Brief Definitive Report

REQUIREMENT FOR TWO *H-2* COMPLEX *Ir* GENES FOR THE IMMUNE RESPONSE TO THE L-GLU,L-LYS,L-PHE TERPOLYMER*

By MARTIN E. DORF, JACK H. STIMPFLING, and BARUJ BENACERRAF

(From the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115 and the McLaughlin Research Institute, Great Falls, Montana 59401)

The genetic study of the capacity to form specific immune responses has revealed that the recognition of antigens by individual animals and inbred strains is governed by the product of dominant genes located in the genome in close relationship with the genes coding for the molecules bearing the major histocompatibility specificities (1, 2). This has been verified in mice (3), guinea pigs (4), rats (5), chickens (6), and rhesus monkeys (7). These immune response genes have been termed histocompatibility, or H-linked *Ir* genes. The presence of the relevant genes permit immune responses to be formed. Individual genes were thought to control the responses to specific antigens (1–7). However, this report demonstrates that at least two genetically separable loci are required for immunological responsiveness to the linear synthetic terpolymer of L-glutamic acid, L-lysine, and L-phenylalanine (GLΦ).

The immune response to the synthetic polypeptide GLΦ was previously characterized by Merryman et al. (8, 9). These investigators demonstrated that the immune response to GLΦ is inherited as a Mendelian dominant character and the gene controlling GLΦ responsiveness was linked to the *H-2* complex. In a previous report (10), using *H-2* recombinant mice, we tentatively mapped the *Ir*-GLΦ gene within the *I* region of the *H-2* complex, more precisely, in the region currently termed *I-B*. This report demonstrates the requirement for at least two *H-2*-linked *Ir*-GLΦ genes for GLΦ responsiveness. Thus, F_1 hybrids derived from selected nonresponder parental strains are shown to be GLΦ responders. Furthermore, these genes have been localized in different regions of the *H-2* complex. Thus, selected recombinant strains demonstrated high levels of anti-GLΦ antibody, although each of the parental strains from which the recombinants were derived were nonresponders.

Materials and Methods

Mice. All inbred mice and F_1 hybrids were produced in our animal facilities or were purchased from the Jackson Laboratories, Bar Harbor, Maine. Animals were between 8 and 30 wk of age at the beginning of immunization. Each experimental group consisted of at least four mice which were bled 7 days after secondary immunization.

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Antigens. The random linear terpolymer L-glutamic acid^{53}-L-lysine^{36}-L-phenylalanine^{11} (sample no. GF6-23-8) was synthesized in Dr. Elkan Blout's laboratory, Department of Biological Chemistry, Harvard Medical School, Boston, Mass. Amino acid analyses gave the values indicated. The specific viscosity of this polypeptide was 0.99 at a concentration of 0.5% in 0.2 M sodium chloride solution at pH 2.7. The terpolymer was emulsified in complete Freund's adjuvant containing 0.5 mg/ml *Mycobacterium butyricum* (Difco Laboratories, Detroit, Mich.). Primary and secondary immunizations with 0.2 ml emulsion containing 100 μg of antigen were carried out intraperitoneally on days 0 and 21, respectively. Mice were bled on day 28. The sera were stored at −20° C until tested.

Antigen-Binding Assay. The humoral response to GLΦ was measured by antigen-binding assay employing the cross-reactive linear terpolymer of L-glutamic acid^{57}-L-lysine^{38}-L-tyrosine^{5} (GLT), a gift from Dr. Paul H. Maurer, Jefferson Medical College, Philadelphia, Pa. GLT was iodinated by the chloromine-T method (11) with carrier-free ^{125}I (New England Nuclear Corp., Boston, Mass.) and separated from inorganic iodide by passage over 0.5 × 25 cm columns of Sephadex G-25F (Pharmacia Fine Chemicals, Piscataway, N. J.). The radioiodinated ligand was diluted with PBS containing 1% normal mouse serum to a concentration of about 2×10^{-8} M for use and had specific activities between 0.2 and 4 Ci/g. Serum samples were diluted 1:5 with PBS for assay. To 25 μl of diluted serum in each well of a Linbro V plate were added 20 μl of radiolabeled ligand solution; the plates were mixed and incubated for at least 60 min at 4° C. Precipitation of the GLT-antibody complexes was achieved with 50 μl of 95% saturated ammonium sulfate (pH adjusted to 7.4 with ammonia). 1-2 h after the addition of the precipitating agent the plates were centrifuged at 800 *g* for 20 min at 4°C and 50 μl of supernate from each well was counted in a Packard gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

Results

After secondary immunization with 100 μg GLΦ in complete Freund's adjuvant, mice carrying the *H-2*^{d} haplotype made high levels of anti-GLΦ antibody. In contrast, mice homozygous for *H-2*^{k} or *H-2*^{b} (including the *H-2*^{b} mutant or variant strains Hz1, 129/J, and M505) made no detectable antibody responses (Table I). Several congenic strains carrying recombinant *H-2* haplotypes were also immunized with GLΦ. All strains carrying the *H-2*^{a} and *H-2*^{h} haplotypes were nonresponders (Table I). It is important to note that many of these recombinant haplotypes contain regions of chromosome derived from the *H-2*^{d} responder haplotype. In addition, four recombinant strains carrying *H-2*^{i} haplotypes were immunized with GLΦ. The 18R and R106 strains failed to make detectable levels of GLΦ antibody. Surprisingly, the other two *H-2*^{i} strains (3R and 5R) were GLΦ responders (Table I). The 3R and 5R responder strains were independently derived from crossovers between the two nonresponder haplotypes, *H-2*^{a} and *H-2*^{p}. The latter strains are congenic resistant lines on the C57BL/10 (B10) background.

Finally, several F_1 hybrids were immunized with GLΦ (Table I). All three hybrids made between responder and nonresponder strains were responders, indicating the dominance of the *Ir*-GLΦ genes. Surprisingly, the (A × B10)F_1 and (A × 18R)F_1 hybrids, which were the product of two nonresponder parental strains, also responded to the GLΦ terpolymer. The complementary genes responsible for the response in the latter hybrids were localized to the *H-2* complex, since the homozygous *H-2*^{a} congenic (A × B10.A)F_1 control mice were nonresponders.

Discussion and Summary

The finding that the mating of two nonresponder strains (A/J with B10 or 18R) produces responder F_1 hybrids is evidence of complementation of the nonrespon-

TABLE I
Immune Response to the GLΦ Terpolymer

Strain*	Parental haplotypes‡	*H-2* regions§						No. tested	Antigen binding (±SE)‖
		K	*I-A*	*I-B*	*I-C*	*S*	*D*		
C57BL/10	b	b	b	b	b	b	b	8	−1.1 ± 2.3
C3H.Sw	b	b	b	b	b	b	b	3	−0.8 ± 5.1
Hzl	ba							4	−0.9 ± 3.2
129/J	bc							4	−1.7 ± 4.0
M505	bd							5	−6.6 ± 2.8
B10.D2	d	d	d	d	d	d	d	9	60.9 ± 4.7
DBA/2	d	d	d	d	d	d	d	4	40.4 ± 7.9
BALB/c	d	d	d	d	d	d	d	4	59.2 ± 2.0
C3H/He	k	k	k	k	k	k	k	5	−6.8 ± 5.3
CBA/H	k	k	k	k	k	k	k	5	1.5 ± 3.2
AKR	k	k	k	k	k	k	k	4	1.7 ± 4.9
B10.A	k/d (a)	k	k	k	d	d	d	7	3.9 ± 2.1
A/J	k/d (a)	k	k	k	d	d	d	11	1.9 ± 1.6
A.AL	k/d (al)	k	k	k	k	k	d	4	1.7 ± 0.6
2R	a/b (h2)	k	k	k	d	d	b	4	−6.8 ± 3.7
4R	a/b (h4)	k	k	b	b	b	b	4	1.0 ± 3.6
15R	a/b (h15)	k	k	k	d	d	b	4	1.4 ± 3.5
3R	b/a (i3)	b	b	b	d	d	d	5	58.5 ± 7.0
5R	b/a (i5)	b	b	b	d	d	d	10	68.3 ± 4.0
18R	b/a (i18)	b	b	b	b	b	d	5	4.7 ± 2.4
R106	b/da (i106)	b	b	b	b	b	da	5	−6.0 ± 3.6
(BALB/c × A/J)F_1	d × a	d/k	d/k	d/k	d/k	d/k	d/k	5	67.7 ± 8.5
(C57BL/6 × DBA/2)F_1	b × d	b/d	b/d	b/d	b/d	b/d	b/d	4	68.3 ± 5.6
(A × 5R)F_1	a × i5	k/b	k/b	k/b	d/d	d/d	d/d	4	55.7 ± 4.4
(A × B10.A)F_1	a × a	k/k	k/k	k/k	d/d	d/d	d/d	5	7.5 ± 2.3
(A × B10)F_1	a × b	k/b	k/b	k/b	d/b	d/b	d/b	5	62.8 ± 5.8
(A × 18R)F_1	a × i18	k/b	k/b	k/b	d/b	d/b	d/b	5	59.5 ± 6.9

† All *H-2*^{h} and *H-2*^{i} recombinant strains are congenic on the C57BL/10 (B10) genetic background.
‡ Parental *H-2* alleles of recombinant strains are separated by slash. The designation of the recombinant haplotypes are indicated in parentheses.
§ Letters indicate parental origin of the genes in each *H-2* region. Vertical bars indicate position of crossing over in recombinant strains.
‖ Mean percentage of radiolabeled GLT ligand bound in Farr assay by a 1:5 dilution of serum ± standard error.

der *H-2*^{a} and *H-2*^{b} alleles. This observation, coupled with the fact that the recombinant 3R and 5R strains (which were derived by recombination between the nonresponder *H-2*^{a} and *H-2*^{b} haplotypes) are GLΦ responders, indicates that at least two distinct *Ir* loci are concerned with responsiveness to GLΦ. Since the two other recombinant strains carrying the *H-2*^{b} haplotype, i.e., the 18R and R106 strains, are GLΦ nonresponders, we must tentatively localize one of the *Ir*-GLΦ genes in the chromosomal region between *I-B* and *D* (see Table I). For convenience, we have designated this *Ir*-GLΦ gene α, and termed its alleles $\alpha(+)$ and $\alpha(-)$. To our knowledge, this represents the first documented data localizing an *Ir* gene in this portion of the *H-2* chromosome.[1] The other *Ir*-GLΦ gene, termed

[1] Merryman, Maurer, and Stimpfling (9) have independently suggested the presence of an *IR*-GLΦ gene in the *I-C* or *S* regions of the *H-2* complex.

β, must lie to the left of the 3R and 5R crossover positions, i.e., to the left of the *I-C* region. Presumably, the β-gene corresponds to the *Ir*-GLΦ gene, which was previously shown to map within the *I-B* region of the *H-2* complex (10). Each of the two *Ir*-GLΦ genes are dominant, however, both the $\alpha(+)$ and the $\beta(+)$ alleles are required for expression of GLΦ responsiveness. Thus, phenotypic nonresponder animals may either lack both responder alleles or possess only the $\alpha(+)$ or $\beta(+)$ alleles. Table II illustrates the genotypes and phenotypes of several representative strains. Additional matings are required to identify $\alpha(-)$ $\beta(-)$ haplotypes. Another interesting result is the demonstration that the $\alpha(+)$ and $\beta(+)$ genes can function in both the cis and trans positions.

In 1972, Stimpfling and Durham (12) first indicated that the immune response to the alloantigen *H-2.2* may be controlled by two interacting genes localized within the *H-2* complex. This was noted using many of the strains described in the current report. However, complementation studies using F_1 hybrids between nonresponders to *H-2.2* were not performed.

Previous reports (5, 6, 13) using other systems under *Ir* gene control have suggested that F_1 hybrids between two low responder parental strains may give responses higher than either parental strain. The most notable example was

TABLE II
Characterization of Ir-GLΦ Genes in Selected H-2 Haplotypes

Strains	*H-2* haplotypes	Genotype		Phenotype (GLΦ response)
		α	β	
A; B10.A	a	+	−	−
B10	b	−	+	−
18R; R106	i18; i106	−	+	−
3R; 5R	i3; i5	+	+	+
(A × B10)F_1; (A × 18R)F_1	a × b; a × i18	+/−	−/+	+

described by Zaleski et al. (13), who demonstrated genetic control of this phenomenon was linked to the *H-2* complex.

The observation that two distinct H-linked *Ir* genes are required for the immune response to a single antigen raises many questions: (*a*) Do all immune responses controlled by *H-2*-linked *Ir* genes require the cooperation of two distinct loci, as in the case for the response to GLΦ? A systematic study of the responses of F_1 hybrids of all nonresponder *H-2* haplotypes to several antigens under the control of H-linked *Ir* genes is indicated to resolve this issue. (*b*) At what cell level does each gene operate? The recent reports by Mozes et al. (14), indicating that the genetic defect in nonresponder strains to (T,G)-A--L is found in B cells in some strains and in T and B cells in other strains may be explained on the basis of the two genes demonstrated in this study. (*c*) What is the relationship of each *Ir* gene with the production of antigen-specific T-cell helper (15) or suppressor (16) factors and with the genetic control of T-B cell cooperation by the products of the *H-2* complex (17, 18)? Additional studies using the GLΦ system may resolve these questions.

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References

1. Benacerraf, B., and H. O. McDevitt. 1972. Histocompatibility-linked immune response genes. *Science (Wash. D. C.).* **175**:273.
2. Benacerraf, B., and M. E. Dorf. 1974. Genetic control of specific immune responses. *Prog. Immunol. II.* **2**:181.
3. McDevitt, H. O., and A. Chinitz. 1969. Genetic control of the antibody response: relationship between immune response and histocompatibility (H-2) type. *Science (Wash. D. C.).* **163**:1207.
4. Ellman, L., I. Green, W. J. Martin, and B. Benacerraf. 1970. Linkage between the poly-L-lysine gene and the locus controlling the major histocompatibility antigens in strain 2 guinea pigs. *Proc. Natl. Acad. Sci. U.S.A.* **66**:322.
5. Rüde, E., and E. Günther. 1974. Genetic control of the immune response to synthetic polypeptides in rats and mice. *Prog. Immunol. II.* **2**:223.
6. Günther, E., J. Balcarova, K. Hala, E. Rüde, and T. Hraba. 1974. Evidence for an association between immune responsiveness of chicken to (T,G)-A--L and the major histocompatibility system. *Eur. J. Immunol.* **4**:548.
7. Dorf, M. E., H. Balner, M. L. deGroot, and B. Benacerraf. 1974. Histocompatibility-linked immune response genes in the rhesus monkey. *Transplant. Proc.* **6**:119
8. Merryman, C., P. H. Maurer, and D. W. Bailey. 1972. Genetic control of immune response in mice to a glutamic acid, lysine, phenylalanine copolymer. III. Use of recombinant inbred strains of mice to establish association of immune response genes with *H-2* genotype. *J. Immunol.* **108**:937.
9. Merryman, C. F., P. H. Maurer, and J. H. Stimpfling. 1975. Mapping the genes governing the immune response to the GAT^{10} and GLΦ terpolymers to two distinct I subregions of the H-2 complex. *Immunogenetics.* In press.
10. Dorf, M. E., F. Lilly, and B. Benacerraf. 1974. Characterization of a new intra *H-2* recombinant. Separation of the *Ir-RE* and *Ir-GLT* genes. *J. Exp. Med.* **140**:859.
11. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89**:114.
12. Stimpfling, J. H., and T. Durham. 1972. Genetic control by the H-2 gene complex of the alloantibody response to an H-2 antigen. *J. Immunol.* **108**:947.
13. Zaleski, M., H. Fuji, and F. Milgrom. 1973. Evidence for multigenic control of immune response to theta-AKR antigen in mice. *Transplant. Proc.* **5**:201.
14. Mozes, E., R. Isac, and M. J. Taussig. 1975. Antigen-specific T-cell factors in the genetic control of the immune response to poly-(Tyr,Glu)-polyDLala--poly-Lys. Evidence for T- and B-cell defects in SJL mice. *J. Exp. Med.* **141**: 703.
15. Taussig, M. J. 1974. T cell factor which can replace the activity of T cells in vivo. *Nature (Lond.)* **248**:234.
16. Tada, T. and T. Takemori. 1974. Selective roles of thymus-derived lymphocytes in the antibody response. I. Differential suppressive effect of carrier-primed T cells on hapten-specific IgM and IgG antibody responses. *J. Exp. Med.* **140**:239
17. Katz, D. H., T. Hamaoka, M. E. Dorf, P. H. Maurer, and B. Benacerraf. 1973. Cell interactions between histoincompatible T and B lymphocytes. IV. Involvement of the immune response (Ir) gene in the control of lymphocyte interactions in responses controlled by the gene. *J. Exp. Med.* **138**:734.
18. Katz, D. H., M. E. Dorf, D. Armerding, and B. Benacerraf. 1975. The role of products of the histocompatibility gene complex in immune responses. *In* Molecular Approaches to Immunology. Miami Winter Symposia, Vol. 9. E. E. Smith and D. W. Ribbons, editors. Academic Press, Inc., New York.