GENETIC CONTROL OF CYTOLYTIC T-LYMPHOCYTE RESPONSES

II. The Role of the Host Genotype in Parental → F_1 Radiation Chimeras in the Control of the Specificity of Cytolytic T-Lymphocyte Responses to Trinitrophenyl-Modified Syngeneic Cells*

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Several instances of Ir gene control of specific cytolytic T lymphocyte (CTL)[1] responses have been reported. Simpson and Gordon (1) and von Boehmer et al. (2) described two complementing Ir genes that control the CTL response to the HY antigen and map in the I region of the H-2 complex. Schmitt-Verhulst and Shearer (3) observed that spleen cells of mice bearing the H-2^k haplotype develop CTL to trinitrophenyl (TNP)-conjugated syngeneic cells that are specific only for modifications of the H-2K but not H-2D region gene products. This response of H-2^k mice is under the dominant control of H-2-linked Ir genes. In the preceding report (4), we have investigated another genetic defect in the CTL primary response of H-2^k mouse spleen cells to TNP-conjugated cells in vitro. H-2^k spleen cells, in contrast to cells of other haplotypes, develop primary CTL that react solely with TNP-modified syngeneic cells and do not cross-reactively lyse TNP-modified allogeneic spleen target cells. The ability to produce cross-reactive CTL in response to TNP-conjugated H-2^k cells is dominant in spleen cells from (H-2^k × H-2^b)F_1 hybrid mice and does not reflect the inability of TNP-conjugated H-2^k cells to stimulate cross-reactive lysis by the appropriate responder-cell population.

The relevant issue in systems that display major histocompatibility complex (MHC) control of CTL responsiveness is whether the restrictions observed are dictated by the H-2 genes of the responding T cell or by the H-2 gene products in the thymus where T cells differentiate and develop immunocompetence. Bevan (5) and Zinkernagel and associates (6, 7) have independently investigated this important question for CTL responses to cells bearing allogeneic minor histocompatibility antigens or virally infected cells. Such responses are normally restricted to produce CTL that preferentially lyse syngeneic target cells bearing the appropriate antigens. By using semiallogeneic radiation chimeras or thymus grafts, these investigators have observed that the H-2-controlled genetic

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[1] *Abbreviations used in this paper:* B6, C57BL/6; CTL, cytolytic T lymphocytes; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; TNP, trinitrophenyl.

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restrictions that govern the specificity of CTL to virally infected syngeneic cells or to cells bearing minor histocompatibility antigens are not dictated by the genome of the responding cell but rather by the environment where the cell differentiated and, in the studies of Zinkernagel et al. (6, 7), by the H-2 antigens of the thymus graft.

The system described in the accompanying paper (4), where the Ir gene-controlled cross-reactive primary CTL response to TNP-modified syngeneic cells was investigated, is well suited to study the role of the host H-2 antigens on the fine specificity of the T-cell clones that develop during differentiation. Taking into account that primary CTL induced to TNP-modified syngeneic cells from C3H ($H\text{-}2^k$) mice are unable to lyse TNP-modified allogeneic targets, bone marrow cells from "nonresponder" C3H mice were allowed to differentiate in lethally irradiated "responder" $(B6 \times C3H)F_1$ ($H\text{-}2^{b/k}$) recipients. The ability of these chimeric C3H cells [$C3H \rightarrow (B6 \times C3H)F_1$] to display cross-reactive lysis after primary stimulation with TNP-modified C3H cells was assessed. The C3H ($H\text{-}2^k$) CTL which differentiated in a $(B6 \times C3H)F_1$ environment shared similar levels of cross-reactive lysis as the $(B6 \times C3H)F_1$ CTL, demonstrating the effect of the environment where T cells differentiate on the specificity of the primary CTL response to TNP-modified MHC antigens.

Materials and Methods

Mice. 6- to 12-wk-old male mice were used in this study. C57BL/6 (B6), $H\text{-}2^b$; BALB/c, $H\text{-}2^d$; C3H/HeJ, $H\text{-}2^k$; DBA/1, $H\text{-}2^q$; and $(B6 \times C3H)F_1$, $H\text{-}2^{b/k}$ hybrid mice were obtained from The Jackson Laboratory (Bar Harbor, Maine).

Preparation of Chimeras. The general method of Sprent et al. (8) was followed. Bone marrow cells were isolated from B6, C3H, and $(B6 \times C3H)F_1$ mice and washed several times. They were then treated twice with anti-Thy 1.2 antiserum and rabbit complement. 1.5×10^7 treated B6, C3H, or $(B6 \times C3H)F_1$ bone marrow cells were then injected i.v. into lethally X-irradiated (675 R)$(B6 \times C3H)F_1$ hybrid mice. All irradiated mice receiving no bone marrow cells died within 1 wk. F_1 recipient animals were isolated 2 days before irradiation and maintained on antibiotics (Neosporin; Burroughs Wellcome Co., Research Triangle Park, N. C.) for 1 mo after irradiation. Survival was 80–90% in all groups receiving bone marrow cells. Mice were histocompatibility typed and used for study 12–16 wk after irradiation.

H-2 Typing. Spleen cells from each radiation chimera were tissue typed by the microcytotoxicity assay (9) using anti-$H\text{-}2^b$ [$(B10.A \times A)F_1$ anti-B10] and anti-$H\text{-}2K^k$ [$(B10 \times LP.RIII)F_1$ anti-2R] antisera plus complement. The percentage of F_1 lymphocytes present in the spleen of all C3H $\rightarrow (B6 \times C3H)F_1$ radiation chimeras was $<2\%$ (which equals the lysis obtained with normal mouse serum in controls).

Mixed Lymphocyte Culture (MLC). 5×10^5 responder spleen cells were cultured in flat-bottomed microtiter wells (Linbro Chemical Co., New Haven, Conn.) in the presence of 10^6 irradiated (1,500 R) splenic stimulator cells. Cells were cultured in RPMI 1640 medium supplemental with 0.5% normal mouse serum, 2 mM glutamine, 100 U/ml of penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol. After 48 h cells were pulsed for 6 h with 0.25 ml of a solution containing [^{3}H]thymidine (40 mCi/ml). Acid-precipitable DNA was isolated and incorporated counts measured on a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

CTL Induction and the ^{51}Cr Release Assay. Induction of CTL to allogeneic or TNP-modified syngeneic cells, the ^{51}Cr release assay, and statistical methods are described in the preceding report (4). In some experiments, CTL effectors were treated with anti-H-2 antiserum and complement before incubation with ^{51}Cr-labeled target cells. They were incubated for 45 min at room temperature with specific anti-H-2 antiserum, washed, and then incubated for 45 min at 37°C with a 1:5 dilution of preselected rabbit complement. Cells were then washed several times in DNase containing media, counted, and diluted appropriately. The specificity and concentration

TABLE I
Mixed Lymphocyte Responses of Radiation Chimeras

Responder cells	Stimulator cells	Net incorporation*	Stimulation index‡
		cpm	
C3H	C3H	0	1.0
C3H	B6	11,825	12.0
C3H	BALB/c	14,932	15.0
C3H → (B6 × C3H)F	C3H	0	1.0
C3H → (B6 × C3H)F_1	B6	−47	0.9
C3H → (B6 × C3H)F_1	BALB/c	10,995	17.0

* Represents incorporated counts per minute (cpm) in a typical experiment. Counting error was usually <15%. Data represent triplicate cultures with standard error <15% within groups. Control groups included: C3H anti-C3H:1,046 cpm, C3H → (B6 × C3H)F_1 anti-C3H:672 cpm, and (B6 × C3H)F_1 → (B6 × C3H)F_1 anti-C3H:831 cpm. Net incorporated cpm was computed according to the equation: experimental group cpm minus syngeneically stimulated group cpm.

‡ Computed according to the equation: experimental group cpm divided by control group (stimulated with syngeneic cells) cpm.

of antisera and complement that were used are listed in Table IV. Conditions used for antiserum plus complement lysis yielded >95% lysis of appropriate targets as measured by dye exclusion.

Results

MLC Responses of Normal and Chimeric Lymphoid Cells. Table I shows an experiment in which the MLC responsiveness of normal and chimeric lymphoid cells is compared. It can be seen that chimeric C3H → (B6 × C3H)F_1 lymphoid cells are unresponsive to either C3H or B6 stimulator cells but are stimulated by BALB/c cells bearing the unrelated H-2^d haplotype.

Normal C3H lymphoid cells responded to both B6 and BALB/c stimulator cells. Thus, by the criteria of MLC responsiveness, chimeric C3H are tolerant to B6 antigens.

CTL Responsiveness of Normal and Chimeric Spleen Cells. Normal C3H spleen cells can be induced by allogeneic cells to lyse H-2^b or H-2^d tumor or normal spleen targets; however, chimeric C3H → (B6 × C3H)F_1 spleen cells do not lyse H-2^b targets while lysing H-2^d targets well (Table II). Though unable to respond to H-2^b stimulator cells, the chimeric C3H cells were able to respond to TNP-modified B6 stimulator cells. Chimeric C3H cells also responded to TNP-modified C3H cells.

Cross-Reactive Lysis by CTL from Normal and Chimeric Mice. We next asked whether the ability to lyse TNP-modified allogeneic targets after stimulation with TNP-modified syngeneic cells was altered in the C3H → (B6 × C3H)F_1 chimeric mice. Results shown in Table III demonstrate, as in the preceding report (4), that normal C3H CTL induced by TNP-modified syngeneic cells only lyse TNP-coupled syngeneic cells, i.e. they do not demonstrate cross-reactive lysis on TNP-coupled allogeneic cells.

When C3H → (B6 × C3H)F_1 spleen cells are stimulated by TNP-modified C3H spleen cells, CTL are produced that lyse TNP-coupled syngeneic (C3H) and

TABLE II
Cytolytic Activity of CTL from Normal and Chimeric Mice

Group	Responder cells	Stimulator cells	Target cells*	Percent specific release‡
I	C3H	B6	B6	32
	C3H	BALB/c	BALB/c	24
	C3H → (B6 × C3H)F_1	B6	B6	5
	C3H → (B6 × C3H)F_1	BALB/c	BALB/c	25
II	C3H	B6TNP	B6	32
	C3H → (B6 × C3H)F_1	B6TNP	B6	1
	C3H → (B6 × C3H)F_1	B6TNP	B6TNP	30
III	C3H	C3H TNP	C3H	0
	C3H	C3H TNP	C3H TNP	63
	C3H → (B6 × C3H)F_1	C3H TNP	C3H	0
	C3H → (B6 × C3H)F_1	C3H TNP	C3H TNP	53

* Spontaneous release from spleen targets was <40%.
‡ Effector to target ratios used: group I, 15:1; group II, 30:1; and group III, 80:1.

TABLE III
Cross-Reactive Lysis by CTL from Radiation Chimeras

Group	Responder cells	TNP-modified stimulator cells	TNP-modified target cells*	Percent cross-reactive lysis‡
I	B6 (H-2^b)	B6	B6	100 (50)
	B6	B6	BALB/c (H-2^d)	45
	C3H (H-2^k)	C3H	C3H	100 (50)
	C3H	C3H	BALB/c	10
	C3H → (B6 × C3H)F_1	C3H	C3H	100 (50)
	C3H → (B6 × C3H)F_1	C3H	BALB/c	40
II	C3H	C3H	C3H	100 (50)
	C3H	C3H	DBA/1 (H-2^q)	4
	C3H → (B6 × C3H)F_1	C3H	C3H	100 (50)
	C3H → (B6 × C3H)F_1	C3H	DBA/1	30
III	C3H	C3H	C3H	100 (50)
	C3H	C3H	B6 (H-2^b)	2
	C3H → (B6 × C3H)F_1	C3H	C3H	100 (50)
	C3H → (B6 × C3H)F_1	C3H	B6	50

* Spontaneous release of normal spleen targets was <40%.
‡ In group I, effector to target ratios of 40:1 for B6 and C3H, and 80:1 for C3H → (B6 × C3H)F_1 CTL were used. In groups II and III effector to target ratios of 48:1 for C3H and 40:1 for C3H → (B6 × C3H)F_1 were used. Numbers in parentheses represent percent specific release on syngeneic TNP-modified target cells.

TNP-coupled allogeneic (BALB/c, B6, DBA/1) targets. Therefore, the specificity of CTL induced by TNP-modified syngeneic antigens is altered when C3H cells differentiate in a (B6 × C3H)F_1 environment.

H-2 Type of Chimeric Cross-Reactive CTL. Finally, we asked whether a

TABLE IV

Characterization of CTL from Radiation Chimeras by Anti-H-2 Serum

Experiment	Responder cells	TNP-modified stimulator cells	TNP-modified target cells*	Antiserum plus complement treatment of CTL‡		
				Control	Anti-$H\text{-}2^b$	Anti-$H\text{-}2^k$
					%	
1	B6 ($H\text{-}2^b$)	B6	B6	30	3	–
	B6	B6	BALB/c ($H\text{-}2^d$)	20	0	–
	C3H ($H\text{-}2^k$)	C3H	C3H	42	–	–
	C3H	C3H	BALB/c	1	–	–
	C3H → (B6 × C3H)F_1	C3H	C3H	48	48	–
	C3H → (B6 × C3H)F_1	C3H	BALB/c	11	14	–
2	B6	B6	B6	36	0	33
	C3H	C3H	C3H	56	–	–
	C3H	C3H	DBA/1 (H-2)	1	–	–
	C3H → (B6 × C3H)F_1	C3H	C3H	33	30	0
	C3H → (B6 × C3H)F_1	C3H	DBA/1	18	21	0

* Spontaneous release of normal spleen targets was <40%.

‡ Data is percent specific release. Effector to target ratio in experiment 1 was 40:1 and experiment 2, 50:1. Control represents percent specific release of untreated effector cells. Anti-$H\text{-}2^b$ serum: (A × B10.A)F_1 anti-B10 and anti-$H\text{-}2^k$ serum: (B10 × LP.RIII)F_1 anti-B10.A(2R) were used at dilutions of 1:2 to lyse effector cells.

small number of residual (B6 × C3H)F_1 CTL could explain the cross-reactive lysis produced in C3H → (B6 × C3H)F_1 chimeric mice. Results in Table IV demonstrate that anti-$H\text{-}2^b$ antiserum plus complement treatment, although it removes 100% of the B6 CTL which lyse TNP-coupled allogeneic targets after TNP-modified B6 spleen-cell stimulation, does not affect the cross-reactive lysis produced by C3H → (B6 × C3H)F_1 CTL induced by TNP-modified C3H stimulators. Anti-$H\text{-}2^k$ antiserum plus complement treatment completely eliminates functional cross-reactive CTL from the chimeric CTL population. When these data are considered along with the H-2 typing data, showing that >98% of all spleen cells from C3H → (B6 × C3H)F_1 mice bear $H\text{-}2^k$ antigens before CTL induction, it is clear that the cross-reactive lysis seen when C3H → (B6 × C3H)F_1 spleen cells are induced by TNP-modified C3H stimulators is due to CTL of C3H origin, and not to contaminating F_1 cells.

Discussion

Bone marrow cells from $H\text{-}2^k$ C3H mice have been allowed to mature in an irradiated ($H\text{-}2^{b/k}$) (B6 × C3H)F_1 recipient. Spleen cells from these chimeric mice, which were of C3H origin, were found to be tolerant to B6 alloantigen in both the mixed lymphocyte reaction and by cell-mediated cytotoxicity. These chimeric spleen cells when stimulated in vitro with TNP-modified syngeneic cells now demonstrated cross-reactive lysis of TNP-modified allogeneic targets (i.e. TNP-BALB/c, TNP-DBA/1, and TNP-B6 targets). The effector cells exhibiting cross-reactive lysis were only susceptible to lysis by anti-$H\text{-}2^k$ antiserum

plus complement and were unaffected by anti-H-2^b antiserum plus complement treatment; thus eliminating any role for contaminating F_1 effector cells.

The data therefore demonstrate that the pattern of CTL responsiveness generated by primary immunization with TNP-modified syngeneic cells can be altered by the environment in which the T cell differentiated. In these experiments, C3H T cells, which normally are unable to exhibit cross-reactive lysis after primary in vitro immunization with TNP-modified syngeneic cells, exhibit the cross-reactive phenotype if they differentiated in a host that does exhibit cross-reactive lysis.

These findings are in agreement with recent observations of Zinkernagel et al. (6, 7) and Bevan (5). Zinkernagel et al. demonstrated that T cells from radiation chimeras develop virus-specific CTL only when presented with virally infected cells that carry the H-2 antigens of the irradiated recipients. From studies with thymus grafts, Zinkernagel et al. (6, 7) were able to conclude that the radioresistant portion of the thymus bears the H-2 antigens involved in restricting virus-specific killer T-cell precursors. Though the influence of the chimeric host on the specificity of CTL to TNP-modified syngeneic antigens is quite apparent from our studies, we cannot conclude that this effect is due to the host thymus without further studies involving thymic transplants.

Inasmuch as macrophages have been demonstrated to be involved in the induction of immune responses in many systems (10), the possibility that residual (B6 × C3H)F_1 macrophages are responsible for our results must be entertained. It is unlikely, however, that this is the case. Zinkernagel et al. (7) demonstrated that, in long-term irradiation bone marrow chimeras, the lymphoreticular system had been replaced by cells derived from the reconstituting stem cells. In fact, Zinkernagel was required to adoptively transfer chimeric spleen cells into a recently irradiated recipient and then virally infect in order to utilize the host lymphoreticular system for viral antigen presentation. Because we waited 3–4 mo before testing our radiation chimeras, it is unlikely that F_1 macrophages continued to populate the spleen.

Our results could also be explained by loss of a regulatory cell that normally restricts the response to C3H CTL, i.e. suppressing cross-reactive lysis. It could be argued that C3H bone marrow cells developing in a (B6 × C3H)F_1 environment lose these clones of regulatory cells. However, attempts at demonstrating such a regulatory cell so far have been unsuccessful; specifically, treatment of C3H mice with cyclophosphamide or adult thymectomy does not appear to reverse the H-2 restriction (data not shown).

It is still not clear how the host environment, presumably the H-2 antigens of the thymus, influences T-cell maturation to produce the phenotypic response pattern characteristic of the adult animal. Jerne (11) originally proposed that the earliest antigen receptors expressed on lymphocytes are specific for self-H-2 antigens. Precursors of CTL presumably express receptors for self-H-2K and H-2D, and proliferate in the thymic environment in response to these MHC products. During this proliferation, the receptors of these CTL precursors are postulated to undergo either selectional and/or mutational changes, ultimately yielding the fully mature receptor pool with preferential specificity for slight variants of the original self-MHC products as discussed previously (12–16). Although not fully documented, this hypothesis can explain both the underlying

basis for the hierarchy of allogeneic, xenogeneic, and modified self-responses, as well as the responses of chimeric mice to modified self-antigens. Furthermore, this model may have important implications for humoral or DTH-type responses for which responder status is regulated by I region genes. It is still an open issue whether some types of Ir gene control are expressed directly in the T cell or indirectly by I region genes in the thymus, affecting the differentiation of T-cell precursors in a manner similar to H-2K and H-2D for CTL. This latter mechanism may in some cases be interpreted as Ir gene expression in the macrophages or B cells with which the T cell must interact via antigen and Ia. However, we favor the alternative that in many instances Ia antigens themselves on macrophages and/or B cells control T-cell antigen recognition independently of receptor repertoire regulation in the thymus. These issues, which pertain to both K-, D-, and I-restricted responses, remain to be resolved by a combination of the current approach using chimeras and careful cell separation and mixing experiments to define the cell(s) involved in the altered response pattern of lymphocytes whose precursors have differentiated in a "nonself" environment.

Summary

Bone marrow cells from C3H (H-2^k) mice, a strain that does not exhibit cross-reactive lysis of trinitrophenyl (TNP)-modified allogeneic targets, were allowed to mature in heavily irradiated (B6 × C3H)F_1 (H-$2^{b/k}$) recipients, an F_1 hybrid that does demonstrate cross-reactive lysis. Spleen cells from these chimeric mice were removed after 3–4 mo and by H-2 typing shown to be of C3H origin. These cells were found to be tolerant to B6 alloantigens by mixed lymphocyte reaction and cell-mediated cytotoxicity and, when stimulated in vitro with TNP-modified syngeneic cells, now cross-reactively lysed TNP-modified allogeneic targets. These studies demonstrate that the host environment where T cells differentiate influences the specificity of the primary cytolytic T-lymphocyte (CTL) response to TNP-modified syngeneic antigens.

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