weigert, and O. Makela. 1975. Genetics of mouse antibody. II. Recombination between V_H genes and allotype. *Eur. J. Immunol.* 5:778.

7. Eichmann, K. 1975. Genetic control of antibody specificity in the mouse. *Immunogenetics.* 2:491.

8. Lieberman, R. 1978. Genetics of IgCH (allotype) locus in the mouse. *Sem. Immunopath.* In press.

9. Lozner, E. C., D. H. Sachs, and G. M. Shearer. 1974. Genetic control of the immune response to staphylococcal nuclease. I. *Ir*-Nase: Control of the antibody response to nuclease by the *Ir* region of the mouse *H-2* complex. *J. Exp. Med.* 139:1204.

10. Taniuchi, H., and C. B. Anfinsen. 1966. The amino acid sequence of an extracellular nuclease of Staphylococcus aureus. I. Linear order of the fragments produced by cleavage with cyanogen bromide. *J. Biol. Chem.* 241:4366.

11. Anfinsen, C. B., P. Cuatrecasas, and H. Taniuchi. 1971. Staphylococcal nuclease, chemical properties and catalysis. *In* The Enzymes. P. D. Boyer, editor. Academic Press, Inc., New York. 4:177.

12. Sachs, D. H., A. N. Schechter, A. Eastlake, and C. B. Anfinsen. 1972. Antibodies to a distinct antigenic determinant of staphylococcal nuclease. *J. Immunol.* 109:1300.

13. Furie, B., A. N. Schechter, D. H. Sachs, and C. B. Anfinsen. 1974. Antibodies to the unfolded form of a helix-rich region in staphylococcal nuclease. *Biochemistry.* 13:1561.

14. Sachs, D. H., A. N. Schechter, A. Eastlake, and C. B. Anfinsen. 1972. An immunologic approach to the conformational equilibria of polypeptides. *Proc. Natl. Acad. Sci. U. S. A.* 69:3790.

15. Berzofsky, J. A., A. N. Schechter, G. M. Shearer, and D. H. Sachs. 1977. Genetic control of the immune response to staphylococcal nuclease. IV. *H-2*-linked control of the relative proportions of antibodies produced to different determinants of native nuclease. *J. Exp. Med.* 145:123.

16. Berek, C., B. A. Taylor, and K. Eichmann. 1976. Genetics of the idiotype of BALB/c myeloma S117: multiple chromosomal loci for V_H genes encoding specificity for group A streptococcal carbohydrate. *J. Exp. Med.* 144:1164.