LINKAGE OF SUSCEPTIBILITY TO EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS TO THE MAJOR HISTOCOMPATIBILITY LOCUS IN THE RAT*

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Experimental allergic encephalomyelitis (EAE)[1] has been widely studied as a possible experimental model for human demyelinative diseases, the two most common of which are multiple sclerosis and acute disseminated encephalomyelitis. EAE is also the only experimental autoimmune disease for which the exact amino acid sequence of an inciting molecule, basic protein of myelin, is known (1). Nearly identical basic proteins are found in all mammalian myelin studied and an encephalitogenic nonapeptide fragment of the bovine basic protein, which has been both isolated and synthesized de novo, has been shown to be encephalitogenic on a molar basis at least equivalent to the 170 amino acid basic protein (2). Thus, EAE also provides an attractive model for studying the immunological mechanisms of autoimmune diseases in general. In view of the rapidly accumulating evidence of genetic control of immune responses by genes linked to the major histocompatibility locus of the species (3), it is now appropriate to determine whether autoimmunity might also be influenced by specific histocompatibility-linked immune response genes. Two independent groups have observed a significant correlation between certain HL-A specificities and presence of multiple sclerosis (4).[2] Susceptibility to experimental autoimmune murine thyroiditis was recently shown to be genetically linked to the *H-2* locus (5). Since EAE is both a possible model for multiple sclerosis and a prototype experimental autoimmune disease in several species, we are currently studying its genetic control. Data presented here show that susceptibility to the induction of histologically determined EAE by injection of purified guinea pig basic protein is determined by a gene, designated *Ir*-EAE, linked to the major histocompatibility locus in the rat.

Materials and Methods

Rats.—Lewis/Mai (L), Brown Norway (BN), and $LBNF_1$ hybrids were obtained from Microbiological Associates, Inc., Bethesda, Md. All backcross animals were bred in our an-

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[1] *Abbreviations used in this paper:* BN, Brown Norway; CFA, complete Freund's adjuvant; EAE, experimental allergic encephalomyelitis; L, Lewis/Mai.

[2] Arnason, B., and J. Lehrich, personal communication.

imal facilities, using rats from Microbiological Associates as parents. Eight Lewis/Wistar rats obtained from Charles River Laboratories, Wilmington, Mass., were also used. Approximately equal numbers of males and females were studied at 4–6-mo of age. The average weight of all BN backcross animals was 285 ± 67 g (SD) including males and females together. Among littermates, males weighed more.

Determination of H-1 Phenotype.—The *H-1* designation of the major histocompatibility locus of the rat has been used in our figures. It is presumed to be equivalent to the AgB designation (see ref. 6). The *H-1* phenotype of the 42 BN backcross rats was determined on peripheral blood cells by an adaptation of the absorption method described by David and Shreffler (7).

The phenotype of Lewis backcross animals was determined by standard Trypan blue cytotoxicity assay and confirmed by mixed lymphocyte reactivity with $LBNF_1$, Lewis, and BN lymph node cells by a method described in detail elsewhere (8, 9). Both these methods were used because the titer of our Lewis anti-BN serum was low.

Production of EAE.—Rats were immunized in one hind footpad with 0.1 ml of an emulsion containing 10 μg of guinea pig basic protein plus 100 μg $H_{37}RV$ *Mycobacteria tuberculosis*. This complete Freund's adjuvant (CFA) emulsion was prepared in Difco incomplete adjuvant (Difco Laboratories, Detroit, Mich.) and tested for surface tension on distilled water immediately before injection. The purified basic proteins and *M. tuberculosis* were the generous gift of Dr. Marion Kies. Animals were observed daily for clinical signs of neurological dysfunction.

Histological Determination of EAE.—At the time of sacrifice brains and spinal cords were removed by gross dissection and fixed for 4 days in 10% buffered formalin. Care was taken to obtain the cord to its most distal extent. Specimens were then washed, dehydrated through graded alcohol, and embedded in paraffin. Sections were cut at six micra and stained with hematoxylin and eosin. Assorted sections were stained with LFB-cresyl violet for myelin and Bodian for axis cylinders to ensure that the lesions noted were in fact demyelinative. All sections were evaluated single blind and ranked on a scale of 0–4+. Animals designated negative did not have a single perivacular mononuclear infiltrative or demyelinative lesion. Results are reported here as positive or negative.

Skin Tests.—Separate animals injected for production of EAE or with CFA alone were skin tested at day 13 with 10 μg guinea pig basic protein and 10 μg rat S basic protein in 0.1 ml saline. Reactions were evaluated at 4 and 24 h, and selected sights were biopsied and evaluated histologically for the presence of perivascular mononuclear cell infiltration with or without the presence of significant numbers of polymorphonuclear leukocytes. Biopsies designated positive at 24 h had definite evidence of mononuclear cell infiltration without a significant polymorphonuclear cell component. These positive lesions were compared in a single blind fashion with skin test sites from normal animals and animals immunized with CFA alone.

RESULTS

All injected Lewis, $LBNF_1$, and Lewis backcross animals had histological evidence of EAE. Among the BN backcross animals studied, 25 were determined to have inherited the $H\text{-}1^l$ allele from the $LBNF_1$ parent, and 21 of these had histological evidence of EAE (Table I). Among the 17 BN backcross animals which were tissue typed as $H\text{-}1^n/H\text{-}1^n$, not a single lesion was detected by the examiner, who was unaware of the tissue type or even whether the animal represented had been immunized. Not a single BN animal had histological signs of EAE.

Among injected animals all Lewis, 14/17 $LBNF_1$, 6/9 Lewis backcross, and 12/42 BN backcross animals showed clinical evidence of EAE. The first neuro-

TABLE I

Incidence of Histological and Clinical Signs of EAE in Lewis, BN, (Lewis × BN)F_1, and Reciprocal Backcross Rats Immunized with 10 μg Guinea Pig Basic Protein in CFA Containing 100 μg of $H_{37}RV$ M. tuberculosis

Rat strain	*H-1* genotype‡	Signs of EAE*	
		Histological	Clinical
Lewis§	$H\text{-}1^l/H\text{-}1^l$	20/20	20/20
BN	$H\text{-}1^n/H\text{-}1^n$	0/16	0/16
(Lewis × BN)F_1	$H\text{-}1^l/H\text{-}1^n$	17/17	14/17
BN backcross¶	$H\text{-}1^l/H\text{-}1^n$	21/25	12/25
	$H\text{-}1^n/H\text{-}1^n$	0/17	0/17
Lewis backcross	$H\text{-}1^l/H\text{-}1^n$	5/5	2/5
	$H\text{-}1^l/H\text{-}1^l$	4/4	4/4

* 10 BN, 9 (Lewis × BN)F_1, and all Lewis backcross animals were sacrificed at day 14. Others were sacrificed at day 21, although all clinically positive animals had definite paralysis on day 14. Two Lewis, one F_1, and four BN backcross animals with severe paralysis died before the day of sacrifice.

‡ For the backcross rats, *H-1* genotype was inferred from *H-1* phenotype as described in the text.

§ Data from 8 Lewis/Wistar and 12 Lewis/Mai.

¶ Included offspring of F_1 female × BN male and reciprocal matings. Lewis backcross animals were littermate offspring from a single F_1 mother.

logical sign was a decreased tendency of the tail to curl around the examiner's finger, followed sequentially by a limp tip of the tail. The signs progressed proximally with decreasing tone in the distal portions of the tail to a completely flaccid tail. Signs which followed progressively were a waddling gait, hind leg paralysis, incontinence, and death in the most severely affected animals. Weight loss occurred throughout this period and may have been the first indication of disease in affected rats (Fig. 1). The disease reached its maximum extent usually between days 12 and 16 and, if death or sacrific did not ensue, was monophasic with nearly complete clinical recovery in most rats. Lewis rats were the first to manifest clinical signs and were the most severely paralyzed. LBNF_1 animals showed clinical signs 1 or 2 days after the Lewis rats and in most cases the paralysis was less severe. Clinically affected Lewis and BN backcross animals had varying degrees of paralysis including some which were as severely affected as Lewis rats and others with less clinical disease than the least severely affected LBNF_1 animals. All of the BN backcross animals with clinical signs were $H\text{-}1^l/H\text{-}1^n$ by tissue typing. Not a single BN or $H\text{-}1^n/H\text{-}1^n$ BN backcross animal had clinical signs of EAE.

The injected BN backcross animals were weighed repeatedly after immunization. Data in Fig. 1 indicate that the clinically affected animals could be distinguished from the others by a drop in total body weight occurring as early as day 12 after injection. By day 14 these differences were highly significant.

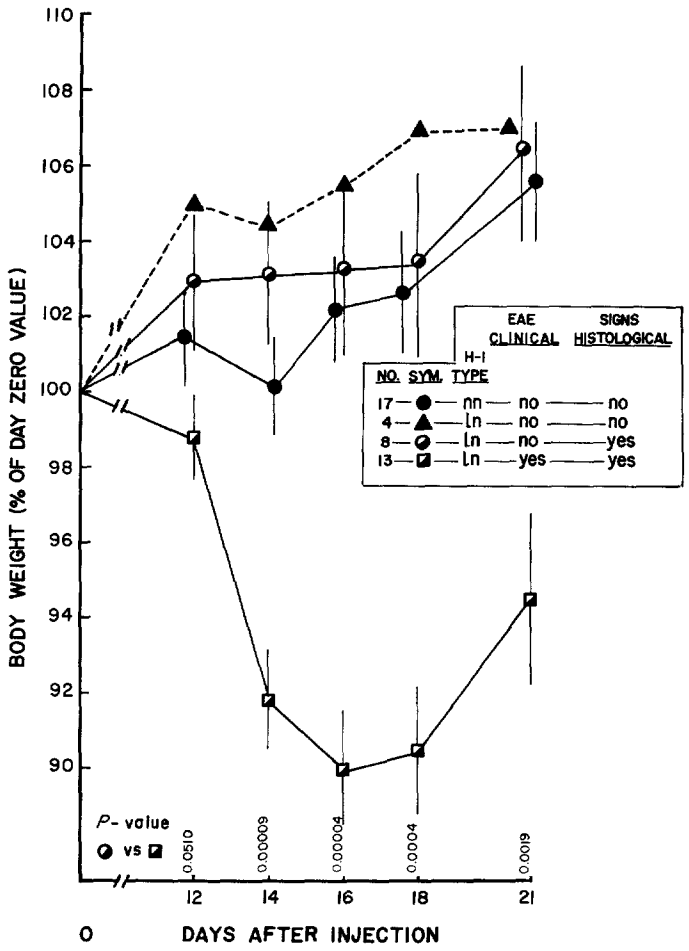

FIG. 1. Body weights of 42 BN backcross animals injected with 10 μg guinea pig basic protein in CFA containing 100 μg $H_{37}RV$ *M. tuberculosis*. Solid circles: *H-1*^{n}/*H-1*^{n} animals (none had EAE); solid triangles: *H-1*^{l}/*H-1*^{n} rats with no signs of EAE; half solid circles: *H-1*^{l}/*H-1*^{n} rats with histological but no clinical signs of EAE; half solid squares: *H-1*^{l}/*H-1*^{n} animals with both clinical and histological signs of EAE. *P* values are presented to the nearest 0.0001 or the first decimal greater than zero.

However, it was not possible to differentiate histologically EAE positive but clinically negative *H-1*^{l}/*H-1*^{n} from unaffected *H-1*^{n}/*H-1*^{n} backcross animals on the basis of weight. The four histologically EAE negative *H-1*^{l}/*H-1*^{n} BN backcross animals did not lose weight.

Skin tests of a separate group of Lewis, BN, and $LBNF_1$ animals injected with the same guinea pig basic protein in CFA revealed that rats of all genotypes could manifest both Arthus and delayed type hypersensitivity to the immuniz-

ing antigen and to rat S basic protein at day 13 (Table II). The two Lewis rats which failed to respond were severely paralyzed and emaciated at the time of skin testing. By clinical evaluation, skin tests had minimal erythema with 4-8 mm induration graded + or ++ on a 4+ scale. Histological evaluation of selected 24 h skin test sites showed definite lesions characteristic of delayed hypersensitivity when either guinea pig or rat S basic protein was the test antigen (Table III). Positive delayed reactions were significantly less intense in BN compared to Lewis rats and this observation was confirmed by histology as described in Table III. The BN animals' reactions to rat S basic protein

TABLE II

Skin Test Reactivity of Lewis, BN, and (Lewis × BN)F_1 Rats Immunized with 10 μg Guinea Pig Basic Protein in CFA Containing 100 μg of $H_{37}RV$ M. tuberculosis

Rat Strain	4 h (Arthus) reaction		24 h (delayed) reaction	
	Guinea pig basic protein	Rat S basic protein	Guinea pig basic protein	Rat S basic protein
Lewis	3/5*	3/5	3/5	1/5
BN	5/5	5/5	8/8	8/8
(Lewis × BN)F_1	5/5	5/5	5/5	4/5

* No. positive/no. tested. All animals were tested on day 13 after injection. The Lewis animals had severe clinical signs of EAE, F_1 animals showed mild but definite paralysis, and the BN animals had no clinical signs of EAE. The skin test dose was 10 μg in saline.

TABLE III

*Histological Evaluation of 24 h Skin Reactions in Rats Immunized with 10 μg Guinea Pig Basic Protein in CFA Containing 100 μg $H_{37}RV$ M. tuberculosis**

Rat strain	Skin test antigen	
	Guinea pig basic protein	Rat S basic protein
Lewis	+++●	+++
Lewis	+++	+++
BN	+++	++●
BN	++	Trace
BN	++●	Trace
BN	++●	Trace
BN	Negative	Trace
BN (unimmunized)	Negative	Negative
BN (immunized with CFA alone)	Negative	Negative
(Lewis × BN)F_1	+	++
(Lewis × BN)F_1	Negative	++

* These represent biopsies from selected animals described in Table II. All specimens were coded and histological evaluation was done by M.J.M. without prior knowledge of the rat strain or test antigen. "Trace" indicates definite evidence of perivascular mononuclear cells, while +, ++, and +++ indicate "mild", "moderate", and "marked" evidence of perivascular mononuclear cells. Lesions marked (●) also had evidence of polymorphonuclear cell infiltration.

were very weak, but were definitely scored positive by single blind examination of the sections. Normal animals or those immunized with CFA alone were skin test negative to both antigens by all criteria.

DISCUSSION

The findings that all Lewis, $LBNF_1$, Lewis backcross, and one half of BN backcross animals were susceptible to the induction of histologically verified EAE indicate that susceptibility was determined by an autosomal dominant gene. The fact that 21/25 BN backcross animals which inherited the *H-1*^{l} allele from the $LBNF_1$ parent were EAE+ while 0/17 *H-1*^{n}/*H-1*^{n} BN backcross rats were EAE+, strongly indicates that susceptibility is linked to the major histocompatibility locus of the rat (*H-1* or AgB). Thus, induction of EAE by injection of purified guinea pig basic protein in the rat satisfies the genetic predictions made from the hypothesis that susceptibility depends on an *H-1*-linked immune response gene, designated *Ir*-EAE, which determines cell-mediated reactivity to an encephalitogenic component of guinea pig basic protein. Lewis animals are designated *Ir*-EAE+ and BN animals *Ir*-EAE-.

The existence of 4/25 *H-1*^{l}/*H-1*^{n} injected BN backcross animals which did not manifest EAE remains to be explained. These could represent examples of recombination between the *Ir*-EAE gene(s) and gene(s) determining serologically defined histocompatibility antigens, a situation analogous to some *Ir* genes in guinea pigs and mice (10). However, no reciprocal recombinants, i.e. *H-1*^{n}/*H-1*^{n} *Ir*-EAE+, individuals were observed. Thus, the possibility that the *Ir*-EAE gene was inherited but not expressed by some BN backcross individuals remains open.

Clinical signs of EAE were present in only 12 of the 21 histologically EAE positive or in 12 of 25 *H-1*^{l}/*H-1*^{n} BN backcross animals. This observation is mathematically consistent with the possibility that there is a second independently segregating gene for susceptibility to clinically defined EAE. However, 3/17 $LBNF_1$ animals were free of clinical signs even though 17/17 had histological evidence of EAE. Therefore, it is more likely that there exists a threshold for expression of clinical signs when the histologically determined EAE is present. We have never observed an injected animal which manifested clinical signs of paralysis without also showing histological evidence of EAE.

Immune response genes control cell-mediated reactions with exquisite antigenic specificity, and the mechanism of gene action may include control of antigen recognition at the T cell level (10). The fact that susceptibility to EAE lesions was *H-1* linked must be considered in terms of the observations that, as compared to *Ir*-EAE− BN rats, Lewis rats develop stronger delayed skin reactions, have higher levels of T lymphocyte DNA synthesis in culture, and are *Ir*-EAE+. These considerations could be interpreted to support the possibility that development of EAE lesions is the first known example of quantitative expression of *Ir*-gene control in cell-mediated immunity. However, other studies

indicate that the nonspecific differences observed between Lewis and BN T lymphocyte reactivity in culture are not genetically linked to the major histocompatibility locus (9, 11). Therefore, in our opinion the *Ir*-EAE gene is more likely to determine reactivity at the level of antigen specificity, while the quantitative expression of this reaction may be influenced by other genes including some which may control thymus-derived cell function. Thus, the precise antigenic specificity of the *Ir*-EAE gene was evaluated with the basic protein preparations available when these experiments were carried out.

Both *Ir*-EAE+ Lewis and $LBNF_1$ rats and *Ir*-EAE− BN rats were immunized with purified guinea pig basic protein and skin tested with the homologous antigen and with purified rat S basic protein. Rat S basic protein is the smaller of the two basic proteins which occur in rat myelin. It differs from the larger protein in that it lacks approximately 40 amino acids in the carboxy-terminal region after tryptophane (12). This deletion contains none of the rat basic protein encephalitogenic site as tested in rats but does include part of the active site for guinea pigs. Animals of each genotype had Arthus and delayed skin reactivity to both antigens. The delayed reactions of the *Ir*-EAE− BN rats were less intense than those of Lewis and $LBNF_1$ rats, a result consistent with the antigen independent differences in thymus-derived cell function between the two strains (9, 11, 13). The fact that BN rats immunized with guinea pig basic protein were definitely positive when tested for delayed hypersensitivity with rat S basic protein as well as the homologous antigen rules out the possibility that the *Ir*-EAE gene only controls specific reactivity to the whole guinea pig basic protein molecule or even to those portions which cross react with the rat S basic protein. The precise antigenic specificity of the *Ir*-EAE gene remains to be determined by direct studies utilizing peptides of defined amino acid sequence.

Recent data of Gasser et al.[3] clearly demonstrate AgB-linked susceptibility to EAE induction in these rat strains after injection of guinea pig spinal cord in CFA. These data are entirely analogous to ours including some EAE negative BN backcross individuals which inherited the major histocompatibility locus of the Lewis strain, but no EAE positive BN backcross individuals which did not inherit this allele. Thus, even when immunized with such a complex antigenic mixture as whole spinal cord, the susceptibility to EAE induction behaves according to predictions based on a single *Ir*-gene linked to the major histocompatibility locus. The possible existence of *Ir*-EAE genes in other species would also offer some explanation for nonuniform reactivity among outbred animals tested with various basic protein fragments (14). The extent to which EAE is a model for human demyelinating diseases such as multiple sclerosis would be strengthened if the correlation of susceptibility and certain HL-A specificities reflects *Ir* gene participation in the human disease.

[3] Newlin, C. M., personal communication.

SUMMARY

Experimental allergic encephalomyelitis (EAE) was induced in rats of various genotypes by injection of 10 μg guinea pig basic protein in complete Freund's adjuvant containing 100 μg H_{37} RV *M. tuberculosis*. Histologically verified EAE was present in 20/20 Lewis, 17/17 (Lewis × BN)F_1, 9/9 Lewis backcross, and 21/42 BN backcross rats. Among the BN backcross animals, 25/42 were determined to carry the major histocompatibility type characteristic of the Lewis strain and 21 of these had EAE. Separate groups of Lewis, BN, and (Lewis × BN)F_1 rats were immunized as described and skin tested on day 13 with 10 μg guinea pig basic protein and rat S basic protein. Animals of each genotype had Arthus and delayed skin reactivity to both antigens. These data are compatible with the hypothesis that susceptibility to EAE in rats is controlled by an autosomal dominant gene linked to the major histocompatibility locus. It is proposed that this is an immune response gene, designated *Ir*-EAE, which controls T cell reactivity directed against a highly encephalitogenic portion of the basic protein molecule.

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