JOURNAL OF VIROLOGY, Sept. 1995, p. 5875–5877 Vol. 69, No. 9
0022-538X/95/$04.00+0

Innate Resistance to Lethal Mousepox Is Genetically Linked to the NK Gene Complex on Chromosome 6 and Correlates with Early Restriction of Virus Replication by Cells with an NK Phenotype

MARGARET L. DELANO† AND DAVID G. BROWNSTEIN*

Section of Comparative Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

Received 21 March 1995/Accepted 7 June 1995

Most inbred strains of mice, including DBA/2 (D2), are highly susceptible to the lethal effects of ectromelia virus, but C57BL/6 (B6) mice are innately resistant. Resistance is controlled by multiple, unlinked, autosomal dominant genes. Of 101 male (B6 × D2)F_1 × D2 backcrossed (N2) mice, 18 died after ectromelia virus challenge and all were homozygous for the D2 allele at the proline-rich protein (*Prp*) locus on distal chromosome 6 ($P < 0.001$). This association was suggested by the patterns of susceptibility to lethal mousepox in recombinant inbred strains derived from B6 and D2 mice (D. G. Brownstein, P. N. Bhatt, L. Gras, and R. O. Jacoby, J. Virol. 65:1946–1951, 1991). The association between the *Prp* locus and susceptibility to lethal mousepox also held for N2 male mice that were castrated as neonates, which increased the percentage that were susceptible to 40. Spleen virus titers were significantly augmented in B6 ($NK1.1^+$) mice depleted of asialo $GM1^+$ or $NK1.1^+$ cells, whereas spleen virus titers were unaffected in D2 ($NK1.1^-$) mice depleted of asialo $GM1^+$ cells. These results suggest that a gene or genes within the natural killer gene complex, adjacent to the *Prp* locus, determine strain variations in resistance to lethal ectromelia virus infection.

Ectromelia virus is a naturally occurring orthopoxvirus of laboratory mice. Most inbred strains of mice, including DBA/2 (D2) mice, are highly susceptible to the lethal effects of ectromelia virus, but C57BL/6 (B6) mice are resistant (4, 23). Resistance is controlled by multiple, unlinked, autosomal dominant genes (4, 6, 7). We have used patterns of resistance and susceptibility to lethal mousepox in 25 recombinant inbred strains of mice derived from B6 and D2 mice to identify subchromosomal regions that may contain resistance genes (7). Three candidate regions were previously tested for linkage with susceptibility to lethal mousepox in (B6 × D2)F_1 × D2 backcrossed (N2) mice, and the proximal portion of chromosome 2, but not the other two regions, was found to contain a dominant resistance gene, provisionally named *Rmp2* (7). A fourth candidate region near the proline-rich protein (*Prp*) locus on distal chromosome 6 was not tested for linkage. Subsequently, the NK (natural killer) gene complex was mapped to the proximity of the *Prp* locus (27). Genes of the NK gene complex are selectively expressed on the surface of NK cells and encode cell surface receptors that appear to be important in cell target recognition (12, 17, 26). NK cells are bone marrow-derived lymphoid cells that mediate early antiviral defenses without prior sensitization and are essential for resistance to lethal ectromelia virus infection (15). Inbred strains of mice that are susceptible to lethal mousepox do not transcribe members of the NK gene complex, whereas B6 mice do (13).

In this study, we tested for linkage between the *Prp* locus and recessive susceptibility to lethal mousepox in intact and castrated N2 mice that were the subjects of a previous linkage study (7). Age, gender, and the presence of gonads determine the proportion of N2 mice that survive challenge infection with ectromelia virus (7, 23). At 8 weeks of age, approximately 80% of intact male N2 mice survive challenge infection with a dose of virus that is lethal for 100% of D2 males and 0% of B6 males, consistent with resistance mediated by multiple, dominant, unlinked genes (7, 23). It is the 20% of susceptible intact male N2 mice that are useful for linkage studies because they are homozygous for recessive D2 susceptibility alleles at most or all resistance loci. The percentage of mice that are susceptible to lethal mousepox increases if N2 mice are castrated as neonates, presumably because some or all resistance genes are regulated by products of the testis (7).

In the first experiment, *Prp* haplotypes were determined in 18 susceptible mice from a total population of 101 male N2 mice which were inoculated subcutaneously with 10^5 PFU of the Moscow strain of ectromelia virus. Genomic DNA was extracted from brains frozen at −70°C within 12 h of death by the method of Krieg (16) and had undergone minimal postmortem degradation as revealed by the absence of smearing in ethidium bromide-stained 0.6% agarose gels after electrophoresis. Control DNA from D2, B6, and (B6 × D2)F_1 mice was included. Ten micrograms of each DNA sample, digested with *Hin*dIII, was Southern blotted by standard methodology (18) and hybridized to the rat Prp33 cDNA probe kindly provided by D. M. Carlson (28). The control mice exhibited the expected restriction fragment sizes; the informative fragment was 2.0 kb and was present in B6 and F_1 mice and absent in D2 mice (2). The 18 mice that were susceptible to lethal mousepox were homozygous for the D2 *Prp* allele, Prp^a (Table 1, experiment 1). On the basis of the expectation that approximately half of these mice should have been heterozygous at the *Prp* locus if there was no resistance gene on distal chromosome 6, this result was highly significant ($P < 0.001$, χ^2 analysis with Yate's correction).

In the second experiment, *Prp* alleles were determined in susceptible and resistant mice in a population of 42 male N2

* Corresponding author. Mailing address: Section of Comparative Medicine, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06510. Phone: (203) 785-2534. Fax: (203) 785-7499.

† Present address: University of Massachusetts at Amherst, Amherst, MA 01003.

TABLE 1. Linkage between susceptibility to lethal mousepox in (B6 × D2)F_1 × D2 male backcrossed mice and the *Prp* locus on chromosome 6

Expt	No. susceptible with Prp^a (D2)/no. susceptible	No. resistant with Prp^b (B6)/no. resistant
1	18/18[a]	ND[b]/83
2	13/13[a]	16/25

[a] $P < 0.001$, compared with expected number with Prp^a, χ^2 analysis with Yate's correction.
[b] ND, not determined.

mice castrated as neonates and infected subcutaneously with 10^5 PFU of ectromelia virus at 4 to 6 weeks of age (7). Seventeen of the mice (40%) died of ectromelia virus infection, and brain DNA from 13 mice was suitable for analysis. The 13 susceptible N2 mice were homozygous for Prp^a ($P < 0.001$; Table 1, experiment 2). Of the 25 resistant mice, 16 (60%) were heterozygous and 9 (40%) were homozygous for Prp^a. These results confirmed that susceptibility to lethal mousepox was conferred by a recessive gene (or genes) on distal chromosome 6 of D2 mice. It also showed that neonatal castration did not demonstrably alter the ability of the dominant resistance allele of this gene to protect mice but that this gene was not the only gene that protected castrated mice.

Results of the second experiment were confirmed by analyzing the same DNA samples for a polymorphic microsatellite, D6Mit150, which has been provisionally mapped 12 centimorgans proximal to the *Prp* locus (11). One-microgram aliquots of genomic DNA were amplified in a Perkin-Elmer model 480 DNA Thermal Cycler with 1.0 μM forward and reverse primers (Research Genetics, Huntsville, Ala.) and AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer's specifications and a published thermocycling protocol for mouse microsatellites (10). PCR products were electrophoresed in a 3.5% NuSieve GTG agarose gel, and D2 and B6 alleles of 150 and 140 bp, respectively, were visualized by ethidium bromide staining. Results were identical to those of the Southern analysis for *Prp* alleles (Fig. 1) indicating that all susceptible mice were homozygous for a segment of distal chromosome 6 from D2 mice provisionally extending from 51 to 63 centimorgans distal to the centromere, which included the NK gene complex.

Results of these linkage studies suggested that a gene or genes within the NK gene complex might be important in innate resistance to mousepox. To determine if NK cells of B6 and D2 mice differed in the capacity to restrict virus replication, we compared ectromelia virus titers in control mice and NK cell-depleted mice during the early phase of infection. Antibodies to asialo GM1 and NK1.1 were used to deplete NK cells in vivo. NK cells of all mouse strains express high levels of asialo GM1 on their surface and are depleted by in vivo treatment with rabbit anti-asialo GM1 (14). Lower levels of surface asialo GM1 are also expressed by alloreactive and cytotoxic T cells and activated macrophages (9, 21, 22, 24, 25); therefore, NK cells may not be the only cells depleted by in vivo serotherapy. NK1.1 is one of the antigens encoded within the NK gene complex (17). Although NK1.1 is expressed by a small subset of $CD4^+$ T cells (1) in addition to NK cells, it is the most specific marker of mouse NK cells currently available. B6 mice are $NK1.1^+$, whereas D2 mice are $NK1.1^-$ (8). In vivo treatment with monoclonal anti-NK1.1 antibody PK136 depletes NK cells in strains that express this antigen (20).

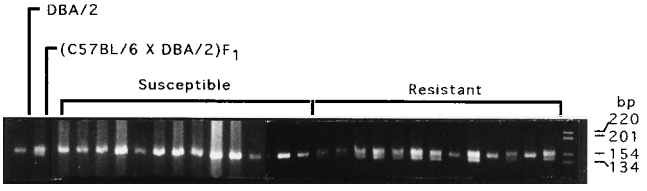

FIG. 1. Comparison of distal chromosome 6 marker locus (D6Mit150) haplotypes between ectromelia virus-resistant and -susceptible castrated (B6 × D2)F_1 × D2 mice. Genomic DNA was extracted from the brains of individual mice, and the PCR-amplified microsatellite was visualized in an ethidium bromide-stained 3.5% NuSieve agarose gel after electrophoresis. All susceptible mice and 13 representative resistant mice are shown. Homozygous D2 and heterozygous (B6 × D2)F_1 are included.

Sixteen B6 mice and 17 D2 mice, males aged 8 to 10 weeks, were divided into control and asialo $GM1^+$ cell depletion groups. Depletion groups were injected intravenously in the tail vein with 0.1 ml of rabbit antiserum to bovine asialo GM1 (Wako Chemicals, Dallas, Texas). Pilot studies indicated that this dose of anti-asialo GM1 and route of administration eliminated splenic NK cell activity in B6 and D2 mice for at least 5 days as measured in vitro against YAC-1 target cells in a 4-h chromium release assay in 5-week-old female B6 and D2 mice (Table 2). Non-NK cell-depleted groups were given normal rabbit serum intravenously. Twenty-four hours after serodepletion, mice were injected intravenously with 10^5 PFU of the Moscow strain of ectromelia virus, and spleens were harvested on post-virus inoculation days two (PID 2) and three (PID 3). Virus titers were determined with BS-C-1 cells as previously described (3). Ectromelia virus titers were significantly higher in the spleens of asialo $GM1^+$ cell-depleted B6 mice than in control B6 mice, whereas virus titers did not differ significantly between asialo $GM1^+$ cell-depleted and control D2 mice (Table 2, experiment 3A). The effect of anti-asialo GM1 pretreatment on ectromelia virus titers in B6 mice was repeated with 28 B6 mice and the same methodology as in the first experiment. Again, there was a highly significant difference between the spleen virus titers of control and asialo $GM1^+$ cell-depleted B6 mice (Table 2, experiment 3B). These results showed that the asialo $GM1^+$ cell population of B6 mice, but not D2 mice, which included NK cells but may not have been specific for NK cells could suppress ectromelia virus titers during the first 72 h of infection, when genetic resistance is being expressed in the spleen (5). This difference in the effects of asialo $GM1^+$ cells on ectromelia virus titers between B6 and D2 mice was unlikely to have been a consequence of high levels of ectromelia virus in D2 mice overwhelming resistance mediated by NK cells because we showed in a previous study that on PID 2 and PID 3, only 2 and 15%, respectively, of spleen cells are infected with virus in intact D2 mice given the same dose of virus by the same route as in the study reported here (5).

To determine if NK cells of B6 mice were the asialo $GM1^+$ cell population responsible for restricting ectromelia virus titers, we depleted mice of NK cells by using monoclonal anti-NK1.1 antibody (hybridoma PK136) kindly provided by Gloria Koo. In a preliminary study, B6 mouse spleen NK cell activity against YAC-1 target cells in a 4-h chromium release assay was eliminated for at least 4 days after intravenous injection of 160 μg of antibody (Table 2, experiment 3C). Eight B6 mice were divided into two equal groups and injected intravenously with 160 μg of anti-NK1.1 or with control ascitic fluid. Twenty-four hours later, mice were injected intravenously with 10^5 PFU of ectromelia virus. Three days after virus infection, the spleens were harvested and virus was quantified. Virus titers were significantly higher in the anti-NK1.1-treated group than in the control group (Table 2, experiment 3C).

Taken together, these results suggest that the gene (or genes) on mouse chromosome 6 that contributes to the resis-

TABLE 2. Effects of anti-asialo GM1 and anti-NK1.1 antibodies on ectromelia virus titers and NK cell activity in the spleens of B6 and D2 mice

Expt	Strain	Treatment	% Lysis against YAC-1[a]	Log_{10} PFU/g ± SD (*n* mice per group)		*P* value[b]
				PID 2	PID 3	
3A	B6	Control	34.5 ± 3.1	7.1 ± 0.6 (4)	7.9 ± 0.6 (4)	
		Anti-asialo GM1	0.1 ± 0.2	7.6 ± 0.5 (4)	8.8 ± 0.2 (4)	0.0121
	D2	Control	17.7 ± 3.3	7.7 ± 0.2 (4)	8.6 ± 0.2 (4)	
		Anti-asialo GM1	0.1 ± 0.2	7.6 ± 0.6 (4)	9.0 ± 0.1 (5)	0.3470
3B	B6	Control		7.0 ± 0.3 (7)	7.7 ± 0.4 (6)	
		Anti-asialo GM1		7.6 ± 0.4 (8)	8.6 ± 0.2 (7)	0.0001
3C	B6	Control	38.7 ± 4.4		7.0 ± 0.4 (4)	
		Anti-NK1.1	0.6 ± 0.4		7.8 ± 0.2 (4)	0.019

[a] Mean (± SD) spleen NK cell activity of four mice per group 4 days after 100 μl of antibody administered intravenously at effector/target ratio of 100:1 in 4-h chromium release assays.
[b] *P* values based on one- or two-way analysis of variance.

tance phenotype of B6 mice is a member of the NK gene complex and is expressed through a cell with an NK phenotype. We provisionally designate this gene *Rmp1*, the name proposed by Wallace and coworkers for an unmapped, gender-independent and presumably gonad-independent resistance gene of B6 mice (23). *Rmp1* is the third gene that controls resistance to lethal mousepox to be localized, the others being on chromosomes *2* (*Rmp2*) and 17 (*Rmp3*), and the second virus resistance gene to be mapped near the NK gene complex. The *Cmv1* locus mediates dominant resistance to lethal mouse cytomegalovirus infection and there is evidence that it is a member of the NK gene complex (20). Mouse strains that are susceptible to lethal mousepox carry the recessive susceptibility allele Cmv^h, whereas B6 mice carry the dominant resistance allele Cmv^l (19). This raises the possibility that *Rmpl* and *Cmvl* are the same gene and that this gene is active against two large, unrelated DNA viruses.

This research was supported by USPHS grants RR02053 and RR07034.

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