The Journal of Immunology

RESEARCH ARTICLE | FEBRUARY 01 1980

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P C Cooper; ... et. al

J Immunol (1980) 124 (2): 790–794.
https://doi.org/10.4049/jimmunol.124.2.790

0022-1767/80/1242-0790$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 124, No. 2, February 1980
Printed in U.S.A.

DELAYED-TYPE HYPERSENSITIVITY TO LIVER F ANTIGEN IN THE MOUSE

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F antigen is a liver cytoplasmic protein to which an autoantibody response can be induced by appropriate alloimmunization. The critical event in the induction of this response is believed to be the recognition of determinants associated with the allovariable region of F antigen by T cells that help B cells respond to determinants common to the immunizing and self F antigen. We have examined the specificity and genetic regulation of T cell responses to F antigen, employing a radiometric ear assay for delayed-type hypersensitivity (DTH). DTH responses were obtained in some strains of mice bearing type 1 F antigen allotype when immunized and challenged with type 2 F antigen, but no type 2 strain responded to type 1 F antigen. All type 1 strains bearing the H-2^k haplotype responded to type 2 F antigen, whereas DBA/2 mice (type 1, H-2^d) failed to respond, and the response was largely directed to determinants associated with the allovariable region of F antigen. Although there is recessive inheritance of the humoral response, some responder X nonresponder F_1 hybrids did give good DTH responses. Our findings with DTH responses are consistent with the autoantibody response to F antigen being triggered in responder strains of mice by T cell recognition of carrier determinants unique to each F antigen allotype, and if nonresponsiveness to F antigen is mediated by suppressor mechanisms, suppression appears to be less effective against DTH responses than against autoantibody responses.

Alloimmunization with liver homogenate by using certain combinations of inbred mouse strains leads to a strong precipitating antibody response against a cytoplasmic protein designated F antigen (1). The anti-F antibodies are autoantibodies, since they react equally with self and the immunizing antigen in double immunodiffusion tests or in competitive radioimmunoassays (1–3). This unusual response to alloimmunization probably reflects the fact that inbred strains of mice possess one of two alloantigenic forms of F antigen. Immunization with the opposite alloantigenic form of F antigen induces T cells, with specificity for the alloantigenic region of the molecule (acting as a carrier determinant), to provide help to B cells with specificity for determinants common to both alloantigenic forms of F antigen (4).

The induction of the autoantibody response to F antigen by alloimmunization is under the control of an H-2-linked immune response (Ir)[1] gene, with strains bearing the H-2^a and H-2^k haplotypes being responders (5–7). However, although most H-2-linked Ir genes are associated with dominant inheritance, responsiveness to F antigen is inherited as a recessive trait (5). F antigen is large (44,000 daltons) compared with other natural antigens that have been used for defining Ir gene function, and the primary structure, conformation, and biologic function of F antigen are as yet unknown. Despite this lack of chemical definition, the response to F antigen is an important model system for investigating autoimmune responses because of the unusual Ir gene control. Furthermore, the alloantigenic region of the molecule thought to be responsible for triggering T helper cells, and thus initiating the autoantibody response, is presumably a minor and readily definable region of the molecule.

In this study we have investigated T cell responses to F antigen by using a radiometric ear assay for delayed-type hypersensitivity (DTH) (8). The results obtained are consistent with T cell recognition of antigenic determinants defined by the alloantigenic region of the F antigen molecule. The strain dependence and the inheritance of DTH responsiveness to F antigen are reported, and the mechanism of regulation of immune responses to F antigen are discussed in the light of these results.

MATERIALS AND METHODS

Mice. Mice bred and reared under specific pathogen-free conditions in the animal house of The Walter and Eliza Hall Institute were obtained at 7 weeks of age. Eight- to 12-week-old female mice were used to assess DTH responses to F antigen. The strains of mice used, together with their F antigen allotype and responder status as established in earlier studies (5, 7) were as follows: CBA/CaH Wehi (CBA, type 1, responder), AKR/J Wehi (AKR, type 1, responder), C3H/HeJGif Wehi (C3H/He, type 1, responder), DBA/2J (DBA/2, type 1, nonresponder), A/J Wehi (A/J, type 2, responder), BALB/c An Bradley Wehi (BALB/c, type 2, nonresponder) and C57BL/6J Wehi (C57BL/6, type 2, nonresponder). BALB/c H-2^k congenic mice were also used.

Preparation of liver extracts and purified F antigen. Livers freshly removed from mice of various strains were homogenized in distilled water (1:1, w/v) and centrifuged at 130,000 × G for 60 min. The resultant supernatant was appropriately diluted and emulsified in complete Freund's adjuvant (CFA) for immunizing mice. Purified F antigen was isolated from the livers of CBA and A/J mice and from one rabbit liver by using a procedure involving $(NH_4)_2SO_4$ precipitation, ion exchange chromatography, and gel filtration chromatography (Anders *et*

Received for publication August 8, 1979.
Accepted for publication November 8, 1979.
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[1] Abbreviations used in this paper: Ir gene, immune response gene; DTH, delayed-type hypersensitivity; ^{125}I-UdR, 5-iodo-2′-deoxyuridine-^{125}I; F-UdR, 5-fluorodeoxyuridine.

al., manuscript in preparation).

Immunization procedures. Mice were immunized to F antigen by injection of allogeneic liver extracts emulsified in CFA. One of two procedures was followed. Initially mice were given 200 mg/kg of cyclophosphamide (Endoxan, Asta) subcutaneously, and 2 days later were injected in the footpads and subcutaneously with appropriate dilutions of liver extract emulsified in CFA. Later experiments included a second identical treatment with cyclophosphamide and liver extract after an interval of 2 weeks.

Isotope. 5-Iodo-2′-deoxyuridine-^{125}I (^{125}I-UdR), specific activity approximately 5 Ci/mg, was obtained from the Radiochemical Centre, Amersham, Bucks, U. K.

Test for DTH. In the radiometric ear assay for DTH (8), 10 μl of purified F antigen (10 μg) were injected intradermally into the left pinna, and the mice were then given 0.1 ml 10^{-3} M 5-fluorodeoxyuridine (F-UdR) i.p., followed 20 to 30 min later by 1 μCi ^{125}I-UdR i.p.; 24 hr later, the mice were killed by cervical dislocation, and the ears were cut off at the hairline and counted in a Packard gamma spectrometer. The DTH response was expressed as the ratio (L/R) of the counts in the left ear to those in the right ear. Ten micrograms of purified F antigen gave a greater DTH response than did 2.5 μg, but higher concentrations were not tested because of the limited amounts of highly purified F antigen available.

Statistics. Arithmetic means, standard errors of means (S.E.M.), and significance of differences with Student's *t*-test were calculated on a programmable HP 97 desk calculator.

RESULTS

Optimal sensitization of CBA mice to A/J F antigen. Initially we chose to examine whether CBA mice (type 1, humoral responders) could respond with a DTH reaction to an ear challenge with purified A/J F antigen after one immunization with varying amounts of A/J liver extract. As can be seen in Figure 1, mice challenged with 10 μg of F antigen 5 days after immunization showed positive ear reactions over the wide range of doses of liver extract used for the initial immunization. The optimal response was obtained after immunization with 25 μl of liver extract, one-eighth of the amount usually employed for inducing antibody responses (7). CBA mice primed with this optimal dose of liver extract responded maximally to ear challenge with purified A/J F antigen 5 days after priming (Fig. 2). There was a lower but still significant response at 7 days, but no detectable response at 3 days.

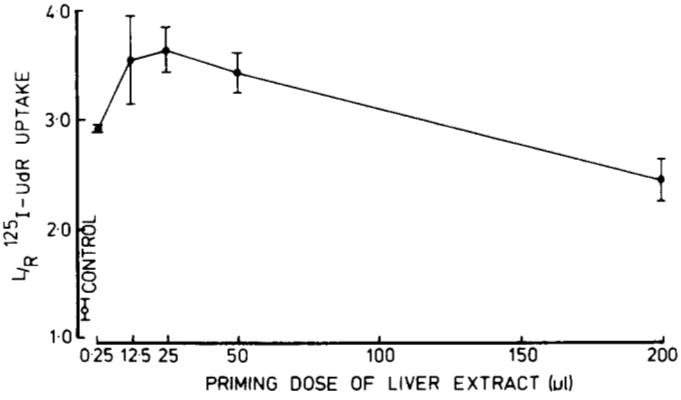

Figure 1. Effect of the amount of A/J liver extract (1:1, w/v) used to immunize CBA mice on the DTH response to an ear challenge with 10 μg purified A/J F antigen. The liver extract was diluted to 200 μl, emulsified 1:1 (v/v) in CFA and injected into footpads and i.p. The control values were obtained from mice treated with CFA alone. Each point represents the arithmetic mean of responses of five or six mice. Vertical bars represent S.E.M.

Strain dependence of liver extract used for priming. Liver extracts prepared from other strains of mice (C57BL/6 and BALB/c) having the same immunogenic type of F antigen (type 2) as A/J mice were equally effective as A/J liver extracts in sensitizing CBA mice to a challenge with purified A/J F antigen (Table I). Liver extracts from C3H/He, DBA/2, or CBA mice, all type 1 F antigen strains, failed to sensitize CBA mice to a challenge with A/J F antigen. Thus, the DTH reaction appears to discriminate between the two immunogenic types of F antigen defined by the antibody response to alloimmunization.

DTH responses to A/J F antigen in other strains of mice. Other strains of mice having type 1 F antigen were tested for their ability to respond with a DTH reaction when immunized with A/J (type 2) liver extract and challenged with purified A/J F antigen. C3H/He and AKR as well as CBA mice, all of H-2^k haplotype, and therefore humoral responders, gave significant DTH reactions, whereas DBA/2, H-2^d haplotype and a humoral nonresponder, failed to give a DTH response (Table

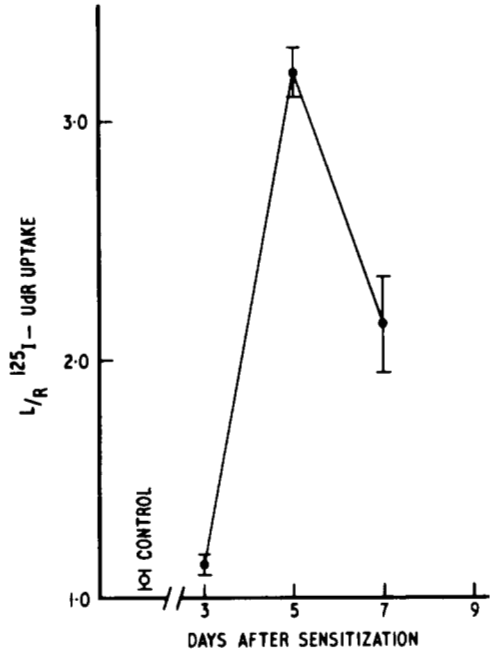

Figure 2. Time required after immunization of CBA mice with A/J liver extract for optimal DTH response to ear challenge with 10 μg purified A/J F antigen. Five or six mice per group. Vertical bars represent S.E.M.

TABLE I

DTH response of CBA mice to allogeneic liver extracts

Liver Extract Used to Immunize[a]	F Antigen Used for Ear Challenge	L/R ^{125}I-UdR Uptake[b]
A/J	A/J	2.98 ± 0.25[c]
BALB/c	A/J	3.12 ± 0.45[c]
C57BL/6	A/J	2.73 ± 0.07[c]
CBA	A/J	1.13 ± 0.12
C3H/He	A/J	1.19 ± 0.07
DBA/2	A/J	1.33 ± 0.09
Control value	A/J	1.12 ± 0.07

[a] Mice were immunized with a single dose of liver extract corresponding to 25 μl.

[b] Arithmetic mean ± 1 S.E.M.; five mice per group.

[c] Significantly different from the control value, $p < 0.01$.

II). A feature of the autoantibody response to F antigen is that responsiveness is inherited as a recessive trait (5). In contrast, DTH responses of (CBA × DBA/2) F_1 hybrids were not equivalent to those of the nonresponder parent (DBA/2), in that some mice responded with L/R ratios equivalent to the highest values obtained with parental responders (CBA), and some mice failed to respond (Fig. 3).

Lack of DTH response to CBA (type 1) F antigen. In contrast to the ability of type 1 responder strains to mount a DTH reaction to type 2 F antigen, no DTH response could be elicited in the reverse direction. Thus, A/J (type 2, humoral responder) mice, which had been immunized either once or twice with CBA liver extract and then ear-challenged with purified CBA F antigen, failed to give ear ratios higher than nonimmunized control mice ear challenged with the same purified F antigen. The dose of liver extract found to be optimal for sensitizing type 1 mice was used for these experiments. In another experiment, A/J mice were immunized with the same range of doses of liver extract as described earlier for characterizing the type 1 response (see Fig. 1), but again no DTH response was elicited after ear challenge with purified type 1 F antigen. BALB/c H-2^k congenic mice, which have the humoral responder H-2^k genotype on the nonresponder background, also failed to give a DTH response to CBA F antigen (data not shown).

TABLE II

Strain differences in DTH responsiveness to A/J F antigen

Strain[a]	Composition of H-2 Region						L/R ^{125}I-UdR Uptake[b]	
	K	IA	IB	IC	S	D	F Ag-immunized	Controls
CBA	k	k	k	k	k	k	2.78 ± 0.14[c]	1.02 ± 0.03
C3H/He	k	k	k	k	k	k	4.46 ± 0.18[c]	1.09 ± 0.04
AKR	k	k	k	k	k	k	2.90 ± 0.25[c]	1.47 ± 0.16
DBA/2	d	d	d	d	d	d	1.11 ± 0.06	0.85 ± 0.02
A/J	k	k	k	d	d	d	1.11 ± 0.09	—[d]

[a] Mice were immunized with two doses of A/J liver extract and ear challenged with purified A/J F antigen.
[b] Arithmetic mean ± S.E.M.
[c] $p < 0.05$.
[d] —, not done.

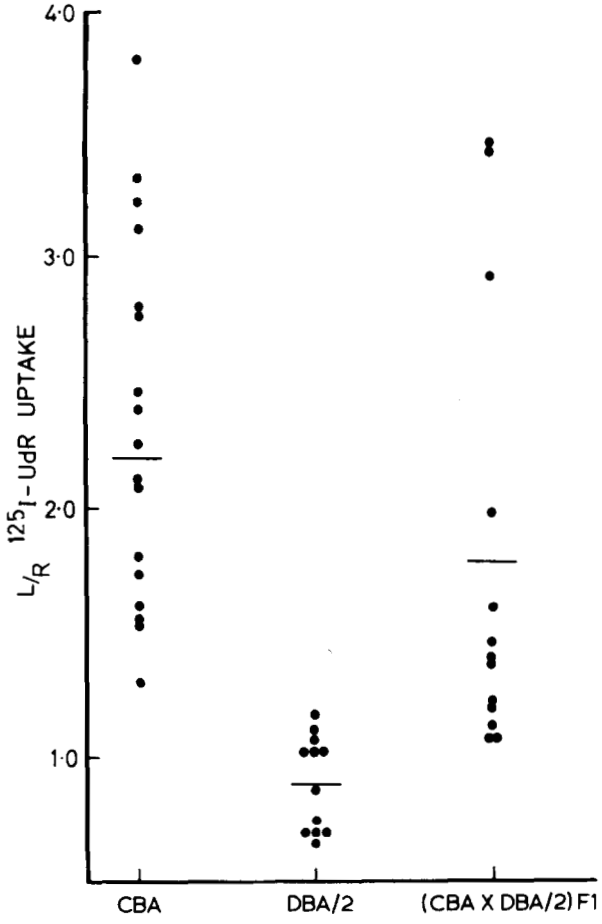

Figure 3. DTH response to A/J F antigen in responder (CBA) and nonresponder (DBA/2) parental strains, compared with responses obtained in F_1 hybrids. Each point represents a single mouse. Horizontal bars represent arithmetic means.

TABLE III

Specificity of the DTH response in CBA mice immunized with A/J liver extract

F Antigen Used for Ear Challenge (10 μg)	L/R ^{125}I-UdR Uptake[a]	
	F Ag-immunized	Controls
A/J	3.36 ± 0.33[b] (5)[c]	1.42 ± 0.14 (4)
CBA	1.49 ± 0.12[b] (5)	1.01 ± 0.06 (5)
Rabbit	1.59 ± 0.10[b] (7)	1.00 ± 0.06 (4)

[a] Arithmetic mean ± S.E.M.
[b] $p < 0.05$.
[c] Number in brackets denotes number of mice per group.

Specificity of the DTH response. CBA mice which were optimally sensitized for a DTH response to type 2 F antigen with two injections of A/J liver extract in CFA were tested for their capacity to give a DTH response to ear challenge with purified syngeneic (CBA/type 1) F antigen and xenogeneic (rabbit) F antigen. Although the DTH responses were lower than those obtained in control mice immunized and challenged with allogeneic F antigen, both syngeneic and xenogeneic F antigen elicited significant responses (Table III), indicating that the specificity of the DTH response was not wholly restricted to determinant(s) defined by the allogeneic difference between the two murine F antigens.

DISCUSSION

Previous studies on the immune response of mice to the liver-specific F antigen have been concerned with the autoantibody response induced by alloimmunization (1, 2, 4–7). In this study we have used the radiometric ear assay of Vadas *et al.* (8) to demonstrate that alloimmunization with liver extracts can also sensitize mice for a DTH response to F antigen. The DTH response had the following features. First, cells involved in the DTH response distinguished the same two types of murine F antigen that were defined by the pattern of autoantibody responses to alloimmunization. Second, DTH responses were only obtained in one direction; that is, type 1 mice responded to Type 2 antigen, but type 2 mice did not respond to type 1 antigen. Third, whereas the antibody response to F antigen was to determinants common to both F antigen types, the DTH response to type 2 antigen was directed largely, but not exclusively, to determinants specific to type 2 antigen. Fourth, and subject to the constraint that responses could be studied in one direction only, the DTH response to F antigen appeared to be under the same H-2-linked genetic control as the humoral response, in that strains of H-2^k haplotype were responders and H-2^d mice (DBA/2) were nonresponders. Fifth, in contrast to the dominant inheritance of nonresponsiveness for the humoral response to F antigen, F_1 hybrids derived from a responder × nonresponder cross were heterogeneous in their response with

some hybrids responding as well as the responder parental mice.

The reason why DTH responses to type 1 F antigen were not detected is not clear. A gene located in the I-A subregion of the H-2 complex determines autoantibody responsiveness to F antigen (6, 7) but another, non-H-2-linked gene is required for a high titer response (6). Although Silver and Lane (5) reported that A strain mice (F antigen type 2) gave antibody responses equal to type 1 responder mice, the A/J mice we have used have responded to alloimmunization with consistently lower and more variable levels of autoantibodies. Thus, these mice may differ at this second locus from the A strain mice used in the studies of Silver and Lane, and from the type 1 responder mice used here. A regulatory effect of this second genetic locus on DTH responses could explain our failure to detect responses in A/J mice or, alternatively, there may be genetic regulation of the DTH response to type 1 F antigen at a locus quite separate from the two loci that regulate the humoral response.

The striking feature of the H-2-linked genetic regulation of the humoral response to F antigen is the recessive inheritance of responsiveness (5). It is therefore of some interest that F_1 hybrids derived from a responder × nonresponder cross were heterogeneous in their DTH responses, with three of the 13 hybrid mice tested responding with values equivalent to the best responses obtained in the responder parent. This contrasts with the total lack of detectable antibody responses in such mice. A number of mechanisms could account for recessive inheritance of immune responsiveness. First, tolerance due to cross-reacting determinants in the immunizing antigen and the gene product of the allele determining nonresponsiveness, as has been implicated in the dominant nonresponsiveness to an erythrocyte antigen (Ea-1) in the mouse (9) and complement component C-5 (10), may occur. Evidence against a cross-tolerance mechanism applying to F antigen has been reported by Silver and Lane (6). Using radiation chimeras, they tolerized lymphoid cells to the antigens coded for by the K and D regions of the H-2 complex of nonresponder mice and then demonstrated that the responder cells were still fully capable of making an anti-F antigen response. Our observation that the dominance of nonresponsiveness is not complete for the DTH response to F antigen is further evidence against a cross-tolerance mechanism.

A second possible mechanism discussed by Silver and Lane (5) for the Ir gene control of anti-F autoantibody responses involved the Ir gene product in the cooperative interaction between T and B cells that is necessary for this T dependent antibody response. In this mechanism, nonresponders would be characterized by a gene product that was defective in this role. It is not clear to us why such a mechanism would lead to dominant inheritance of nonresponsiveness if there was expression of both parental alleles in F_1 hybrids derived from a responder X nonresponder cross. Furthermore, this mechanism cannot explain our observation that many such hybrids are also DTH nonresponders.

The third explanation proposed (6) for the recessive inheritance of responsiveness to F antigen is that nonresponsiveness is due to active suppression. The same H-2 linked Ir gene control of DTH and antibody responses is consistent with regulation by suppressor cells. However, if suppressor mechanisms are operating to regulate the immune response to F antigen, they appear less effective against DTH responses than against antibody responses, in that some responder × nonresponder F_1 hybrids did give a good DTH response. This may be due to an alteration of the balance between suppression and induction by the particular assay we used for assessing DTH responses. An altered balance between induction and suppression has been postulated previously for other systems, to explain high responses in some mice bearing low responder genotypes (11, 12). The use of cyclophosphamide and a low dose of immunizing antigen were two factors that may have favored induction rather than suppression of responsiveness in our assay system for DTH (13, 14). However, when we examined the effect of antigen dose in F_1 hybrids by immunizing with 8 times the usual amount of liver extract, a similar range of DTH responses was obtained (data not shown). Thus, a low antigen dose leading to escape from suppression does not explain the high responses in some F_1 (responder × nonresponder) hybrids. The use of cyclophosphamide in our system was essential to detect a DTH response to F antigen, but an examination of the effect of lower doses of cyclophosphamide has not been carried out. In a study of the antibody responses to the hapten 2,4,6-trinitrophenyl conjugated to mouse serum albumin, Urba and Hildemann (15) found that all anomalous high-responding mice were females. The nature of this sex-linked effect, which was especially evident in low × high responder F_1 hybrids, has not been defined, but it may be operative in the DTH responses to F antigen, since all the animals studied here were females.

The observation reported here that alloimmunization with F antigen induces a DTH response largely, although not exclusively, directed to the allovariable region of F antigen supports the hypothesis that the allovariable region of the molecule provides a carrier function for the induction of autoantibodies (1). However, recognition of the alloantigenic region is not obligatory for the induction of autoantibodies, since we have identified anti-F antibodies in the serum of some unimmunized mice and in some type 2 mice immunized with highly purified type 2 F antigen (Anders *et al.*, manuscript in preparation).

Thus, there appears to be more than one mechanism whereby tolerance to F antigen can be broken. The radiometric ear assay for DTH employed in this study provides a convenient method for studying T cell recognition of F antigen *in vivo*, and together with assays for autoantibody responses, should help to resolve the mechanisms that regulate immune responses and tolerance to this unusual autoantigen.

Acknowledgments. We wish to acknowledge the excellent technical assistance of Mrs. Cheryl Dickins and the assistance of Dr. J. F. A. P. Miller and Miss Jenny Gamble with the assay for DTH.

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