DOMINANT NONRESPONSIVENESS IN THE INDUCTION OF AUTOIMMUNITY TO LIVER-SPECIFIC F ANTIGEN*

By DONALD M. SILVER AND DAVID P. LANE

(From the I.C.R.F. Tumor Immunology Unit, Department of Zoology, University College, London)

Species possessing functional humoral and cellular immune systems do not normally undergo autoimmune responses. Many disease states, however, result in the induction of an immune response directed against self-components. Clearly the ability to produce autoantibodies under certain conditions exists, but normally some mechanism functions to prevent this from occurring. It has been suggested that this tolerance to self involves the continuous surveillance of certain components of the immune system which renders them nonfunctional in inducing autoimmunity (1).

Strong support for this concept of tolerance derives from a system described recently concerning a liver-specific alloantigen, designated as F antigen, which is not an autoimmunogen but can induce the production of autoantibodies (2). F antigen is present in the livers of all mammals tested. In the mouse, two immunologically distinct forms of the antigen exists. Certain mouse strains will respond to liver extracts from appropriate donors with the production of antibody that appears directed entirely against the F molecule. What is striking is that these antibodies will combine with the F antigen present in a liver extract from the animal producing the antibodies. But, liver extracts from one strain are unable to induce an immune response to F antigen in mice of that strain.

It was postulated (2) that F antigen consists of at least two immunologically identifiable moieties, an antibody combining site which is identical in all strains and a carrier moiety which is the alloantigenic part of the molecule responsible for its recognition. Furthermore, it was suggested that animals are tolerant to their own carrier moiety, but can recognize and respond to carrier moieties on F antigen of appropriate donors.

The mechanism governing this tolerance induction, however, has not been elucidated. Indeed, since F antigen is found almost exclusively within the liver (2), it seems that except under conditions of liver damage an adult animal would not be exposed to F antigen. Clearly, all mice that respond to F antigen possess B cells producing the antibody. It was inferred then, that tolerance to an animal's own F antigen resides in the T cell population (3). At the same time, it was shown (3) that T cells serve as helper cells in the induction of an immune response to F antigen from appropriate donors.

Even though liver extracts from every strain examined will induce the production of anti-F antibodies in at least one other strain, not all mouse strains

* This investigation was supported by U. S. Public Health Service Grant No. CA02152 from the National Cancer Institute and a postgraduate research grant from the Medical Research Council.

are able to respond to F antigen. The genetic basis of this nonresponsiveness was studied as a possible model to explain self-tolerance to F antigen. Data will be presented to the effect that nonresponsiveness is a dominant trait, and that there exist two genes, one linked to the *H-2* locus of the mouse and the other not, involved in the ability to respond to F antigen from appropriate donors.

Materials and Methods

Animals. Male and female mice of strains A, AKR, BALB/c, B10.A, C3H/He, C57BL/10, CBA, and DBA/2 were obtained from the Imperial Cancer Research Fund breeding unit at Mill Hill, London, England. F_1 hybrid mice obtained from matings between female CBA and male DBA/2 and female C57BL/10 and A male mice were raised in our laboratories, as were a backcross generation between female (CBA × DBA/2)F_1 hybrids and CBA males.

Antisera. Mice were injected intraperitoneally with 0.5 ml of an equal volume mixture of crude liver extract and Freund's complete adjuvant (CFA).[1] The crude liver extract was prepared by removing livers, washing in phosphate-buffered saline (PBS), homogenizing with an equal volume of distilled water, and centrifuging for 10 min at 2,500 rpm. The supernate was removed and stored at −20°C until use. In the early experiments mice were injected at weekly intervals. Later it was determined that two injections, spaced by an interval of 4 wk, produced identical results. Bleedings were made from individual mice 6 days after the injection specified. In some cases the repeated injections in CFA induced the formation of ascites that was tapped and stored at −20°C with the other groups of sera.

Serology. Serum titers of anti-F antibodies were determined using the radioimmunoassay described by Lane and Silver.[2] Purified F antigen from CBA livers was prepared according to the method of Lane and Silver[2], then labeled with ^{125}I (sp act 15 mCi/mmol) using the chloramine-T method (4). To 0.05 ml of ^{125}I F antigen (in PBS plus 2% normal mouse serum [NMS]) was added 0.02 ml antiserum diluted in PBS plus 2% NMS. The mixture was allowed to stand at room temperature for 1 h after which time 0.1 ml of a rabbit antimouse immunoglobulin serum diluted 1:2.5 in PBS was added to each tube. After a further incubation for 15 min at room temperature, the assay was placed at 4°C overnight, then spun at 6,000 rpm for 30 min. The supernates were removed and the individual tubes counted for 1 min on a well-type scintillation counter. All of the assays reported in this paper were run with the same anti-F antiserum as a positive control. The titer was taken as the reciprocal of the highest dilution of serum that bound specifically labeled F antigen. Specific binding was compared with the positive control serum and the background detected in the presence of NMS alone. It has been shown[2] that the NMS control produced a result identical to that of serum from CBA mice repeatedly injected with CBA liver extracts in CFA.

H-2 Typing. *H-2* typing was performed by cytotoxic assay using anti-H-$2K^k$ and anti-H-$2K^d$ sera kindly supplied by Dr. J. Woody. Spleens were removed from all animals and single cell suspensions made in Eagle's medium with 10% fetal calf serum to 2×10^6 cells/ml. To 0.02 ml of spleen cells was added 0.02 ml anti-H-2 antiserum at various dilutions. The mixtures were incubated at 37°C for 30 min after which time 0.02 ml guinea pig serum (undiluted) was added, followed by an additional incubation for 30 min at 37°C. The mixtures were centrifuged for 3 min at 1,000 rpm after which time the supernatant fluid was removed and replaced with one drop of trypan blue in saline (0.2% wt/vol). The suspension was transferred to a microscope slide, and a differential count was made of stained and unstained cells. The positive controls were DBA (H-$2K^d$) and (CBA × DBA/2)F_1(H-$2K^{k/d}$) spleen cell suspensions and the negative control a suspension of CBA (H-$2K^k$) spleen cells. The results were scored as positive killing in those samples showing 30% or more dead cells compared with controls of less than 5% cells staining with trypan blue in each case.

Results

It was originally reported (2) that certain strains of mice, when immunized with liver extracts from other strains, produced antibodies against F antigen

[1] *Abbreviations used in this paper:* CFA, complete Freund's adjuvant; NMS, normal mouse serum; PBS, phosphate-buffered saline.

[2] Lane, D. P., and D. M. Silver. The purification and properties of a murine liver-specific alloantigen 'F'. Manuscript submitted for publication.

which is present in the livers of all mice. No antibody was produced by immunizing mice with liver extracts from the same inbred strain. These data, presented by Fravi and Lindenmann (2), have been confirmed and extended to include strains AKR and C57BL/10 (Table I). All of the mouse strains listed were immunized with liver extracts from every other strain presented. Although three of the strains did not respond to any of the liver preparations, their own liver extracts were able to immunize other strains as shown in Table II. Since all F molecules contain the same antibody combining site, it was suggested (2) that the strains listed in each column of Table II share a carrier region of their F molecules. This region allows A mice to recognize F antigen from CBA, C3H, AKR, and DBA/2 mice as immunogenic and also CBA, C3H, and AKR mice to recognize F antigen from strains A, C57BL/10, and BALB/c.

Serological Analysis of the Response of Mice to F Antigen. It has been reported[2] that in response to weekly immunizations with C57BL/10 liver extracts, C3H mice respond with the production of anti-F antibody increasing from titers of 256 after three immunizations to 10,000 after six. If mice able to respond to liver extracts from appropriate strains are immunized on day 0 and day 28 with the extract in CFA, the titers detected after the second immunization reach the level of 10,000. This titer has been detected in sera of CBA, C3H, and AKR mice responding to liver extracts from A mice and vice-versa.

Nonresponding strains fail to show any significant antibody titer (≤ 10) under any of the regimens so far employed. Similarly, any responder strain immunized repeatedly with liver extracts from its own strain fails to show any anti-F antibody titer. Likewise, any responding strain immunized with liver extracts from any strain listed in its column in Table II fails to show any anti-F antibody titer.

Genetic Analysis of the Differences in Responsiveness between Responder and Nonresponder Mice. When (CBA × A) F_1 hybrid mice were immunized with liver extracts from either parent no response was detected, even though both parents are able to respond to liver extracts from the other parent (2). Fravi and Lindenmann showed that liver extracts from (CBA × A) F_1 hybrids could immunize mice of both parental strains, indicating that both carrier types were expressed in the F_1 hybrids. The expression of a carrier type was postulated to lead to the induction of tolerance, thus explaining why mice will not make antibodies when immunized with their own F antigen, but will made antibodies that combine with their own F antigen if presented in a liver extract from a strain possessing a different carrier portion of the molecule.

In the present study the immune responses of (CBA × DBA/2) F_1 and (C57BL/10 × A) F_1 hybrids were studied. Each of these crosses is derived from a responder and a nonresponder strain of identical carrier type (Table II). (CBA × DBA/2) F_1 hybrids were immunized on day 0 and 28 with liver extracts from A mice, while (C57BL/10 × A) F_1 mice received extracts from C3H mice. 6 days after the second immunization all mice were bled and sera tested for reactivity in the radioimmunoassay. The results in Table III indicate that in a cross of a (responder × nonresponder) of identical carrier type, nonresponsiveness is dominant.

Appropriate backcross mice were bred from female (CBA × DBA/2) F_1 hybrids and male CBA mice in order to determine the minimal number of independently

TABLE I
Strain Distribution of the Ability to Respond to F Antigen

Responder strains	Nonresponder strains
A	BALB/c
AKR	C57BL/10
CBA	DBA/2
C3H	

TABLE II
*Immunogenicity of Liver Extracts**

Extracts immunogenic in A mice	Extracts immunogenic in AKR, CBA, and C3H mice
AKR	A
CBA	BALB/c
C3H	C57BL/10
DBA/2	

* Mice were immunized with a 0.5-ml mixture of equal volumes of liver extract and CFA.

segregating loci at which there are allelic genes exerting an influence on the ability to respond to F antigen. The proportion of mice showing CBA-like responses should equal $(1/2)^n$, where n is the number of loci involved. The results of these immunizations are presented in Table IV. Of the 15 sera tested, 4 showed responses identical to the CBA parent, which is consistent with two loci controlling the ability to respond to F antigen with titers of 10,000. According to the Chi square test, the present value of 4/15 fits with a two locus hypothesis ($\chi^2 = 0.0166$, for one degree of freedom, $0.85>P>0.90$). Three other mice, however, responded with antibody titers lower than those of the CBA parent, but greater than those of the F_1 hybrids. In all, half of the backcross mice were able to make some response to F antigen, implying that one locus was sufficient to allow at least a low antibody response to F antigen.

Linkage to the H-2 Complex. Previously, it has been described that there exists a series of immune response (*Ir*) genes linked to the *H-2* complex of the mouse which are involved in the ability to recognize and respond to many antigens (5). All of the backcross mice listed in Table IV were *H-2* typed for the presence of H-2K^d and H-2K^k antigens to determine whether the ability to respond to liver extracts from strain A mice was associated with the major histocompatibility locus of the mouse. It can be seen from Table IV that such an association indeed did exist. All of the mice producing significant titers of anti-F antibody were typed as H-2K$^{k/k}$. The location of the other locus, the possession of which allowed H-2K$^{k/k}$ mice to produce high titers (10,000) of anti-F antibodies, was not determined.

The linkage to *H-2* led to the prediction that a nonresponder strain possessing homozygously the *H-2* locus from a responder strain should produce low, but significant titers of anti-F antibodies. This was confirmed using B10.A mice immunized with liver extracts from C3H donors. B10.A is a subline of C57BL/10

TABLE III
Serological Response of (Responder × Nonresponder) F_1 Hybrids

Liver extract	Strain immunized*	Numer of mice	Titer‡
A	CBA	10	>10,000
	DBA/2	10	≤10
	(CBA × DBA/2)F_1	4	≤10
C3H	A	10	>10,000
	C57BL/10	10	≤10
	(C57BL/10 × A)F_1	5	≤10

* Mice were immunized on days 0 and 28 with a 0.5-ml mixture of equal volumes of liver extract and CFA. Mice were bled 6 days after the second injection.

‡ Titer taken as the reciprocal of the highest dilution of antibody showing significant binding of the labeled F antigen in the radioimmunoassay.

in which the *H-2A* complex has been substituted for *H-2B*, mice of the B10.A line differ from C57BL/10 mice at the *H-2* locus, but the two lines are otherwise very similar if not identical. When B10.A mice were immunized with liver extracts from C3H mice, they responded with titers of 100 compared with the titers of <10 detected in sera from C57BL/10 mice immunized with the same liver extracts.

Discussion

The immune responsiveness of different mouse strains to the liver-specific alloantigen 'F' is governed by multiple genes. Apart from those genes coding for the antibody specificity, two other genes are involved in the ability to recognize and respond to F antigen. One of these genes is linked to the *H-2* locus of the mouse, while the other is not. To respond to F antigen from appropriate donors with a low antibody titer, it is necessary to possess a gene found linked to the *H-2* locus of strains A, AKR, CBA, and C3H. Production of high anti-F titers is dependent upon the presence of the non-*H-2*-linked gene and the *H-2*-linked gene. The backcross data of Table IV imply that the non-*H-2*-linked gene is not able to effect the production of anti-F antibody in the absence of the gene linked to the *H-2* complex. It has previously been shown that the genes coding for the two forms of F antigen are not linked to the *H-2* locus (3).

At first glance it would seem that the genetic basis for the response to F antigen is similar to that governing responsiveness to antigens under single *Ir* gene control (5). What distinguishes the system described above is the necessity for two genes in order to effect a high responder phenotype and the dominance of nonresponsiveness in the F_1 hybrids which were derived from matings of parents possessing identical F molecules. The ability to respond is a dominant trait of the *Ir* loci.

It is difficult to escape the conclusion that the presence of a gene or its gene product, which is linked to the *H-2* locus of the nonresponder DBA/2 strain, results in the nonresponsiveness to F antigen. None of the backcross mice possessing the *H-2* allele from the DBA/2 strain were able to respond. No data are yet available as to the region of the *H-2* complex where this gene is coded.

TABLE IV
Genetic Analysis of the Ability to Respond to F Antigen

Strain immunized*	Numer of mice	Titer‡	*H-2*
CBA	10	>10,000	$K^{k/k}$
DBA/2	10	≤10	$K^{d/d}$
(CBA × DBA/2)F_1	4	≤10	$K^{k/d}$
(CBA × DBA/2)F_1 × CBA	4	>10,000	$K^{k/k}$
	3	≤100	$K^{k/k}$
	8	≤10	$K^{k/d}$

* Mice were immunized on days 0 and 28 with a 0.5-ml mixture of equal volumes of strain A liver extract and CFA. Mice were bled 6 days after the second injection.

‡ Titer taken as the reciprocal of the highest dilution of antibody showing significant binding of the labeled F antigen in the radioimmunoassay.

Although soluble molecules inducing supression of immune responses have been reported (6), it must be pointed out that the gene in nonresponders may code for an *H-2* alloantigen. Iverson and Lindenmann (3) have presented data suggesting that the carrier portion of the F molecule is expressed on the surface of spleen cells. Their evidence, however, on tolerance induction to the carrier moiety was based on their finding that liver extracts from strain C57BL/10 constituted a "third" helper type. This has not been confirmed in the present study. Extracts from strain C57BL/10 liver behave identically to those from A and BALB/c, both in their immunogenicity and in their ability to specifically induce a secondary response in mice primed to F antigen from strains A or BALB/c. It will be necessary to repeat these tolerance experiments, for they offer a model to explain the origin of self-tolerance to the carrier portion of the F molecule.

There are at least three possible explanations for the dominance of nonresponsiveness to F antigen. One is that a gene product of the *H-2D* allele cross-reacts with the antibody combining specificity of the F molecule, thereby inducing self-tolerance at the B-cell as well as the T-cell level (for that matter, this product may cross-react with the carrier portion of the molecule so the nonresponder possesses both carrier types on [possibly] its spleen cells). Such dominant nonresponsiveness mediated by a tolerance mechanism would be similar to that described by Cinader et al. (7) for the inheritance of immune responsiveness to complement component C-5.

Another possibility is that the gene product of the *H-2D* allele is defective at the B-cell level and involved in effective cooperation between T cells and B cells in producing anti-F antibodies. Finally, as mentioned above, a gene linked to the *H-2D* locus may code for a suppressor molecule. Phenotypically the induction of suppressors and the induction of self-tolerance are identical in their effects. Indeed, it has been suggested that the mechanism of tolerance induction is the formation of suppressors (8).

The demonstration that the presence of a homozygous $H\text{-}2K^k$ locus is sufficient to permit the induction of an antibody response to F antigen raises the question of specificity. Which genetic locus is involved in the recognition of one carrier moiety of the F molecule from the other? It is important to stress that this

recognition is by T cells of an alloantigenic difference. Three strains carrying the *H-2K*^{k} allele respond to extracts from A, C57BL/10, and BALB/c livers. A mice respond to liver extracts from CBA, AKR, C3H, and DBA/2 mice. The *H-2* allele of A mice derives from a recombination between the *H-2K* and *H-2D* alleles at the *Ir1-C* locus (9). Since all strains that respond to F antigen share the H-2K^k, *Ir1-A*, *Ir1-B* loci, it may be that this region is critical in effecting a response. Although recent work has demonstrated the role of this region in T- B cell cooperation, the question of the specific T-cell receptor remains unanswered (10).

Summary

The liver-specific F antigen, although not an autoimmunogen, can induce the production of autoantibodies in responder strains. The ability to respond is under the control of two genes, one linked to the *H-2* locus of mice, the other not. Responders possessing both genes produce high anti-F titers, while the *H-2*-linked gene alone permits a significant but low antibody response. (Responder × nonresponder) F_1 hybrids derived from parents possessing identical F molecules are nonresponders, in contrast with the dominance of responsiveness in *Ir* gene systems. The presence of the *H-2* locus from nonresponders appears involved in the inability to respond. This is discussed in terms of self-tolerance and suppression.

Received for publication 11 August 1975.

References

1. Howard, J. G., and N. A. Mitchison. 1975. Immunological tolerance. *Prog. Allergy.* 18:43.
2. Fravi, G., and J. Lindenmann. 1968. Induction by allogeneic extracts of liver-specific precipitating autoantibodies in the mouse. *Nature (Lond.)* 218:141.
3. Iverson, G. M., and J. Lindenmann. 1972. The role of a carrier determinant and T cells in the induction of liver-specific autoantibodies in the mouse. *Eur. J. Immunol.* 2:195.
4. Hunter, R. 1970. Standardization of the chloramine-T method of protein iodination. *Proc. Soc. Exp. Biol. Med.* 133:989.
5. Benacerraf, B., and H. O. McDevitt. 1972. Histocompatibility-linked immune response genes. *Science (Wash. D. C.).* 175:273.
6. Tada, T., K. Okumura, and M. Taniguchi. 1973. Regulation of homocytotropic antibody formation in the rat. VIII. An antigen-specific T cell factor that regulates anti-hapten homocytotropic antibody response. *J. Immunol.* 111:952.
7. Cinader, B., S. W. Koh, and D. Naylor. 1969. Tolerance-mediated inheritance of immune responsiveness. *Int. Arch. Allergy Appl. Immunol.* 35:150.
8. Gershon, R. K. 1974. T cell control of antibody production. *Top. Immunobiol.* 3:1.
9. Shreffler, D. C., and C. S. David. 1975. The H-2 major histocompatibility complex and the I immune response region: genetic variation, function, and organization. *Adv. Immunol.* 20:125.
10. Katz, D. H., M. Graves, M. E. Dorf, H. Dimizio, and B. Benacerraf. 1975. Cell interactions between histoincompatibile T and B lymphocytes. VII. Cooperative responses between lymphocytes are controlled by genes in the *I* region of the *H-2* complex. *J. Exp. Med.* 141:263.