

GENETIC CONTROL OF THE IMMUNE RESPONSE TO STAPHYLOCOCCAL NUCLEASE

I. *I_R*-NASE: CONTROL OF THE ANTIBODY RESPONSE TO NUCLEASE BY THE *I_R* REGION OF THE MOUSE *H-2* COMPLEX

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Staphylococcal nuclease is a well-characterized extracellular enzyme produced by *Staphylococcus aureus* (1). The amino acid sequence and three-dimensional crystallographic structure of this protein have been determined (2, 3). Furthermore, a variety of peptide fragments of this enzyme have been characterized (4-6). Previous immunochemical studies of nuclease (Nase)¹ have shown that antibody populations specific for individual antigenic regions of the molecule can be isolated by means of successive immunoabsorption to peptide fragments of decreasing size (7), and that inactivation of enzymatic activity by such antibodies provides a very sensitive means of quantitating the antibody-antigen interaction (8).

In the course of immunization of inbred mice with Nase, a marked difference was observed in the ability of two different strains to produce antibodies under identical conditions of immunization. Since Nase offers certain immunochemical advantages compared to other native proteins for which genetic control of immune responsiveness has been described (9-11), a detailed investigation of possible differences in response potential was made using inbred, congenic resistant, and recombinant lines of mice. This report presents evidence for genetic control of levels of antibody production to Nase in mice and describes the localization of the gene(s) responsible for this control within the major histocompatibility complex (*H-2*).

Materials and Methods

Mice.—Adult male mice, 8-12 wk of age, were used throughout the experiments. DBA/2 and BALB/c mice were obtained from the Animal Production Unit, National Institutes of Health, Bethesda, Md. B10.A(4R) recombinant mice were kindly provided by Dr. Jack H. Stimpfling, The McLaughlin Institute, Great Falls, Mont. B10.A(2R) and B10.A(5R) were obtained from Mr. S. Pooley, Mammalian Genetics and Animal Production Section, National

¹ *Abbreviations used in this paper:* *H-2*, histocompatibility complex; *I_R*, immune response; Nase, nuclease; (Phe, G)-Pro-L, poly (LPhe, LGlu)-polyLPro- -polyLLys; (T, G)-Pro-L, poly (LTyr, LGlu)-polyLPro- -polyLLys.

Cancer Institute, Bethesda, Md. Other mouse strains were purchased from the Jackson Laboratory, Bar Harbor, Me. [(DBA/1 × SJL/J)F₁ × (DBA/1 × SJL/J)F₁]F₂ mice were raised in our animal rooms from stock purchased from the Jackson Laboratory.

Antigens.—Staphylococcal nuclease (Nase) was prepared and purified by one of us (DHS) as previously described (7), using facilities kindly made available by Dr. C. B. Anfinsen in the Laboratory of Chemical Biology, National Institute of Arthritis, Metabolic and Digestive Diseases, National Institutes of Health, Bethesda, Md.

Poly (L-Tyr, L-Glu)-poly-L-Pro- -poly-L-Lys, abbreviated (T, G)-Pro- -L, was a gift from Dr. Edna Mozes, Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel.

Immunizations.—Mice were immunized with a single intraperitoneal injection of 100 μg of Nase in 0.1 ml NaCl emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.), and bled from the retroorbital plexus 3 wk later.

The F₂ progeny mice immunized with Nase were injected intraperitoneally with 10 μg of (T, G)-Pro- -L in complete Freund's adjuvant 3 wk after Nase immunization. These animals were boosted with 10 μg of soluble (T, G)-Pro- -L 3 wk later and bled 2 wk after boosting (12).

Assays for Anti-Nase and Anti-(T, G)-Pro- -L Antibodies.—20 μl of a 5 μg/ml solution of Nase in 0.1% bovine serum albumin was mixed with 10 μl of the antiserum being tested in the well of a siliconized microtiter plate (Cooke Engineering, Alexandria, Va.) and incubated for 5 min at room temperature. 10 μl of this mixture was then added to a cuvette containing 1.0 ml of a 50 μg/ml solution of heat-denatured DNA (13) in 0.025 M Tris Buffer, pH 8.8, containing 0.01 M Ca. The change in OD₂₆₀ was then recorded on a Gilford Model 2000 multiple sample absorbance recorder equipped with a Beckman DU spectrophotometer. Generally three antiserum samples and one control sample, in which serum was replaced by saline in the initial incubation, were run simultaneously. The units of enzymatic activity (one unit = 1.0 OD₂₆₀ U/min) in the experimental cuvettes were then subtracted from the units of activity in the control cuvettes, and the difference was corrected according to the value obtained that day for a standard goat antinuclease antiserum of known activity. Antiserum activity was expressed as units of Nase inactivated per milliliter of serum.

Sera of F₂ progeny mice were assayed for anti-(T, G)-Pro- -L activity by passive microhemagglutination as previously described (1).

H-2 Typing.—The H-2 type of the [(DBA/1 × SJL/J)F₁ × (DBA/1 × SJL/J)F₁]F₂ mice was determined by the hemagglutination method of Kaliss (14). Anti-H-2^a and anti-H-2^b alloantisera were prepared by reciprocally immunizing SJL/J and DBA/1 animals with 2 × 10⁷ lymphoid cells (spleen, lymph nodes, and thymus) given intraperitoneally at weekly intervals for five injections. Animals were bled 1 wk after the last immunization.

RESULTS

Antibody Response to Nase in Inbred Mouse Strains.—Pooled or individual sera from a variety of inbred mouse strains were assayed by the Nase enzymatic inactivation method. The results, shown in Table I, indicate that the inactivation capacity of the sera from different strains could be divided into two categories without overlap: those that inactivated more than 10 U of Nase, and those that inactivated less than 4 U of the enzyme. From the distribution of responses shown, ability to make anti-Nase antibody conformed to the H-2 type of the animals. Thus, of the strains tested, H-2^{a, d, k, s} mice were all high responders to Nase, whereas H-2^{b, q} animals were low antibody producers. Furthermore, this association of responsiveness with H-2 type was verified by

the observation that two groups of congenic resistant mouse strains C57BL/10, B10.A, and B10.D2; and C₃H/HeJ and C₃H.SW, which differ only at *H-2* within each group, also differed in their response potential to Nase (compare in Table I, line 3 with lines 2 and 7, and line 4 with line 9).

H-2 Linkage of the Immune Response Gene Controlling Antibody Response to Nase in SJL/J and DBA/1 mice.—Because the results presented in the preceding section suggested possible *H-2* linkage of the *Ir* gene controlling antibody responsiveness to Nase, classical genetic verification of this observation was attempted using SJL/J, DBA/1, (DBA/1 × SJL/J)F₁, and [(DBA/1 × SJL/J)F₁ × (DBA/1 × SJL/J)F₁]F₂ mice. These two parental strains seemed

TABLE I
*Anti-Staphylococcal Nuclease Antibody Responses in Different Mouse Strains**

Mouse strain	<i>H-2</i> type	Units of nuclease (Inactivated/ml serum ± SE)
A/J	a	17.9 ± 1.0
B10.A	a	11.8 ± 1.0
C57BL/10	b	3.9 ± 0.8
C3H.SW	b	1.4
DBA/2	d	14.0
BALB/c	d	15.8
B10.D2	d	16.4 ± 1.2
AKR/J	k	10.3
C3H/HeJ	k	11.0
C57.BR	k	14.5 ± 1.1
DBA/1	q	1.3 ± 1.1
SJL/J	s	17.9 ± 1.2

* Four to five mice of each strain were injected intraperitoneally with 100 μg Nase in complete Freund's adjuvant. Mice were bled 3 wk later and their sera were assayed for anti-Nase activity by the enzymatic inactivation method. Sera were assayed from individual mice in those cases where standard errors are shown. Otherwise, the assays were from serum pools.

particularly appropriate for this genetic study because they have previously been shown to be high and low responders for two non-*H-2*-linked *Ir* genes (11, 12), including the *Ir-3* gene controlling response to (T,G)-Pro-*L* in SJL/J and DBA/1 mice (12). For a multideterminant immunogen such as Nase (7, 15), it seemed possible that responsiveness could be *H-2* linked in some strains, but not necessarily linked to the major histocompatibility complex in the SJL/J and DBA/1 combinations. SJL/J, DBA/1, and the F₁ and F₂ progeny of these two strains were immunized by the standard protocol. These sera were assayed for anti-Nase activity, and the animals typed for *H-2*. The same F₂ animals were subsequently immunized and boosted with 10 μg of (T,G)-Pro-*L*, bled, and their sera were titered for anti-(T,G)-Pro-*L* antibody by hemagglutination. An identical immunization schedule was performed

using SJL/J mice (high responders to both immunogens) to exclude possible antigenic competition between Nase and (T,G)-Pro-*-L* (data not shown). Results of individual Nase and (T,G)-Pro-*-L* antibody responses for 19 F₂ progeny, as well as their respective *H-2* types are summarized in Table II. The mean Nase responses (excluding mouse 8) of the three *H-2* classes of F₂ progeny, as well as the parental and F₁ responses are graphically illustrated in Fig. 1. The anti-Nase response potential of all the F₂ mice tested (with one

TABLE II
H-2 Type, Anti-Staphylococcal Nuclease, and Anti-(T, G)-Pro-*-L* Antibody Responses in (DBA/1 × SJL/J)F₂ Mice*

Mouse	<i>H-2</i> type	Units of nuclease (Inactivated/ml serum)	Anti-(T,G)-Pro- <i>-L</i> hemagglutination titer
1	s/s	18.2	1:16
2	q/s	18.2	1:16
3	s/s	16.1	1:32
4	q/s	14.6	1:16
5	s/s	18.4	1:64
6	s/s	16.0	1:4
7	s/s	18.4	NT
8	q/q	11.1	1:8
9	q/s	13.0	1:4
10	q/s	10.1	1:16
11	q/q	2.1	1:16
12	q/s	13.0	1:32
13	q/q	3.1	1:8
14	s/s	19.3	1:8
15	q/s	18.6	1:32
16	q/s	17.7	1:8
17	q/s	18.4	1:32
18	q/q	3.5	1:8
19	q/s	16.0	1:16

* Mice were injected intraperitoneally with 100 μg Nase in complete Freund's adjuvant and bled 3 wk later. Their sera were assayed for anti-nuclease activity by the enzymatic inactivation method.

NT, not tested.

exception) correlated well with *H-2* type. The sera of all s/s and q/s animals inactivated more than 10 U of Nase, whereas sera from the mice that typed q/q inactivated less than 4 U of enzymatic activity. One mouse (no. 8) which repeatedly typed q/q was a high responder. This animal is being progeny tested for possible genetic recombination. The anti-(T,G)-Pro-*-L* hemagglutination titers of the 19 mice are shown in the right column of Table II. Neither the *H-2* type nor the Nase response potential correlated with responsiveness to (T,G)-Pro-*-L*. This confirms the published results of Mozes et al. (12) that the *Ir-3* gene controlling antibody response to (T,G)-Pro-*-L* is not *H-2* linked.

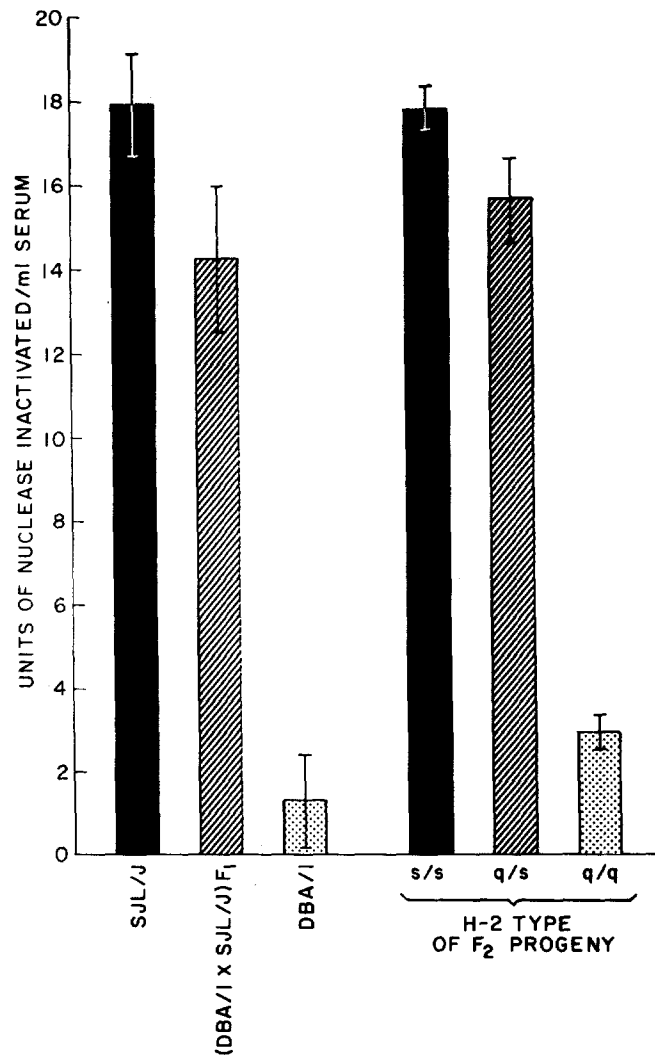


FIG. 1. Anti-Nase antibody responses in SJL/J, DBA/1, (DBA/1 × SJL/J)F₁, and (DBA/1 × SJL/J)F₂ mouse strains, ±SE. The responses of the F₂ progeny are separated according to their H-2 type and, for comparison purposes, illustrated with the same shading as the corresponding parental or F₁ or H-2 type.

Intra-H-2 Localization of Ir-Nase.—In order to map the *Ir* gene controlling responsiveness to Nase within the major histocompatibility complex, use was made of the B10.A(2R), B10.A(4R), and B10.A(5R) recombinant strains in which three distinct crossovers have been demonstrated within H-2 (16, 17). C57BL/10, B10.A, and the three recombinant strains were immunized with

Nase. Sera from the individual mice were assayed for anti-Nase activity. The results are shown in Fig. 2, along with the corresponding *H-2* types and diagrams of the crossovers of the recombinants. B10.A(2R) mice were the highest responders among the recombinants tested, whereas the B10.A(4R) and B10.A(5R) were low responders. These results map the gene controlling antibody response to Nase to the same region as the gene regulating responsiveness to mouse IgG myeloma (17) and distinct from the region(s) controlling responses to mouse IgA myeloma (17), and the A-*-L* series of branched chain synthetic polypeptides (18).

MOUSE STRAIN	H-2 TYPE	MAJOR HISTOCOMPATIBILITY COMPLEX					UNITS OF NUCLEASE INACTIVATED/ml SERUM \pm S E
		H-2K	Ir-I	Ir-IgG	Ss-Slp	H-2D	
C57BL/10	b	■	■	■	■	■	3.9 \pm 0.8
B10.A	a	□	□	□	□	□	11.8 \pm 1.0
B10.A(2R)	h ^{"2"}	□	□	□	□	■	8.0 \pm 1.9
B10.A(4R)	h ^{"4"}	□	□	■	■	■	2.0 \pm 0.5
B10.A(5R)	i	■	■	■	□	□	1.6 \pm 0.6

FIG. 2. Anti-Nase antibody responses of C57BL/10, B10.A, B10.A(2R), B10.A(4R), and B10.5(5R) congenic resistant mouse strains. Schematic diagrams of the major histocompatibility complex of each strain are illustrated to show the relationship of the anti-Nase response to the *Ir-IgG* subregion of *H-2*.

DISCUSSION

Genetic control of antibody responses in mice has been demonstrated for a number of defined synthetic immunogens composed of multichain (12, 19-21) and linear polypeptides (22-24). For more complex immunogens, genetic regulation of responsiveness in this species has been reported for limiting immunogenic doses of certain proteins (9, 10). This dose-dependent expression of *Ir* genes was attributed to immunological stimulation by those determinants which were both most immunopotent and under the control of a specific *Ir* gene (25). It is possible therefore, that heredity influences responsiveness to many complex immunogens, although such control may go undetected due to the animal's response potential to different antigenic moieties not being controlled by a particular *Ir* gene. This hypothesis has been verified using the multichain synthetic polypeptide poly (L₁Phe, L₁Glu)-polyL₁Pro- -polyL₁Lys [(Phe, G)-Pro- -L] (21), and more recently with the "loop" peptide of lysozyme

(11). Thus, although both the SJL/J and DBA/1 mouse strains responded to (Phe, G)-Pro- α -L, the antibodies synthesized by the former strain were specific for Pro- α -L, whereas the antibodies from the latter strain were directed mainly toward the (Phe, G) portion of the molecule (21). In the case of lysozyme, all mouse strains tested (with one exception) responded to the intact lysozyme molecule. However, the antibodies produced by some mouse strains were directed toward determinants other than the "loop" region of the enzyme (11).

With regard to such possible dissection of a complex macromolecule into components to which responsiveness may or may not be under genetic control, it is important to note that the relative roles of carrier and haptenic determinants in the Ir-controlled response to an antigen remain unclear. It seems possible, for example, that a genetic defect in the ability of an animal to recognize a carrier determinant might preclude response against a number of haptenic determinants even though the animal was capable of responding to such haptenic determinants on an appropriate carrier (26-28). On the other hand, there may also be genetic defects in ability to recognize haptenic determinants regardless of carrier, and these may also be controlled by the *I τ* region.

Staphylococcal nuclease may have advantages over other multideterminant antigens in terms of potential for answering these questions. Unlike the synthetic polypeptide antigens, nuclease is a naturally occurring protein and has inherent enzymatic activity, which may correlate with the invasiveness of the staphylococcal pathogen (29). During evolution, the development of the immune system must have been influenced by the need to recognize and react against just such naturally occurring foreign proteins. Thus nuclease should serve as an accurate model for dissecting early events in the afferent phase of immune recognition. The fact that the humoral response to the entire immunogen is genetically controlled in mice, and that the locus of control resides in the *H-2* complex, should make possible a variety of functional studies, such as, for example, specific cellular cooperation (30).

In addition, the extensive biochemical and immunologic characterizations which have been previously carried out on this molecule make possible a variety of correlations between the antigen's structure and functional aspects of its interactions with antibodies and cells. The amino acid sequence and three-dimensional structure are known (2, 3), and indicate that while there is considerable ordered secondary structure in the intact molecule, all of the ordering forces are noncovalent since there are no disulfide bridges. It is probably for this reason that specific cleavage of nuclease has been found to produce peptide fragments capable of refolding to assume the native conformation (4-6). The availability of such polypeptide fragments bearing isolated antigenic determinants of the intact protein provides a tool for examining individual portions of the immunogen for carrier or haptenic functions. It will be interesting to ascertain whether there is one determinant responsible for genetic control of recognition of the entire molecule (i.e. a carrier determinant) or whether the

humoral responses to a variety of individual antigenic determinants (i.e. haptenic determinants) are under separate genetic control. Very sensitive methods for assessing antibody to such haptenic determinants of nuclease are already available (7) and, in essence, involve inhibition by peptides of the antibody-mediated inactivation of enzymatic activity described above. In order to examine carrier determinants we are presently developing methods of assessing cell-mediated immunity to nuclease in vitro. Preliminary results indicate that there is also genetic control of this aspect of immunity similar to that which we have reported here for humoral immunity.²

The *Ir* region has been recently subdivided into two distinct subregions. One subregion contains *Ir-1* which controls responsiveness to multichain synthetic polypeptides built on a polyDLAla—polyLlys backbone and probably the gene controlling responsiveness to mouse IgA myeloma (17, 18). A second region distinct from *Ir-1* which maps to the *Ss-Slp* side of *Ir-1*, has been identified using B10.A recombinant mouse strains. This locus was found to control antibody responses to mouse IgG myeloma (17). From the results obtained by immunization of B10.A(2R), B10.A(4R), and B10.A(5R) recombinant mice (see Fig. 2), which represent three distinct crossovers within *H-2*, the *Ir* gene(s) controlling responsiveness to nuclease also appears to be localized to the *Ir*-IgG region of *Ir* within the major histocompatibility complex.

While it might be tempting to attribute some functional significance to the fact that genetic control of immune responsiveness to nuclease has been found to map within the same chromosomal segment as the control of immune responsiveness to IgG myeloma proteins, it seems more likely to us that this localization is merely coincidental. The map distance between the *H-2K* region and the *Ss-Slp* region of the *H-2* complex (Fig. 2) is large enough to include numerous *Ir* genes (31). In the absence of known allelism between *Ir* genes, the only means of localizing such genes within this region is the use of mice with recombinant *H-2* types in which the recombinational event divides responsiveness patterns. Furthermore, only if the two parental strains leading to a recombinant show different responses to a given immunogen can the recombinant be helpful in localizing the relevant *Ir* gene. If all *Ir* genes were distributed randomly throughout the *Ir* region then the number which would be found to map to the left or to the right of any given crossover would depend only on the position of the crossover within this chromosomal segment. Thus the only significance we would attribute to the mapping of *Ir*-Nase to the same region as *Ir*-IgG is that it indicates that the crossover leading to the B10.A(4R) recombinant occurred sufficiently to the left of *Ss-Slp* to permit more than one (and possibly many) *Ir* genes to be localized to the right of that crossover but within the *Ir* region.

² G. M. Shearer, C. Garbarino, E. C. Lozner, D. H. Sachs, and W. D. Terry. Genetic control of the immune response to staphylococcal nuclease. II. *H-2* limited *Ir* gene control of cell mediated immunity in the mouse. Manuscript in preparation.

SUMMARY

A number of inbred and congenic resistant strains of mice were immunized with staphylococcal nuclease (Nase). Antibody responses were measured in the sera of the animals by a sensitive method involving inhibition of enzymatic hydrolysis of DNA. High responder strains included A/J, DBA/2, BALB/c, AKR/J, C57BR, and SJL/J. DBA/1 and C57BL/6 mice were low responders. The strain distribution of anti-Nase response potential was compatible with the relevant immune response gene(s) being linked to the murine major histocompatibility complex. Linkage of this response to *H-2* was demonstrated by the findings that: (a) the congenic C3H/HeJ and C3H.SW mice were respectively high and low responders; (b) the congenic lines B10.A and B10.D2 were high responders, whereas the C57BL/10 strain was a poor responder; and (c) anti-Nase response potential of F₂ progeny from DBA/1 × SJL/J matings correlated with their *H-2* type. Three B10.A recombinant lines were used to map this *Ir* gene within *H-2*. B10.A(4R) was a high responder to Nase, whereas B10.A(2R) and B10.A(5R) were both low responders. We wish to propose the name *Ir-Nase* for the gene(s) controlling antibody responsiveness to this immunogen. Our data indicate that *Ir-Nase* is located within the same chromosomal segment of the *H-2* complex as is *Ir-IgG*.

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