Genetic Studies of the Murine Corneal Response to *Pseudomonas aeruginosa*

RICHARD S. BERK,* KIRK BEISEL,[1] AND LINDA D. HAZLETT[2]

Department of Immunology and Microbiology[1] and Department of Anatomy,[2] Wayne State University School of Medicine, Detroit, Michigan 48201

The murine genetic control of resistance to *Pseudomonas aeruginosa* eye infection previously has been demonstrated to be regulated by two complementing dominant genes, *PsCR1* and *PsCR2*. The *PsCR1* locus apparently is not associated with the *H-2* complex, whereas the *PsCR2* locus could not definitively be associated with *H-2*. In this study we attempted to demonstrate a possible *H-2* linkage of the *PsCR2* locus. A panel of inbred congenic strains varying with either the *H-2* haplotype or genetic background from inbred partners of C57BL/10, C3H, A, and BALB/c strains were characterized for their *P. aeruginosa* infectivity phenotypes. These studies indicated that the *PsCR2* locus is not associated with the *H-2* locus. Furthermore, variations of the *H-2* haplotype did not change the resistance patterns observed in these strains. However, BALB.B and BALB.K congenic lines were resistant to *P. aeruginosa* eye infection, whereas BALB/cJ mice were susceptible. Examination of hybrids (BALB.K × BALB/cJ)F₁ and (BALB.B × BALB/cJ)F₁ demonstrated that an autosomal dominant gene(s), *PsCR*, confers resistance. Segregation analysis for the *H-2* haplotype and the *PsCR* gene in offspring of backcross matings with the BALB/cJ parental strain suggested that this *PsCR* gene is not linked to the *H-2* complex and has an inheritance pattern of a single locus or several tightly linked loci.

The use of inbred mice by some investigators has led to an increased interest in genetic control of natural resistance to infection (2, 4, 6, 8, 12). However, there appears to be virtually nothing in the literature on genetic control of eye infections in experimental animals. A number of studies recently have been performed in our laboratory to examine the response of the murine cornea to challenge with *Pseudomonas aeruginosa* (3, 10, 11; R. S. Berk, M. A. Leon, and L. D. Hazlett, Rev. Infect. Dis., in press). These studies have shown that the Swiss Webster, DBA/1J, and DBA/2J strains are naturally resistant to intracorneal infection and that the infection remains localized and spontaneously heals within 3 to 4 weeks (11). On the other hand, the BALB/cJ, C57BL/6, C3H, and NZB strains are susceptible, since the infection leads to extensive necrosis, eye shrinkage (phthisis), and blindness 12 to 15 days after infection (11; Berk et al., in press). An intermediate ocular response also has been observed in the A mouse congenic lines. Initial genetic studies (3; Berk et al., in press) indicate that natural resistance of the cornea may be controlled by two or more autosomal dominant genes, at least one of which is located outside the *H-2* complex. Intracorneal

challenge of F₁ hybrids of the susceptible BALB/cJ and C57BL/6 backgrounds exhibited natural resistance to infection. On the basis of these complementation studies and (BALB/cJ × C57BL/6)F₂ data, we conclude that each susceptible strain bears one autosomal gene and that a dominant gene is required at each of the two loci to express resistance. We have provisionally designated the C57BL/6 resistance gene as *PsCR1* and the BALB/cJ resistance gene as *PsCR2*. The purpose of this manuscript is to determine whether *PsCR2* is located within or outside the *H-2* complex.

MATERIALS AND METHODS

Bacterial cell cultures. Stock cultures of *P. aeruginosa* ATCC 19660 stored at 25°C on tryptose agar slants (Difco Laboratories, Detroit, Mich.) were used for inoculation of 50 ml of broth medium containing 5% peptone (Difco) and 0.25% Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). The culture was hemolytic and proteolytic and produced lecithinase and exotoxin A. Cultures were grown on a rotary shaker at 37°C for 18 h, centrifuged at 27,000 × *g* for 20 min (4°C), washed with saline, and suspended in 0.9% sterile nonpyrogenic saline (Travenol Laboratories, Inc., Deerfield, Ill.) to a concentration of 2.5 × 10¹⁰ colony-forming units per ml,

using a standard curve relating viable counts to optical density at 440 nm.

Infection of animals. Inbred congenic lines of all F_1, F_2, and backcross matings were bred in our own animal facilities or purchased from Jackson Laboratories, Bar Harbor, Maine). Strain A/He was purchased from Cumberland View Farms, Clinton, Tenn. In all experimental studies involving either F_1 or F_2 animals, both male and female progeny were used in approximately equal numbers. Mice were infected at 5 to 6 weeks of age (18 to 22 g). Before infection, they were lightly anesthetized with ether and placed beneath a stereoscopic microscope. The corneal surface of the left eye was incised (three 1-mm-long incisions) with a sterile 26-gauge needle, taking care not to penetrate the anterior chamber. A bacterial cell suspension (5 μl), containing a final concentration of 1.25 × 10^8 colony-forming units, was topically delivered onto the surface of the incised corneas, using a micropipette (Oxford Laboratories, Foster City, Calif.) with a sterile, disposable tip. Control animals received a similar wound and 5 μl of sterile saline. All experimental data represent the results of two or more independently performed experiments.

The ocular response was macroscopically evaluated at 24 h after bacterial challenge and then at weekly intervals over a 3- to 6-week time period and compared with the contralateral eye and the saline control eyes. In addition, the eyes were monitored intermittently for the presence of bacteria by gently swabbing the cornea with a sterile cotton swab and inoculating tryptose agar plates. The plates were incubated at 37°C for 24 to 48 h. All plates showing growth contained pure cultures of *P. aeruginosa*.

Antisera. Preparation of *H-2* antisera has been described previously (16). Animals from the (BALB.K × BALB/cJ)F_1 × BALB/c and (BALB.B × BALB/cJ)F_1 × BALB/cJ backcrosses were typed to determine their respective *H-2* haplotypes. The antisera used in this study were (BALB/c × A.TL)F_1 anti-A/J (anti-H-2K^k), (A × DBA/2)F_1 anti-B10.A(4R) (anti-H-2D^b), and (BALB/K × A.BY)F_1 anti-A.ATR1 (anti-H-2^d).

H-2 typing. Individual animals were typed for their respective *H-2* haplotype by using the PVP hemagglutination method of Stimpfling (23) as modified by Shreffler et al. (21).

RESULTS

In a previous paper, we used (BALB/cJ × DBA/2)F_1 hybrids to describe the *PsCR1* locus (Berk et al., in press). We established that this resistance locus is not *H-2* linked. Since both mouse strains share the same *H-2^d* haplotype, we could not determine whether *PsCR2* is *H-2* linked or not. In an attempt to answer this question, we screened several inbred mouse strains. First, examination of congenic lines of C3H (Table 1) indicated that all of the six lines tested were susceptible regardless of their *H-2* haplotype.

Similar studies with 11 B10 (i.e., C57BL/10) congenic lines indicated that all of the test

TABLE 1. *Response of C3H congenic lines to intracorneal challenge with P. aeruginosa[a]*

Mouse strain	H-2 haplotype	Susceptible response
C3H/HeJ	k	71/71
C3H.SW/Sn	b	9/10
C3H.JK/Sn	j	34/34
C3H.NB/Sn	p	19/19
C3H.Q	q	12/12
C3H/HeSn	k	51/51

[a] All mice received a topical application of 1.25 × 10^8 colony-forming units of *P. aeruginosa*.

strains were uniformly susceptible (Table 2). In addition, a few of the mice died from a systemic infection resulting from the intracorneal challenge. Congenic lines of the A strain were also tested because previous unpublished studies from our laboratory indicated that the A/J mouse strain exhibited an intermediate response to the bacterial challenge. In the present study, three of the four congenic strains and a subline of the A strain (A/He) all exhibited the same intermediate response of either eye loss or spontaneous recovery (Table 3). Thus, 41 of 64 A.BY(*H-2^b*) mice and 1 of 20 A.SW(*H-2^s*) mice were susceptible. Similar results were obtained with A.CA(*H-2^f*) and A/He(*H-2^a*) mice, whereas only the A/J(*H-2^a*) mice were almost all susceptible (23 of 24), and six A/J mice died of the infection. These results indicate that the *H-2* locus does not seem to exert an effect on resistance or susceptibility. The anomalous results with the A/J strain are due to an expected fluctuation in response, more so than normally observed with mouse strains which are wholly resistant or susceptible in response.

In a previous study (10, 11), the BALB/cJ strain was found to be completely susceptible to intracorneal challenge, and from the congenic studies described herein with mice of the B10, C3H, or A background, we were expecting that BALB.B and BALB.K congenic lines would also be uniformly susceptible. However, when these two lines were examined, their responses were uniformly resistant. Thus, 51 of 51 BALB.B mice and 101 of 102 BALB.K mice exhibited resistance. The kinetics of recovery of these two congenic lines seemed to differ from other naturally resistant strains such as the DBA/1J and DBA/2J, which show gradual and eventually complete recovery within around 3 weeks postchallenge. The BALB.B and BALB.K strains do not usually recover until about 5 to 6 weeks postchallenge and during much of this time appear as though they will exhibit susceptibility and lose their sight.

These delayed recoveries indicate that there

TABLE 2. *Response of B10 congenic lines to intracorneal challenge with P. aeruginosa*[a]

Mouse strain	*H-2* haplo-type	Susceptible response
B10	*b*	20/20
B10.D2	*d*	17/17
B10.M	*f*	14/14[b]
B10.WB	*j*	7/8
B10.BR	*k*	20/20
B10.F	*p*	14/14[c]
B10.Q	*q*	8/8
B10.RIII	*r*	16/16
B10.S	*s*	19/19
B10.PL	*u*	6/6[b]
B10.SM	*v*	9/9[c]

[a] All mice received a topical application of 1.25 × 10^8 colony-forming units of *P. aeruginosa*.
[b] One died.
[c] Two died.

TABLE 3. *Response of A congenic lines to intracorneal challenge with P. aeruginosa*[a]

Mouse strain	*H-2* haplotype	Susceptible response
A.BY	*b*	41/64[b]
A.CA	*f*	15/29
A.SW	*s*	9/20
A/J	*a*	23/24[c]
A/He[d]	*a*	8/23

[a] All mice received a topical application of 1.2 × 10^8 colony-forming units of *P. aeruginosa*.
[b] Three died.
[c] Six died.
[d] Cumberland mice.

is an *H-2* association with resistance. The delay in recovery of the BALB.B and BALB.K mice compared with that of the DBA/2J mice may be due to expression of different alleles either at one locus or as the result of different intergenic interactions. However, the previous data, described earlier in this manuscript, argue against this interpretation. To examine this discrepancy, we initiated studies with F_1 hybrids and backcrossed animals. The first study of this type was to determine the response of (BALB.B × BALB/cJ)F_1 and (BALB.K × BALB/c)F_1 mice, which were both resistant. These results imply that the gene(s) controlling resistance is dominant. The results of reciprocal crosses between males and females incorporated in this study indicate that this gene is not sex linked.

Using backcrosses, we initiated segregation analyses to determine *H-2* linkage and the number of genes involved by mating the F_1 hybrids of BALB.B and BALB.K to BALB/cJ with the BALB/cJ parental strain [(BALB.K × BALB/cJ)F_1 × BALB/cJ]. The phenotypes of the backcross progeny yielded an approximately 1:1:1:1

segregation pattern of the following phenotypes (Table 4): of the 58 (BALB.K × BALB/cJ)F_1 × BALB/cJ backcross progeny, 12 animals were susceptible and homozygous for the *H-2* complex (i.e., *H-2$^{d/d}$*), 19 animals were resistant and homozygous (*H-2$^{d/d}$*), 12 animals were susceptible and heterozygous (*H-2$^{k/d}$*), and the remaining 15 mice were resistant and heterozygous (*H-2$^{k/d}$*). Similar studies with 54 (BALB.B × BALB/cJ)F_1 × BALB/cJ backcrosses yielded the following results: 16 mice were susceptible and homozygous (*H-2$^{d/d}$*), 11 were resistant and homozygous (*H-2$^{d/d}$*), 15 were susceptible and heterozygous (*H-2$^{b/d}$*), and 12 were resistant and heterozygous (*H-2$^{b/d}$*). The 1:1:1:1 ratio is indicative of two loci segregating independently of each other. Thus, the *H-2* complex and the *PsCR* gene are not linked. Chi-square analyses of these data [$\chi^2 = 2.43$ for (BALB.K × BALB/cJ)F_1 × BALB/cJ, and $\chi^2 = 1.28$ for (BALB.B × BALB/cJ)F_1 × BALB/cJ] are in accord with the hypothesis that *H-2* is not linked to the *PsCR* gene and that there is only one gene or group of tightly bound loci that controls resistance in the BALB.B and BALB.K lines. This latter point was substantiated by the 1:1 segregation pattern for BALB.K of 24 resistant mice versus 34 susceptible mice. A 1:1 segregation pattern was also observed for BALB.K of 31 resistant mice versus 23 susceptible mice.

DISCUSSION

In a previous study, matings of two susceptible strains, BALB/c and C57BL/6, resulted in progeny that are resistant to *P. aeruginosa* eye infection (3; Berk et al., in press). Segregation analysis of (BALB/c × C57BL/6)F_2 animals suggests that two loci, *PsCR1* and *PsCR2*, are involved in the regulation of resistance. Also, dominant alleles are required at both loci to avoid phthisis bulbi. Genetic studies suggest that the

TABLE 4. *Phenotypic analysis of backcrosses between F₁ hybrids and BALB/cJ*

Cross	Phenotype		Genotype[a]	No. of animals
	H-2 K^k	Resistance		
(BALB.K × BALB/c)F₁ × BALB/c	−	−	*H-2$^{d/d}$ rr*	12
	−	+	*H-2$^{d/d}$ Rr*	19
	+	−	*H-2$^{k/d}$ rr*	12
	+	+	*H-2$^{k/d}$ Rr*	15
(BALB.B × BALB.c)F₁ × BALB/c	−	−	*H-2$^{d/d}$ rr*	16
	−	+	*H-2$^{d/d}$ Rr*	11
	+	−	*H-2$^{b/d}$ rr*	15
	+	+	*H-2$^{b/d}$ Rr*	12

[a] rr, Susceptible; Rr, resistant.

PsCR1^r gene of C57BL/6 is not *H-2* linked, whereas the linkage of the *PsCR2^r* gene cannot be determined. Since both the DBA/2J (resistant) and BALB/cJ (susceptible) strains carry the *H-2^d* haplotype, the *PsCR2* locus may possibly be linked to the *H-2* complex. To determine the *PsCR2* association with the *H-2* complex, a panel of *H-2* congenic inbred lines was characterized for its resistance to *P. aeruginosa* infection. The B10.D2 strain, which carries the *H-2^d* haplotype and the C57BL/10 background gene, was chosen as the most relevant strain for examination of the possible *H-2* linkage of the *PsCR2* locus. The results of the infectivity experiments demonstrated that B10.D2 animals were susceptible to infection. The lack of gene complementation necessary for resistance observed in the B10.D2 strain argues against an *H-2* linkage of the *PsCR2* locus. All of the strains examined which carried various different *H-2* haplotypes did not vary phenotypically from the parental strain contributing the background genes.

The major histocompatibility complex appears to play only a minor role, if any, in host resistance to bacterial infection. Resistance to infection by *Corynebacterium kutscheri* (12, 17), *Mycobacterium lepraemurium* (6, 7, 13), *Mycobacterium tuberculosis* (1), *Listeria monocytogenes* (4, 5, 8, 22), *Rickettsia tsutsugamushi*, *Rickettsia akari* (2, 9), and *Salmonella typhimurium* (8, 14, 15, 18–20) has been demonstrated to be genetically regulated. In general, non-*H-2* genes have the predominate role in regulation of host resistance to other organisms. Data from our experiments suggest that only non-*H-2* loci control resistance to *P. aeruginosa* eye infection.

Interestingly, we found that BALB.B(*H-2^b*) and BALB.K(*H-2^k*) congenic lines were resistant to *Pseudomonas* eye infection, whereas the BLAB/cJ(*H-2^d*) mice were susceptible. These results led us initially to consider a possible *H-2* role in the resistance differences among the BALB.B, BALB.K, and BALB/cJ strains. However, all other reported studies of the genetic influence on bacterial infectivity argue against an *H-2* role. Genetic segregation studies were done to determine the linkage of this *PsCR* locus with the *H-2* complex. In the F$_1$ animals derived from matings between BALB/c and BALB.B or BALB.K mice, all F$_1$ offspring were resistant to the eye infection, regardless of whether the BALB.B or BALB.K parents were male or female. Gene *PsCR*, which controls resistance to *Pseudomonas* infection, is thus autosomal and dominant in inheritance. By a characterization of backcross animals [i.e., (F$_1$ × BALB/cJ) offspring for their respective resistance phenotypes and *H-2* haplotypes], the *PsCR* gene of BALB.B

and BALB.K strains, as determined by segregation analysis, is not linked to the murine major histocompatibility complex. In the backcross animals, an approximate 1:1 ratio of resistant to susceptible animals was observed, thereby suggesting that this resistance phenotype is controlled by a single locus, *PsCR*, or by a group of tightly linked loci. Because we cannot determine at this time whether this gene is representative of a different allele form of either the *PsCR1* locus, the *PsCR2* locus, or actually a third locus, no numbered designation will be added to the *PsCR* symbol for this gene. The difference of this *PsCR* locus in the BALB.B and BALB.K strains from the BALB/cJ strain may be a result of variational differences in sublines of the BALB/c strain, since BALB.B and BALB.K strains were derived from the BALB/cKh subline (F. Lilly, personal communication).

From previous gross observations, we can now see that, depending on the strain of inbred mouse, there appear to be several types of responses to intracorneal challenge with *P. aeruginosa*. For example, DBA/1J and DBA/2J strains exhibit recovery of a clear cornea in 100% of the animals within approximately 3 to 4 weeks postchallenge (3; Berk et al., in press). BALB.B and BALB.K mice take 5 to 6 weeks to exhibit a clear cornea and during the first 4 weeks exhibit severe corneal infection. On the other hand, the A congenic lines yield an intermediate response in that the corneas of some mice spontaneously recover in a manner similar to the DBA/1J and DBA/2J strains, whereas in others, the infected eye becomes phthisical or the animal dies of systemic infection. To determine whether there has been a change in BALB/c gene *PsCR2*, which complements with *PsCR1* from the B6 strain, complementation studies between the BALB/cKh and B6 strains may prove to be interesting. These studies are in progress in our laboratory.

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