CYTOTOXIC T-CELL RESPONSES IN MICE INFECTED WITH INFLUENZA AND VACCINIA VIRUSES VARY IN MAGNITUDE WITH *H-2* GENOTYPE*

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Mice primed with influenza viruses generate at least two categories of cytotoxic T cells (1, 2). One component is highly cross-reactive for all type A influenza viruses (1–4), and may be specific for the shared internal matrix (M)[1] protein which is now known to be expressed on cell surface (5–7). The second set of T cells reacts only with the virus used for immunization and is probably recognizing determinents on the hemagglutinin (H) molecule (8, 9). Other virus-immune T-cell responses have been subdivided in quite a different way, on the basis of associating various specificities with the H-2K and H-2D genetic regions (10). Clonal analysis of this type has not been attempted previously for influenza, principally because we lacked a sufficient range of *H-2* different target cells which could be suitably infected with the virus. Such experiments are especially worth pursuing for the T-cell subset which is cross-reactive for all type A influenza viruses. The biological specificity and relevance of this effector population have been seriously questioned (8) though, for most investigators (2–4, 9), these lymphocytes apparently comprise the majority of the host response.

The development of SV40-transformed tumor cells from H-2 recombinant mice has now enabled us to map influenza-immune T-cell responses within the *H-2* gene complex. Two new points emerge. The first is that the H-2K^b allele is associated with a total failure in the generation of influenza-immune effector T cells. The second is the existence of a possible *Ir* gene effect that modulates cytotoxic T-cell responses to both influenza and vaccinia viruses at H-2D^b.

Materials and Methods

Mice. The CBA/J and B10.Br (kkkkkk), C57BL/6J (B6) and C57BL/10J (B10) (bbbbbb), A/J and B10.A (kkkddd), B10.D2 (dddddd), B10.A(5R) (bbbddd) and the various F1 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. The C3H.OH (dddddk) mice and some of the B10 recombinant strains, including the B10.A(2R) (kkkddb), B10.A(3R) (bbbddd), B10.A(4R) (kkbbbb), B10.A(5R) and B10.HTT (ssskkd) were either bred at the Wistar Institute or provided by Dr. G. M. Shearer of the National Cancer Institute. The BALB/c mice were purchased from the Institute for Cancer Research, Fox Chase, Philadelphia.

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[1] *Abbreviations used in this paper:* H, influenza virus hemagglutinin antigen; HA, virus hemagglutinating unit; *H-2*, mouse major histocompatibility complex; LU, lytic unit; M, influenza virus matrix protein; N, influenza virus neuraminidase antigen; PFU, virus plaque-forming unit; SV40, simian virus 40.

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Target Cells. The L cell (C3H, kkkkkk), P815 (DBA/2, dddddd), MC57G (B6, bbbbbb) and NA (A/J, kkkddd) target cells have been described previously (1, 11). Cell lines were established by transforming C3H.OH, B10.A(2R), B10.A(4R) and B10.A(5R) kidney cells with SV40 virus. These cells are referred to as C3H.OHSV, 2RSV, 4RSV, and 5RSV.

Viruses. The preparation and source of the influenza A virus strains PR8 [A/Puerto Rico/8/34 (HON1)] and HK [A/Hong Kong/× 31 (H3N2)] and the vaccinia virus (WR isolate) have been described previously (1, 2, 12). Mice were dosed intraperitoneally with from 120–300 HA U of influenza virus, the interval between primary and secondary challenge ranging from 9 to 50 days. Target cells were incubated for 1 h with 6–15 HA U per 10^6 cells. The dose of vaccinia virus given intravenously to mice was 5×10^6 plaque-forming units (PFU), and target cells were exposed to 1 PFU per cell.

Cytotoxic Assays. Alloreactive T cells were generated in vitro by using a standard technique, and effector function was determined in a conventional 6 h ^{51}Cr release assay (13). The influenza and vaccinia-immune T-cell assays have also been described previously in detail (1, 12), the lymphocytes and ^{51}Cr-labeled targets being incubated togther for 12–14 h at 37°C. Results are expressed as specific ^{51}Cr release relative to incubation in medium alone (1), or as lytic unit (LU) values calculated by the formula:

$$\frac{\text{target cells per well} \times 1{,}000}{\text{spleen cells causing 33\% specific } ^{51}\text{Cr release}}$$

The number of spleen cells causing 33% specific ^{51}Cr release was determined by graphing values for four effector:target ratios (14), usually ranging from 12:1 to 100:1.

Results

H-2 Restriction of Cross-Reactive Influenza-Immune T Cells and Absence of Response at H-2K^b. Mice were first primed with one influenza A virus, and memory T-cell populations were then challenged with a heterologous type A virus. This procedure has been shown previously to stimulate only the cross-reactive T-cell subset (1, 15). Effector lymphocytes did not discriminate between target cells infected with serologically different influenza A viruses (PR8, HON1 and HK, H3N2), but clear evidence was found for *H-2* restriction operating at the D locus (Table I). The surprise was, however, that the B10.A(5R) mice generate a strong response associated with H-2D^d, but not with H-2K^b.

TABLE I

H-2 Restriction of Cytotoxic T-Cell Activity which is Cross-Reactive for Different Influenza A Viruses

Immune spleen cells	H-2 Type		Specific ^{51}Cr release (100:1)								
			L Cells (kk)			P815 (dd)			MC57G (bb)		
	K	D	PR8*	HK	N	PR8	HK	N	PR8	HK	N
						%					
C3H.OH	d	k	30	37	1	45	45	0	0	0	0
B10.HTT	s	d	3	0	0	35	29	0	2	3	0
B10.A(5R)	b	d	0	0	0	65	61	0	2	7	0
B6	b	b	1	0	0	5	5	0	39	28	0
(BALB/c × B6) F1	d/b	d/b	6	6	1	50	45	0	26	23	0
(A/J × B6) F1	k/b	d/b	68	72	0	54	47	2	35	45	0

* Mice were primed with PR8 (HON1), challenged 9 days later with HK (H3N2), and killed after an additional 4 days.

TABLE II

Absence of Influenza-Specific T Cells at H-2K^b and Defective Responsiveness (but not Antigenicity) at H-2D^b in Recombinant Mice

Exp.	Mouse strain*	H-2		LU Values for HK (H3N2)-infected target cells‡					
		K	D	L Cell	MC57G	P815	2RSV	5RSV	NA
1	B6	b	b	<1	32	<1	25	<1	<1
	B10.Br	k	k	1,000	<1	<1	100	2	330
	B10.HTT	s	d	<1	<1	40	<1	32	50
	B10.A(4R)	k	b	500	2	<1	100	<1	250
	BALB/c	d	d	<1	<1	32	<1	16	32
	(BALB/c × B6) F1			<1	50	100	50	63	100
				L Cell	MC57G	C3H.OHSV		2RSV	5RSV
2	B6	b	b	<1	25	<1		40	<1
	B10.Br	k	k	1,000	<1	16		200	<1
	B10.D2	d	d	<1	<1	16		<1	20
	B10.A(2R)	k	b	66	<1	<1		40	<1
	B10.A(3R)	b	d	<1	<1	<1		<1	125
	B10.A(5R)	b	d	<1	<1	<1		<1	50
				L Cell	MC57G	2RSV	NA		
3	B10	b	b	<1	32	32	<1		
	B10.Br	k	k	32	<1	16	13		
	B10.A(2R)	k	b	40	<1	32	32		
	B10.A(5R)	b	d	<1	<1	<1	20		

* Mice in the top two experiments were first primed with PR8 (HON1), challenged with HK (H3N2) 1 mo later, and sampled after an additional 4 days. The order of virus administration was reversed in the third experiment.

‡ L cell, K^k-D^k; MC57G, K^b-D^d; P815, K^d-D^d; 2RSV, K^k-D^b; 5RSV, K^b-D^d; NA, K^k-D^d; C3H.OH, K^d-D^k.

Both the validity of the *H-2* restriction phenomenon and the existence of a defect at H-2K^b were confirmed in further experiments (Table II). Neither B10.A(5R) nor B10.A(3R) mice (which differ in the I-J region) generate influenza-immune T cells capable of lysing the MC57G (H-2^b) target cell. Furthermore, lymphocytes from B6 and B10 mice do not interact with influenza-infected B10.A(5R) cells. There is thus an apparent failure of both response and recognition at H-2K^b, even though the target cells express the H-2K^b alloantigen (Table III).

Differential Response to Influenza Virus at H-2D^b. The second observation of interest is that B10.A(2R) and B10.A(4R) mice do not generate a significant influenza-immune T-cell response at H-2D^b, although there is no defect at H-2K^k (MC57G and L cell targets, Table II). This differs from the situation for the B10.A(5R), however, as effector T cells from *H-2^b* mice are strongly cytotoxic for the B10.A(2R) target cell (Table II). A cold-target competitive-inhibition experiment further established that suitable antigenic determinants are expressed at H-2D^b on the virus-infected MC57G (H-2^b) target, which are recognized by B6 influenza-immune T cells (Fig. 1). Thus, there is a failure of responsiveness when H-2D^b is associated with *H-2K^k* and with *I-A^k*, but not with *H-2K^b* and *I-A^b*.

TABLE III

Detection of H-2K^b and H-2D^b Antigens by Alloreactive T Cells

Effector population		Ratio*	Specific ^{51}Cr release %		
Stimulator	Responder		MC57G K^b-D^b	5RSV K^b-D^d	P815 K^d-D^d
B10.A(5R) (K^b-D^d)	B10.A (K^k-D^d)	20:1	65	26	0
		5:1	35	14	0
B10.A(2R) (K^k-D^b)	B10.A (K^d-D^d)	20:1	70	0	10
		5:1	38	0	3

* Killer:target ratio in a 6-h ^{51}Cr release assay (13).

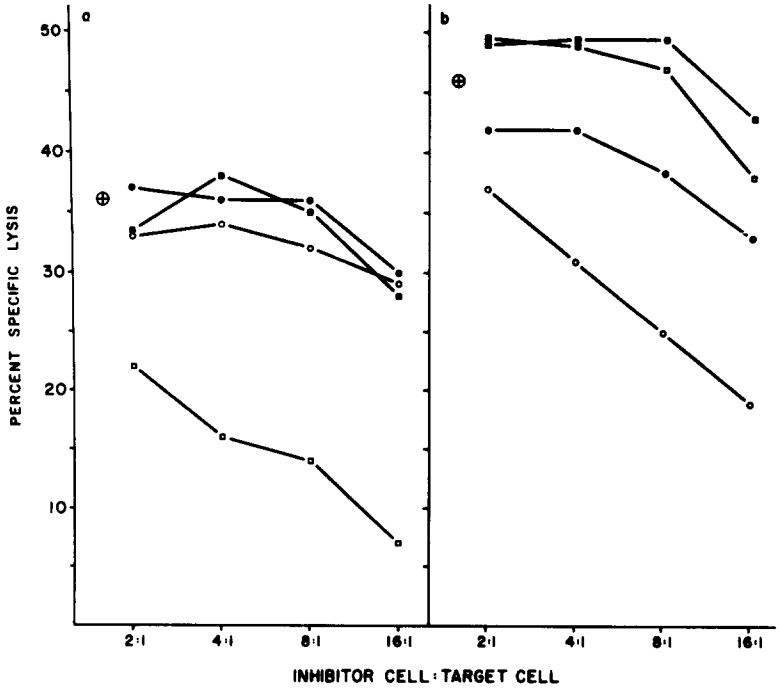

FIG. 1. Secondary B6 (K^b-D^b) and (BALB/c × B6) F1 (dd × bb) influenza-immune T cells were generated by priming with PR8 influenza virus and challenging 6 wk later with the HK influenza virus. Effector spleen populations were tested 4 days later in a cold-target competitive inhibition assay (1, 2). (a) B6 effectors assayed on ^{51}Cr-labeled HK-infected MC57G (bb) target cells (100:1). (b) (BALB/c × B6) F1 effectors assayed on ^{51}Cr-labeled HK-infected NA (kd) target cells (75:1). Competitor cells were uninfected (●, ■) or HK-infected (○, □) 2RSV (■, □) (kb), and 5RSV (●, ○) (bd). ⊕ indicates lysis in the absence of any competitor cells. The obvious inhibition of cytotoxicity on MC57G cells caused by virus-infected 2RSV, but not 5RSV, competitors indicate that this *H-2^b* target is lysed by virus-immune T-cell populations recognizing H-2D^b. Similarly, both 5RSV and NA are shown to be recognized by cytotoxic T cells operating at H-2D^d, a point which is necessary for control purposes but is already established in Tables I and II.

TABLE IV

Vaccinia-Immune T-Cell Response is Found Reciprocally for H-2K^b but not for H-2D^b

Exp.	Mouse strain*	H-2 Type		LU Values for vaccinia-infected targets					
		K	D	L Cell kk	MC57G bb	P815 dd	2RSV kb	4RSV kb	5RSV bd
1	B10	b	b	3	50	2	20	–	50
	B10.Br	k	k	200	2	<1	125	–	<1
	B10.D2	d	d	3	2	40	3	–	50
	B10.A(2R)	k	b	170	<1	<1	170	–	<1
	B10.A(4R)	k	b	125	2	<1	250	–	2
	B10.A(5R)	b	d	2	50	8	3	–	100
2	B10.A(2R)	k	b	40	<1	–	20	20	<1
	B10.A(5R)	b	d	<1	13	–	<1	<1	25

* Mice were primed i.v. with vaccinia virus 6 days previously.

Vaccinia Virus and the H-2^b Haplotype. Control experiments were done with vaccinia virus to determine whether MC57G and the SV40-transformed B10.A(5R) cell line could act as targets at H-2K^b for any virus system. A strong vaccinia-immune cytotoxic T-cell response was seen at both H-2K^b and H-2D^b in the B10 strain (Table IV). However, an identical defect to that found in the influenza model was observed for both the B10.A(2R) and B10.A(4R). Little, if any, effector function was recognized for H-2D^b in these H-2 recombinant strains, though there was no failure in expression of virus-induced antigenic changes associated with H-2D^b in the B10.A(2R) target.

Two possible explanations were considered for the *K, I-A*-related defect modifying the response at H-2D^b. The first is that genes in the K-end in some way influence generation of the receptor repertoire (16) of the T-cell clone(s) associated with H-2D^b. The second is that helper T cells operating at I-A^b, but not at I-A^k, function to facilitate the cytotoxic T-cell response associated with H-2D^b (17, 18). The latter seems more likely to be true. Normal spleen and lymph node cells from (A/J × B6) F1 mice can be stimulated to generate cytotoxic T cells at H-2D^b when exposed to vaccinia virus in an irradiated B6, but not in a B10.A(4R) recipient (Table V). These F1 responder T cells can thus functionally interact with vaccinia virus in the context of H-2D^b, but cannot be stimulated in a K^{k}I-A^{k}D^b environment. This experiment also shows that the defect associated with K^k and I-A^k is recessive, a point which we could not otherwise establish with the available target cells.

Discussion

Secondary influenza-immune T-cell responses, which may be specific for the shared M protein determinant (5–7), exhibit the same *H-2* restriction spectrum found previously for other virus systems (19). The total absence of responsiveness associated with H-2K^b is, however, unique for the conventional infectious viruses studied so far (20), though similar phenomena have been described for the murine tumor viruses and for the male H-Y antigen (21–23). Little can be said concerning the possible mechanism, as we do not know whether there is a

TABLE V

Stimulation of F1 Lymphocytes with Vaccinia Virus in Parental or B10.A(4R) Recipients

Transferred population*	Irradiated recipient	Specific ^{51}Cr release (50:1)‡							
		L Cell (kk)		MC57G (bb)		2RSV (kb)		5RSV (bd)	
		Inf.	N	Inf.	N	Inf.	N	Inf.	N
		%							
(A/J × B6) F1	B6	22	21	73	0	64	13	68	0
(K^k-D^d ×	A/J	83	21	6	0	61	14	58	0
K^b-D^b)	B10.A(4R)	81	16	15	6	66	7	0	0

* Mice were irradiated (950 rads), given 6.0×10^7 spleen and lymph node cells i.v. 18 h later and dosed i.v. with vaccinia virus after an additional 3 h.

‡ Target cells were either infected with vaccinia virus (Inf.) or uninfected (N).

defect in the T-cell receptor repertoire or if antigen is presented in an inadequate way. Evidence to support the latter idea is available from the Friend leukemia model, where *H-2* gene products associated with responsiveness are selectively incorporated into the virion (21).

In either case, the existence of such a defect associated with a potentially lethal (and highly contagious) virus disease strengthens earlier speculation that infectious processes have exerted major pressures on the evolution of H-gene polymorphism (24). Both heterozygosity at a particular *H-2* locus, and the existence of a duplicated system at *H-2K* and *H-2D*, would operate to minimize the possibility of a total lack of T-cell responsiveness to any given virus. Such a concept is, as pointed out before (19), equally valid for altered-self and for dual recognition models.

Earlier experiments with ectromelia virus, which (unlike influenza) is a major cause of disease in laboratory mice, failed to detect any *H-2*-related failure in T-cell responsiveness (10, 20). Alleles associated with defective response may thus have been selected out. Wild mice normally exist in environmentally restricted "demes," that exhibit a much more limited range of *H-2* types than is recognized for the species as a whole (25). Endemic infectious processes may also observe distinct boundaries for susceptible species that live in close proximity but have little real contact as documented, for instance, for leptospirosis in the Malayan rain forest (26). Selection may thus operate to maximize the frequency of genes associated with resistance to the set of pathogens encountered commonly in a particular environmental niche. A novel pandemic may eliminate some demes, but leave many intact. Selection for polymorphism would thus operate at the total population level. The situation for a more widely ranging species, such as man, may be quite different. Evolutionary pressures may favor heterozygosity, and thus polymorphism, in the population at large (24).

The absence of significant virus-immune T-cell generation at H-2D^b in B10.A(4R) (kkbbbb) but not in B10 (bbbbbb) mice probably represents an *Ir*-gene effect, similar to those described for responses to trinitrophenyl-modified cells and to the male Y antigen (22, 27). The H-2 recombinants necessary for

distinguishing whether the *K* or the *I-A* locus is involved do not yet exist. However, the phenomenon is seen for both the infectious processes studied. These systems differ in that mice primed with vaccinia virus, but not with influenza virus, generate a strong cytotoxic T-cell response associated with H-2K^b. It thus seems likely that any collaboration between proliferating T-cell subsets (17, 18) would involve I-A^b rather than K^b.

Exposure to vaccinia virus leads to generation of potent effector T cells at K^k but not at D^b in the B10.A(4R) environment, even when the lymphocyte population stimulated (A/J × B6) F1 is capable of responding at D^b in a B6 recipient and the relevant antigen is expressed on B10.A(4R) target cells. The defect is thus in the capacity to stimulate. Two quite different explanations may be proposed. One possibility is that the T-cell operating at D^b has receptors for both I-A^b and D^b, as well as for viral antigen, and all of these are required for induction of the response but only the latter two are necessary for mediating cytotoxic effector function. The Ia in question may be expressed on macrophages or on a secreted factor (28).

The alternative idea that T-T help (17, 18) is involved raises questions concerning the specificity of the helper T-cell subset. The vaccinia- (and influenza-) immune T-cell response at K^k is potent in the B10.A(4R). Thus, if T-T help is mandatory for all cytotoxic T-cell responses (17, 18), a helper population exists which can facilitate generation of effector T cells at K^k but not at D^b. How might such discrimination operate?

One possibility is that the helper T cells are recognizing idiotype. However, the non-H-2 genetic background is the same for B10.A(4R) and B10 so the lymphocyte receptor idiotypes (29, 30) specific for viral components should be identical for cytotoxic T cells functioning at K^k, K^b, and D^b, unless we are considering recognition of distinct altered-self structures. Perhaps helper T cells which interact with the self-recognition component operating at K^k, but not at D^b, are generated in the context of I-A^k. This would explain why the response to both influenza and vaccinia viruses is depressed. A further possibility is that the helper T cell shows specificity for Ia determinants which are expressed on the virus-immune population functioning at K^k but not at D^b. We know of no information concerning such an exclusion of Ia presentation on T-cell subsets. However, the presence of I-region antigens on thymus-derived lymphocytes is now established (31), and helpers and suppressors are known to express I-A and I-J coded components, respectively (32).

Summary

Secondary effector T-cell populations generated by cross-priming with heterologous influenza A viruses operate only in H-2K or H-2D compatible situations, when assayed on SV40-transformed target cells infected with a range of influenza A viruses. The H-2K^b allele is associated with a total failure in the generation of influenza-immune cytotoxic T cells, though this is not seen for the primary response to vaccinia virus. In both influenza and vaccinia development of effector T cells operating at H-2D^b is greatly depressed in B10.A(2R) (kkkddb) and B10.A(4R) (kkbbbb), but not in B10 (bbbbbb), mice. However, there is no defect in viral antigen expression at either H-2K^k or H-2D^b in B10.A(2R) target

cells. This apparently reflects some inadequacy in the stimulator environment, as (A/J × B6) F1 T cells can be induced to respond at H-2D^b when exposed to vaccinia virus in an irradiated B6 but not in a B10.A(4R) recipient. The present report, together with the accompanying paper by Zinkernagel and colleagues, records the first rigorous demonstration of both a nonresponder situation and a probable *Ir*-gene effect for conventional infectious viruses. Possible implications for the evolution of *H-2* polymorphism and mechanisms of *Ir* gene function are discussed.

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