NON-H-2 RESTRICTION OF EXPRESSION OF
PASSIVELY TRANSFERRED DELAYED SENSITIVITY*

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It is well established in the mouse (1–3) that the lysis of virus-infected target cells by virus-sensitized T cells is very inefficient unless target cells and T cells are compatible at the K or D region of the H-2 complex. This phenomenon is known as H-2 restriction, and it has been shown to apply to the T cell-mediated lysis of target cells displaying foreign antigens, such as haptens (4–6), minor histocompatibility antigens (7, 8), and the male specific antigen (9, 10). This and other evidence has been used to formulate a hypothesis (1) that explains the recognition of antigen by antigen-reactive lymphocytes on the basis of altered self-H-2. According to this hypothesis, extrinsic antigens are not seen as distinct entities by effector lymphocytes but are seen, instead, in combination with H-2-encoded surface molecules, either by a single recognition site or by dual recognition sites on the lymphocyte plasma membrane. Cell to cell interactions in the inductive events of the immune response are also known to be H-2 restricted in that they depend on compatibility at the I subregion of the H-2 complex (11, 12). It has been shown, moreover, that H-2 restriction of immunological function occurs in the in vivo setting. In this regard, there is evidence that the passive transfer of delayed-type hypersensitivity (DTH)[1] to certain viruses (13, 14), proteins (15, 16), and haptens (15–17) is significantly restricted when donor and recipient mice are incompatible at the K, D, or I region of the H-2 complex, depending on the model used.

Acquired immunity in the mouse to infection with the bacterial pathogen *Listeria monocytogenes* is invariably associated with the development of a state of DTH to *Listeria* antigens. Moreover, the generation and decay of DTH are concordant with the generation and loss of sensitized T cells that are capable of adoptively immunizing normal recipients against a lethal *Listeria* challenge infection. Because anti-*Listeria* immunity is ultimately expressed by activated macrophages with increased microbicidal capacity, it has been hypothesized (18–21) that the activation of host macrophages is mediated by sensitized T cells and that the events that occur at the site of a DTH reaction are identical to those that take place at foci of infection. It has been suggested that it is at the foci of infection where lymphocytes must recruit and activate macrophages for immunity to be expressed. It is important for an analysis of the expression of anti-*Listeria* immunity, therefore, to determine whether the passive transfer of delayed sensitivity to antigens of this organism is H-2 restricted, particularly

[1] *Abbreviations used in this paper:* DTH, delayed-type hypersensitivity; PBS, phosphate-buffered saline; PPD, purified tuberculin derivative.

in view of already published evidence that shows (22) that the passive transfer of protective anti-*Listeria* immunity is H-2 restricted.

We show that restriction of the passive transfer of DTH to *Listeria* antigens is not necessarily determined by incompatibility at the H-2 locus. We show that severe restriction is also observed when reciprocal passive transfer of DTH is performed between strains sharing the same H-2 haplotype and between parental strains and their F_1 hybrids.

Materials and Methods

Animals. C3H-SW/SnJ (H-2^b congenic to C3H/He), C57BL/KsJ (H-2^d), and AKR/J (H-2^k) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. C57BL/6 (H-2^b), C57BL/6 H-2K (H-2^k), DBA/2 (H-2^d), BALB/c (H-2^d), A/Tru (H-2^a), C3H/He (H-2^k), AB6F$_1$ (A/Tru × C57BL/6 Tru), CB6 (BALB/c × C57BL/6 Tru), and B6D2F$_1$ (C57BL/6 Tru × DBA/2 Tru) mice were supplied by the Trudeau Institute Animal Breeding Facility, Saranac Lake, NY. These animals were free of viral infections according to tests routinely performed by the Animal Diagnostic Testing Service of Microbiological Associates, Bethesda, MD. Male and female 6–9-wk-old mice were used in experiments. Spleen cell transfers were performed with sex-matched mice.

Adult (8–10 wk old) congenitally athymic male nude (*nu/nu*) mice and their heterozygous (*nu/+*) littermates on BALB/c background were obtained from the Trudeau Institute Animal Breeding Facility. These caesarean-derived axenic mice were maintained in sterile isolators. They were provided with sterile vitamin-enriched food and acidified water (1% HCl) *ad lib.*

Bacteria. *L. monocytogenes* (strain EGD) was grown to log phase in Trypticase-soy broth and dispensed in 1-ml volumes and stored at −70°C. For each experiment, a sample was quickly thawed and diluted in a standard fashion in 0.9% sodium chloride solution for intravenous inoculation. Bacterial growth in the spleen and liver was followed against time by plating 10-fold serial dilutions of whole organ homogenates on Trypticase-soy agar. Colonies were counted after 24-h incubation at 37°C. The intravenous inoculum was 1–2 × 10^3 bacteria in a volume of 0.2 ml of saline.

Antigens. The *Listeria* antigens used in this study were kindly provided by Dr. P. J. Patel of the Trudeau Institute. Briefly, *Listeria* was grown past log phase at 37°C in dialyzed Trypticase-soy broth. The culture was subjected to centrifugation at 12,000 *g* for 30 min to remove bacteria, and the resulting supernatant was subjected to filtration on an Amicon Filter 10 (Amicon Corp., Scientific Sys. Div., Lexington, MA) to remove material <10,000 mol wt. The resulting medium was precipitated with 75% ammonium sulfate at 4°C, and the precipitate was redissolved in distilled water and dialyzed and sterilized by filtration. It was then lyophilized. The lyophilized preparation was dissolved in an appropriate volume of phosphate-buffered saline (PBS) before use. Purified tuberculin derivative (PPD) from Parke-Davis, Detroit, MI, and *Yersinia enterocolitica* antigens were used for specificity controls. The *Yersinia* antigens were prepared from the WA strain of *Y. enterocolitica* (23). The organism was grown for 24 h at 37°C in Trypticase-soy broth. The bacterial suspension was washed three times by centrifugation and resuspended in distilled water and subjected to ultrasound for 1 min. After centrifugation, the supernatant was lyophilized for storage and redissolved in an appropriate volume of saline for injection.

Delayed Sensitivity. Delayed reactions were elicited in a right hind footpad by injecting 20 μg of *Yersinia* antigens, 20 μg of *Listeria* antigens, or 5 μg of PPD in 0.05 ml of PBS. Footpad swelling was measured against time with dial calipers (Schnelltaster, H. C. Kröplin, GMbH, Schlüchtern, Hessen, Germany) that are capable of measuring 0.05-mm increments in thickness. The injection of 20 μg of *Yersinia* antigens in the footpad of day-5 *Yersinia*-infected mice resulted in a swelling of 6 U that peaked in 6 h and almost disappeared by 24 h. The footpad injection of 5 μg of PPD resulted in a delayed hypersensitivity measuring 4 U in mice appropriately infected with Calmette-Guerin bacillus.

Adoptive Immunization. Adoptive immunization of recipient mice with spleen cells from 6-day *Listeria*-infected donors was performed as described previously (21). Briefly, immune donors were treated subcutaneously at day 5 of infection with 10,000 μg of penicillin G and given

drinking water containing 500 mg/liter of ampicillin. After 24 h, spleens were removed, diced into small pieces, and extruded gently through a 60-mesh stainless steel screen. After filtration through gauze, the spleen cells were washed twice in PBS-1% fetal calf serum, and 10^8 cells were infused intravenously into normal recipients. Spleen cell viability was >90%. The plating of homogenates of the spleen cells on Trypticase-soy agar showed that there was <100 bacteria per spleen at the time of transfer. 1 h after the spleen cell transfer, delayed sensitivity was elicited in the recipients by injecting *Listeria* antigens in the right hind footpad.

Treatment with Anti-Thy-1.2 Antibody. Lyophilized anti-Thy-1.2 monoclonal IgM antibody (Sera Lab, Accurate Chemical & Scientific Corp., Westbury, NY) was dissolved in a volume of distilled water equal to the original volume of ascites. The antibody solution was aliquoted and stored at −70°C until needed. When tested against mouse thymocytes, the antibody had a cytotoxic titer of >1:50,000. Spleen cells were treated at 5×10^7 with a 1:1,000 dilution of the antibody for 45 min at 4°C. The cells were then washed and incubated for 30 min at 37°C in the same volume of a 1:10 dilution of rabbit serum (Low-Tox-M rabbit complement, Accurate Chemical & Scientific Corp.) as a source of complement. After washing twice in PBS-fetal calf serum, the cells were resuspended to the desired concentration in PBS for intravenous infusion.

Histology. Hind footpads were fixed in 10% formalin and embedded in glycol methacrylate (24). Sections of 2-μ thickness were stained with methyl green pyronin (25).

Results

Characteristics of the Listeria DTH Reaction. Previous studies have established (21, 26) that the capacity to mount a DTH reaction to injection of *Listeria* antigens is first evident on day 2 of a sublethal *Listeria*-immunizing infection, increases progressively to peak on day 6 of infection, and then declines to a low but stable level on day 20. All of the passive transfer results presented in this paper were obtained with spleen cells harvested from donor mice at the time of peak development of DTH on day 6. Moreover, it was important to confirm, under our experimental conditions, that the inflammatory reaction to *Listeria* antigens was genuine DTH. The kinetics of a DTH reaction elicited in a hind footpad of donor mice on day 6 of infection are shown in Fig. 1. It can be seen that aside from an immediate short-lived increase in footpad thickness caused by the injection of a relatively large volume of PBS in which the antigens were dissolved, the sensitivity reaction to *Listeria* antigens did not begin to develop until between 3 and 6 h after giving antigen. The reaction increased in size to peak at 18 h and then progressively decayed. No sensitivity reaction was elicited by *Yersinia* antigens or PPD.

Confirmation that *Listeria* DTH is mediated by T cells (27, 28) is supplied in Fig. 2, where it can be seen that DTH failed to develop in *Listeria*-infected athymic nude mice. Fig. 2 shows, in addition, that the spleen cells from B6D2F$_1$ immune donors that passively transfer DTH to normal syngeneic recipients were destroyed by incubation with anti-Thy-1.2 antibody and complement. There can be little doubt, therefore, that the inflammatory reaction elicited when *Listeria* antigens are injected into a *Listeria*-sensitized mouse, displays the essential characteristics of a classical DTH reaction. Moreover, plastic-embedded sections of 18-h footpad reactions showed that the cellular infiltrate was predominantly mononuclear in nature and was almost completely devoid of basophils.

Passive Transfer of DTH between H-2-incompatible Congenic Strains Is Restricted. Evidence for H-2 restriction of the passive transfer of DTH is supplied in Table I, which shows the results of attempts to passively transfer DTH to *Listeria* antigens between two congenic strains that differ with respect to their H-2 haplotype. When reciprocal passive transfer was performed between C57BL/6^b and C57BL/6-H2K^k
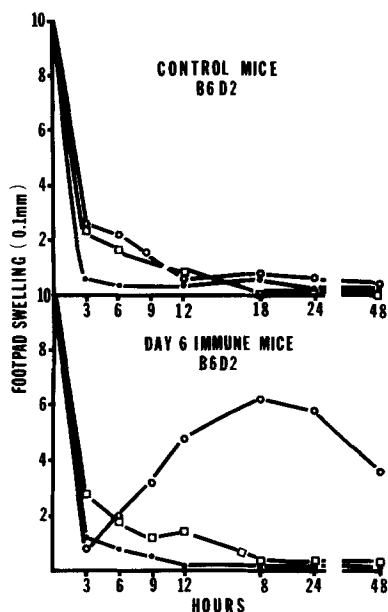
Fig. 1. The kinetics of development of the DTH reaction to *Listeria* antigens elicited in the footpads of mice on day 6 of an immunizing *Listeria* infection. Control mice of *Listeria*-immune mice were inoculated in the right hind footpad with 20 μg of *Listeria*, 20 μg of *Yersinia* antigen, or 5 μg of purified tuberculin. A DTH reaction developed only in *Listeria*-immune mice, and only in response to specific antigen. (Five mice per-group). ○, *Listeria* AG; □, *Yersinia* AG; ●, PPD.

mice, the level of DTH expressed was substantially less than that expressed when passive transfer was performed between syngeneic mice. This represents evidence, therefore, for H-2 restriction of the passive transfer of DTH. However, the results presented in the following section show that genes in addition to those of the H-2 locus can cause equally severe restriction.

Passive Transfer of DTH between Parental Strains and F_1 Hybrids and between H-2-compatible Strains. The in vitro demonstrations of H-2 restriction of T cell-mediated lysis of virus-infected or hapten-coupled target cells has been interpreted as meaning that sensitized lymphocytes only recognize antigen in conjunction with self-H-2-encoded products. This in vitro evidence seems irrevocable. It has been suggested (15) in this connection that the observed H-2 restriction of passively transferred DTH might be caused by the inability of the transferred, sensitized lymphocytes to recognize the eliciting antigen presented by H-2-incompatible recipient macrophages at the site of antigen injection. If this were so, it would follow that there should be no H-2 restriction of the passive transfer of DTH between parental strains and their F_1 hybrids, as suggested by the results of others (13, 15), or between strains sharing the same H-2 haplotype. The results in this section show that this is not the case. It can be seen first in Table II that DTH was severely restricted in its expression when it was passively transferred reciprocally between parent mice and F_1 hybrids. This restriction was evident when reciprocal passive transfer was performed between C57BL/6 mice and each of three different F_1 hybrids containing the C57BL/6 genome. Second, Table III shows that severe restriction of DTH also resulted when it was passively transferred reciprocally between strains sharing the same H-2 haplotype. In this
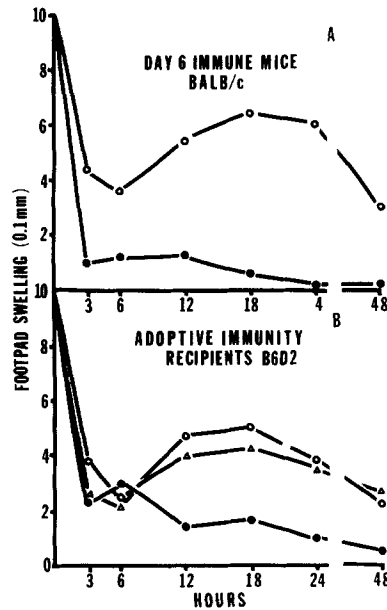
Fig. 2. Evidence that *Listeria* DTH is T cell mediated. (A) a DTH reaction to intrafootpad *Listeria* antigen could not be elicited in athymic nude mice on day 6 of a *Listeria* infection but could be elicited in their heterozygous littermates. Means of five mice. O, nu/+; ●, nu/nu (B) the cells that passively transfer DTH from *Listeria*-sensitized donors to syngeneic recipients are T cells, as evidenced by their destruction by incubation with anti-Thy-1.2 antibody plus complement. The kinetics of the adoptive DTH reaction were almost identical to those of the recipients' DTH reaction. Means of five mice. O, control cells; △, complement; ●, complement + anti-Thy-1.2.

TABLE I

Transfer of Delayed-Type Hypersensitivity between Syngeneic and Allogeneic Mice

Spleen cell transfer		DTH: 24-h footpad increase		Probability
Donor	Recipients	DTH ($\times$ 0.1 mm)	Percentage	
C57BL/6^b	C57BL/6^b	4.6 ± 0.5	100	<0.001
C57BL/6^b	C57BL/6-H2K^k	1.8 ± 0.8	39	
C57BL/6-H2K^k	C57BL/6-H2K^k	4.0 ± 0.7	100	
C57BL/6-H2K^k	C57BL/6^b	2.7 ± 0.5	68	<0.01

experiment, six donor-recipient strain combinations sharing the same H-2 haplotype displayed restriction. Therefore, so far as the results in Table III are concerned, there can be no doubt that the restriction observed in all cases was not determined by incompatibility at the H-2 locus.

Discussion

The purpose of the experiments described in this paper was to confirm, with the *Listeria* model of DTH, the published findings of others (13–17) that the expression of passively transferred DTH is H-2 restricted. Confirmatory evidence was obtained by

TABLE II

Transfer of Delayed-Type Hypersensitivity between Parents and F_1 Hybrids

Spleen cell transfer		DTH: 24-h footpad increase		
Donors	Recipients	DTH (× 0.1 mm)	Percentage	Probability
C57BL/6[b]	C57BL/6[b]	4.6 ± 0.5	100	
	AB6[ab]	1.0 ± 0.7	29	<0.001
	CB6[bd]	2.0 ± 0.7	43	<0.001
	B6D2[bd]	2.2 ± 0.6	48	<0.001
CB6[bd]	CB6[bd]	4.8 ± 0.8	100	
	C57BL/6[b]	1.8 ± 0.6	38	<0.001
B6D2[bd]	B6D2[bd]	4.6 ± 1.1	100	
	C57BL/6[b]	1.6 ± 0.5	35	<0.001
AB6[ab]	AB6[ab]	4.2 ± 0.4	100	
	C57BL/6[b]	1.8 ± 0.4	43	<0.001

TABLE III

Passive Transfer of Delayed-Type Hypersensitivity between Strains Sharing the Same H-2 Locus

Experiment	Spleen cell transfer		DTH: 24-h footpad increase		Probability
	Donors	Recipients	DTH (× 0.1 mm)	Percentage	
1	C57BL/6[b]	C57BL/6[b]	4.1 ± 0.7	100%	<0.01
	C3HSW[b]	C57BL/6[b]	2.0 ± 0.6	48%	
	C3HSW[b]	C3HSW[b]	4.3 ± 0.9	100%	<0.01
	C57BL/6[b]	C3HSW[b]	2.1 ± 0.9	49%	
2	C3H-He[k]	C3H-He[k]	3.9 ± 0.7	100%	<0.001
	AKR/J[k]	C3H-He[k]	0.8 ± 0.5	20%	
	AKR/J[k]	AKR/J[k]	3.5 ± 0.5	100%	<0.001
	C3H-He[k]	AKR/J[k]	1.1 ± 0.7	31%	
3	C57BL/6-H2K[k]	C57BL/6-H2K[k]	3.8 ± 0.6	100%	<0.001
	C3H-He[k]	C57BL/6-H2K[k]	1.2 ± 0.6	31%	
	C3H-He[k]	C3H-He[k]	3.7 ± 0.8	100%	<0.01
	C57BL/6-H2K[k]	C3H-He[k]	1.5 ± 0.7	40%	
4	C57BL/6-H2K[k]	C57BL/6-H2K[k]	3.0 ± 0.7	100%	0.01
	AKR/J[k]	C57BL/6-H2K[k]	1.3 ± 0.9	43%	
	AKR/J[k]	AKR/J[k]	3.6 ± 0.4	100%	0.02
	C57BL/6-H2K[k]	AKR/J[k]	2.2 ± 1.0	61%	
5	BALB/c[d]	BALB/c[d]	4.2 ± 0.4	100%	<0.01
	DBA/2[d]	BALB/c[d]	2.5 ± 0.7	59%	
	DBA/2[d]	DBA/2[d]	3.8 ± 0.8	100%	<0.001
	BALB/c[d]	DBA/2[d]	1.5 ± 0.5	39%	
6	C57BLKS[d]	C57BLKS[d]	3.0 ± 0.4	100%	<0.001
	BALB/c[d]	C57BLKS[d]	1.4 ± 0.5	47%	
	BALB/c[d]	BALB/c[d]	3.7 ± 1.0	100%	0.001
	C57BLKS[d]	BALB/c[d]	1.1 ± 0.6	30%	

showing that the expression of DTH to *Listeria* antigens was restricted when it was passively transferred reciprocally between C57BL/6 and congenic C57BL/6-H-2K mice. It was also found, however, that DTH was at least as severely restricted in its expression when it was transferred reciprocally between parental mice and their F_1 hybrids and between different strains of mice sharing the same H-2 haplotype. Taken together, therefore, the results presented here show unequivocally that severe restriction of the expression of passively transferred DTH can result from genetic differences in addition to those determined by the H-2 locus. It is apparent that other published studies of restriction of passively transferred DTH rarely used H-2-compatible donors and recipients with different genetic backgrounds, but used, instead, congenic strains that differ with respect only to the H-2 locus or subregions of it. An investigation of restriction with H-2-congenic strains would not reveal restriction caused by non-H-2 genes, unless these strains also differ with respect to non-H-2 genes. This must remain a real possibility, in view of the knowledge (29) that certain H-2-recombinant strains can differ with respect to genes on a long segment of chromosome 17 next to the H-2 locus as well as with respect to genes on other chromosomes (30–32).

The nature of the non-H-2 genes responsible for restriction when DTH is passively transferred between different strains with the same H-2 has yet to be determined. It is important to point out, however, that all of the strain combinations shown in Table III differed with respect to their Ig-1 loci. This could be significant, in view of recent demonstrations (33–36) that the Ig-1 locus is involved in the restriction of certain T cell-mediated functions. Again, all strain combinations in Table III, except one, have different Mls loci. Therefore, gene products of both or either of these loci might have been responsible for the restriction observed. What needs to be realized in this connection is that a failure of intravenously infused sensitized T cells to mediate a delayed inflammatory reaction at a site of extravascular antigen might result from a failure of these T cells to perform efficiently in any one of a number of intravascular functions that need to occur in sequence before the DTH reaction can develop. For example, before passively transferred sensitized T cells can react with antigen in a footpad, they must interact intimately with vascular endothelial cells to migrate actively from blood to the extravascular site of antigen. Thus, although H-2 compatibility between donor and recipient might well be needed for cellular interactions at the site of extravascular antigen, compatibility at other loci might be needed for the T cells to interact efficiently with vascular endothelium. On the other hand, if host-vs.-graft, or graft-vs.-host reactions can take place during the 24-h period of the assay, it would have the effect of significantly reducing the magnitude of the DTH reaction in H-2-incompatible recipients by reducing the number of sensitized T cells in circulation (37–39). This would also occur when F_1-sensitized T cells are infused into parental mice, although not in reverse direction. It is not the purpose of this paper, however, to explain the mechanisms responsible for allogeneic restriction of the expression of passively transferred DTH. Its purpose, instead, is to show the complexity of in vivo models of restriction of immunological function by providing evidence that severe restriction can result from non-H-2 differences between donor and recipient animals.

Summary

The results of this study of allogeneic restriction of passively transferred delayed sensitivity to *Listeria* antigens serve to illustrate the complexity of in vivo models. They

show that the H-2 restriction observed when delayed-type hypersensitivity was transferred between H-2-congenic strains was no more severe than the restriction observed when delayed-type hypersensitivity was transferred between parental and F_1 mice and between different strains sharing the same H-2 haplotype. It is obvious that genes, in addition to those of the H-2 locus, can be responsible for allogeneic restriction in vivo.

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