ALLORESTRICTED CYTOTOXIC T CELLS

Large Numbers of Allo-H-2K^b-restricted Antihapten and Antiviral Cytotoxic T Cell Populations Clonally Develop In Vitro from Murine Splenic Precursor T Cells

By JÖRG REIMANN, DIETER KABELITZ, KLAUS HEEG, AND HERMANN WAGNER

From the Department of Medical Microbiology and Immunology, University of Ulm, Ulm, Federal Republic of Germany

T cells recognize 'foreign' antigens in the context of a private specificity within the first or second external domain of a class I or II major histocompatibility complex (MHC)[1] molecule. The large diversity of receptor specificities clonally expressed by functional T cells is thought to be selected during the ontogeny of the individual in such a way that only self-tolerant and self-restricted receptor phenotypes appear among mature T cells (reviewed in 1). This view does not explain the presence of allorestricted receptor phenotypes in the peripheral T cell pool; the finding of measurable numbers of these receptor specificities among mature T cells would thus be as enigmatic as the presence of alloreactivity itself.

Two general strategies have been used to detect allorestricted T cells in various in vitro systems. (*a*) Alloreactive T cells were removed before coculture of the responding T cells with antigen-bearing stimulator cells of the respective allogeneic H-2 type (2–6); the specific allorestricted T cell reactivity thus generated was often considerably lower than the reactivity towards the respective antigen presented by syngeneic stimulator cells (4, 5), and some investigators have even failed to detect such allorestricted receptor phenotypes (7, 8). (*b*) Alternatively, allorestricted T cell reactivities were generated directly from nonfractionated peripheral T cell populations in limiting dilution systems (9, 10); these studies have demonstrated substantial numbers of allorestricted peripheral T cells by specificity analyses at the clonal level that distinguished alloreactive and allorestricted receptor phenotypes. We have chosen the latter experimental approach to demonstrate a surprisingly large pool of splenic precursor cells in the mouse that could clonally develop into cytotoxic T lymphocyte (CTL) populations with specific allorestricted lytic reactivity in a modified limiting dilution system in vitro (11). Under the in vitro conditions used, estimated frequencies of precursors

This work was supported by the Deutsche Forschungsgemeinschaft (SFB112). Address correspondence to J. Reimann, Department of Medical Microbiology and Immunology, University of Ulm, Oberer Eselsberg, D-7900 Ulm (Donau), Federal Republic of Germany.

[1] *Abbreviations used in this paper:* Con A, concanavalin A; CLP, cytotoxic lymphocyte precursor; CTL, cytotoxic T lymphocyte; FCS, fetal calf serum; HSV, herpes simplex virus; LDA, limiting dilution analysis; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MLR, mixed leukocyte response; moi, multiplicity of infection; PBS, phosphate-buffered saline; TNP, trinitrophenyl.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/85/08/0592/15 $1.00
Volume 162 August 1985 592–606

for CTL populations inducible in a primary in vitro mixed leukocyte reaction (MLR) to allo-H-2 plus hapten, ranged from 1/30 to 1/300 in the six investigated allogeneic strain combinations set up with CBA, BALB/c, and C57BL/6 mice. Split-well analysis revealed that one-third to three-fourths of the generated CTL populations (selected for a high probability of clonality) lysed the respective trinitrophenyl (TNP)-modified but not TNP-unmodified allogeneic targets, i.e., were allorestricted.

The interpretation of these data suffered from potential drawbacks: (*a*) the hapten TNP was used as antigenic determinant; although most antihapten–self CTL responses exhibit predominant lytic reactivity restricted by a single class I H-2 gene product (12–14), TNP derivatization of cell surface products might result in the generation of neodeterminants that resemble alloantigens (15); and (*b*) the resticting allogeneic MHC molecule was not defined. To control these experimental parameters we studied the allogeneic CTL responses between normal H-2K^b (B6) and mutant H-2K^{bm1} (bm1) mice. The bm1 H-2K^b mutant differs from the parental strain C57BL/6 (B6) only at amino acid positions 152, 155, and 156 of the H-2K molecule (16). Since this mutation is of the "gain and loss" type, B6 mice respond to allogeneic, conformational, bm1-specific determinants on the K^b molecule, and vice versa. Antigens presented in the context of the allogeneic (wild-type or mutant) K^b molecules were either haptenic (TNP) determinants, or herpes simplex virus (HSV) determinants. Here, we show that large numbers of CTL clones were inducible from a population of normal splenic T cells that recognized haptenic or HSV determinants in the context of an allogeneic K^b molecule. A companion paper describes corresponding data in a human limiting dilution system.[2]

Materials and Methods

Mice. Normal C57BL/6 (B6) and mutant H-2k^{bm1} (bm1) mice were bred under standard pathogen-free conditions in the animal colony of the Department of Medical Microbiology and Immunology, Ulm University. Breeding pairs of these mice were kindly provided by Dr. C. Melief (Amsterdam, The Netherlands). All mice were 8–10 wk old.

Culture Medium. The culture medium was Click/RPMI 1640 medium (Biochrom, West Berlin) supplemented with 10% (vol/vol) of a selected batch of fetal calf serum (FCS) (Biochrom), 10 mM Hepes buffer, 5×10^{-5} M 2-mercaptoethanol, 1 μg/ml indomethacin, 50 mM methyl-α-D-mannoside, and antibiotics. In limiting dilution cultures we further added 15–20% (vol/vol) of a selected batch of concanavalin A (Con A)-stimulated rat spleen cell supernatant (see below) plus 4 U/well of recombinant human interleukin 2 (IL-2) (batch 89050/848001 RIK, kindly provided by Dr. D. Armerding, Sandoz Research Institute, Vienna) to the medium.

Cell Suspension. Spleen cells were freed from erythrocytes by a brief suspension in 0.84% ammonium chloride solution. The responding spleen cells were always passed through nylon wool columns (17); 20–30% of the loaded cell number was eluted from the nylon wool column after a 1 h incubation at 37°C.

Con A–stimulated Spleen Cell Supernatant. Spleen cells (3×10^6/ml) from outbred rats were incubated for 24 h in culture medium containing 5 μg/ml Con A (Pharmacia, Inc., Uppsala, Sweden). The supernatant was collected, spun at 1,000 *g* for 10 min, filtered through a 0.22 μm filter, and stored in aliquots at −20°C until use. Individual batches of

[2] Kabelitz, D., Jörg Reimann, Wolf-Rüdiger Herzog, Klaus Heeg, and Hermann Wagner. Allorestricted cytotoxic T cells (CTL). Large numbers of human peripheral blood precursor T cells clonally develop into allorestricted antiviral CTL populations in vitro. Manuscript submitted for publication.

supernatants were screened for IL-2 activity on an IL-2-dependent cell line (18) and compared with a reference preparation (i.e., a semipurified preparation from the phorbol ester–stimulated mouse thymoma subclone EL4.1 [19]) that was assigned to contain 100 U/ml of IL-2 activity.

Limiting Dilution System. Cultures were set up in 96-well round-bottom microtiter trays (Greiner, Nürtingen, FRG). Responder cells were mixed at titrated densities with irradiated (1,500 rad) stimulator spleen cells (2×10^5/well). 2,4,6-Trinitrophenyl (TNP) modification of stimulator and target cells was based on the method of Shearer (20): Stimulator cells were incubated in 3 mM trinitrobenzene sulfonate (Serva Feinbiochemica, Heidelberg, FRG) dissolved in phosphate-buffered saline (PBS), pH 7.2, and target cells were incubated with 10 mM trinitrobenzene sulfonate. This was followed by four washes in PBS containing 10% (vol/vol) FCS. Stimulator cells were virus infected by incubating spleen cells with heat-inactivated (30 min, 56°C) Herpes simplex virus (strain LENETTE, kindly provided by Dr. D. Falke, Mainz, FRG) at about two multiplicities of infection (moi) per cell for 45 min at 37°C. Cells were washed three times thereafter. Virus-infected target cells were prepared by incubating 3-d lipopolysaccharide (LPS)-activated spleen cells with the same (not inactivated) virus preparation overnight at ~2 moi per cell. Limiting dilution cultures were set up in a 0.15 volume and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. In all experiments, cultures were restimulated at day 1 or 2 of incubation (21) by adding 0.1 ml fresh culture medium containing 20% (vol/vol) conditioned medium and 10^5 stimulator cells. Cultures were set up using a minimum of four different responder cell concentrations with 12–96 replicates of each.

Cytotoxicity Assay. Cytotoxic activity was measured by removing three replicates (0.06 ml) from each microculture and adding to each 0.14 ml of culture medium containing 10^3 ^{51}Cr-labeled, 48–72 h splenic LPS or Con A blast target cells. Targets (5×10^6 cells in 0.5 ml PBS/10% FCS) were labeled with 400 μCi (14.8 MBq) $Na^{51}CrO_4$, for 90 min at 37°C, after which cells were washed four times before use in the cytotoxicity assay. Under these conditions targets gave a total count of 2–5 counts per cell per minute. Con A blast targets gave a spontaneous release of 10–25%; LPS blast targets gave a spontaneous release of 15–30%. Cultures were incubated for 5 h at 32°C before removal of 0.1 ml supernatant for gamma radiation counting. The percent specific release was calculated as [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100. Total counts were measured by resuspending target cells; spontaneously released counts by assaying cultures containing only irradiated stimulator cells (but not responder cells). In all experiments, methyl-α-D-mannoside (final concentration, 50 mM) was present in microcultures, washing fluids, and in the assay. Frequencies of cytotoxic lymphocyte precursors (CLP) were calculated using the procedure of Taswell (22); the probability of clonality was determined as described by Miller (23). Note that, for CLP frequencies, all cultures with lysis values greater than the mean spontaneous release plus two standard deviations were included in the analysis; for the analysis of lytic specificity patterns, only cultures with >10% specific lysis of at least one target were analyzed.

Controls for Scoring of Lytic Patterns. In control experiments we estimated the frequency of scoring errors, as described (11, 24). The rate of false positives was 1–4%; the average difference in measured lysis values of replicates tested independently against the same target (±SD) was 4 ± 3%.

Results

Experimental Approach. The limiting dilution analysis (LDA) of CTL responses to haptenic (TNP) and HSV determinants recognized in association with allogeneic mutant (bm1) or normal (B6) H-2K^b molecules is described. In addition to the estimates of CLP frequencies, we determined by split-well analysis the lytic specificity patterns expressed by CTL populations selected for a high probability of clonality. We used a restimulation protocol (11, 21) in which feeding of microcultures at day 1 or 2 of in vitro incubation (with fresh culture medium,

fresh conditioned medium, and the respective irradiated stimulator cells) increased the detected CLP frequencies considerably. Starting at day 7 or 8 of incubation, individual microcultures in these limiting dilution experiments were repeatedly tested at 2–3 d intervals for specific lytic activity by split-well analysis (from which CLP frequency estimates were derived), and restimulated thereafter.

CTL Populations Generated Under Limiting Dilution Conditions in Response to TNP Presented in the Context of Allogeneic H-2K^b Molecules. Coculture of limiting numbers of splenic responder T cells from (normal) B6 or mutant (bm1) mice with an excess of allogeneic, irradiated, TNP-modified stimulator spleen cells from bm1 or B6 mice generated CTL populations that displayed specific lytic reactivity.

In Table I, frequency estimates for CLP from normal H-2K^b B6 mice activated by TNP-modified mutant bm1 stimulator cells in three representative experiments are listed. In nylon wool–passaged B6 spleen cell populations cultured in the B6–anti-bm1^{TNP} MLR under limiting dilution conditions, 1/120 to 1/400 CLP gave rise to a CTL clone that lysed bm1^{TNP} targets. The estimated frequencies for CLP of CTL clones that lysed unmodified allogeneic bm1 targets were two- to threefold lower, and estimated CLP frequencies of the CTL response, read out against syngeneic (B6) TNP-modified targets, were considerably lower (the latter cytotoxic response did not consistently titrate in every experiment).

A comparable picture emerged from the LDA of the reverse bm1–anti-B6^{TNP} MLR, although estimated frequencies for CLP of CTL clones that lysed TNP-modified syngeneic/allogeneic and/or unmodified allogeneic targets were consistently lower (Table I).

TABLE I

Large Fraction of Splenic CLP Is Induced to Clonal Development by TNP-modified Allogeneic (Mutant or Wild-type) H-2K^b-bearing Stimulator Cells

MLR*		Target‡	Estimated CLP frequency§		
Responder	Stimulator		Exp. 1	Exp. 2	Exp. 3
B6	bm1^{TNP}	bm1^{TNP}	1/384	1/127	1/170
		bm1	1/618	1/187	1/488
		B6^{TNP}	NT‖	1/396	1/775
bm1	B6^{TNP}	B6^{TNP}	1/536	1/572	1/1,325
		B6	1/947	1/756	1/2,208
		bm1^{TNP}	NT	1/960	1/1,705

* Limiting numbers of nylon wool–nonadherent splenic responder cells from normal B6 and mutant bm1 mice were cocultured with irradiated TNP-modified allo-H-2K^b-bearing stimulator spleen cells in the presence of growth factor(s) for 7 to 13 d (see Materials and Methods).

‡ The lytic response was read out against allogeneic TNP-modified/unmodified and syngeneic, TNP-modified Con A blast targets.

§ Frequencies of CLP that gave rise to a clone cytolytic for the target indicated. In all experiments, split-well analysis was done by dividing the culture volume in three equal aliquots. Cultures were always assayed sequentially two to three times at 2–4 d intervals; only the highest calculated CLP frequencies are included in the table.

‖ The lytic response did not titrate.

The lytic response was read out against the stimulating allogeneic target, either unmodified or TNP-modified, and against the TNP-modified syngeneic target. Microcultures were selected that contained CTL populations which showed >10% lysis of at least one of the three tested targets and displayed >60% probability of clonality according to limiting dilution statistics (23). The lytic specificity analyses of these selected CTL populations are shown in Figs. 1 and 2. Three patterns of specific lysis can be distinguished: (*a*) alloreactive pattern (i.e., lysis of unmodified and TNP-modified allogeneic targets, but not of TNP-modified syngeneic targets); (*b*) TNP-reactive pattern (i.e., lysis of TNP-modified syngeneic and allogeneic targets, but not of unmodified allogeneic targets); and (*c*) allorestricted pattern (i.e., lysis of TNP-modified allogeneic targets, but no lysis of TNP-modified syngeneic targets or unmodified allogeneic targets). In the B6–anti-bm1^{TNP} MLR (Fig. 1), 27% (7/26) of all lytic microcultures showed an allorestricted pattern of lysis; in the reverse bm1–anti-B6^{TNP} MLR (Fig. 2), 26% (6/23) of all lytic reactivities were classified as allorestricted. Thus, at least one-fourth of all patterns of specific lysis expressed by CTL populations with a high probability of clonality could be classified as allorestricted in these two types of MLR.

CTL Populations Generated Under Limiting Dilution Conditions in Response to HSV in the Context of Allogeneic H-2K^b Molecules. Experiments were performed that used HSV instead of TNP determinants as the foreign antigen. Limiting numbers of responding splenic T cells from normal (B6) or mutant (bm1) mice were thus cocultured with an excess of irradiated, virus-infected allogeneic spleen

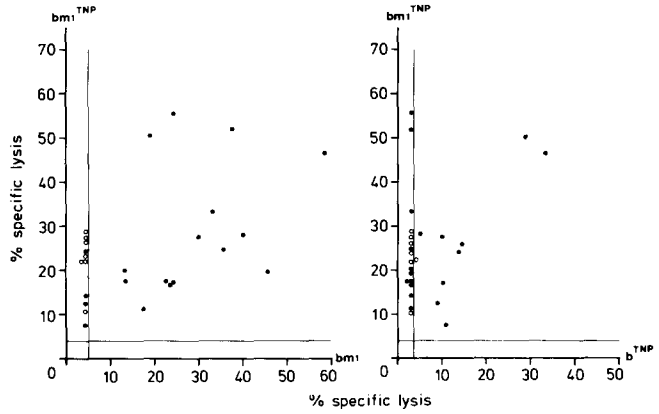

FIGURE 1. Normal splenic B6 responder cocultured at limiting numbers with TNP-modified mutant bm1 stimulator cells generate allorestricted CTL populations. Nylon wool–nonadherent B6 spleen cells were stimulated with TNP-modified bm1 spleen cells under limiting dilution conditions using the restimulation protocol (see Materials and Methods). Individual cultures were read out against allogeneic (bm1) TNP-modified and unmodified Con A blast targets, as well as syngeneic (b) TNP-modified Con A blast targets, at day 10 of in vitro incubation. Only microcultures with (*a*) >60% probability of clonality, and (*b*) >10% specific lysis of at least one of the three tested targets were included in either panel. 7 (○) of the 26 microcultures in this selected panel (27%) showed an allorestricted pattern of lysis. Frequencies: anti-bm1, 1/488 (95% confidence limits [CL], 1/361 to 1/749; $\chi^2 = 0.411$; $P = 0.94$); anti-bm1^{TNP}, 1/170 (95% CL, 1/129 to 1/248; $\chi^2 = 0.996$; $P = 0.68$); anti-b^{TNP}, 1/775 (95% CL, 1/589 to 1/1132; $\chi^2 = 0.872$; $P = 0.96$).

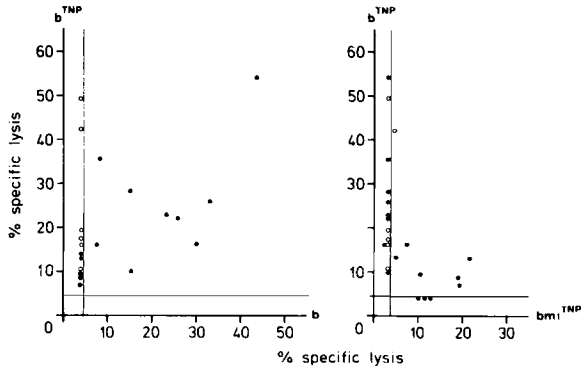

FIGURE 2. Mutant bm1 splenic responder cells cocultured at limiting numbers with TNP-modified normal B6 stimulator cells generate allorestricted CTL populations. Nylon wool–nonadherent bm1 spleen cells were stimulated with TNP-modified B6 spleen cells under limiting dilution conditions using the restimulation protocol. Individual cultures were read out against allogeneic (b) TNP-modified or unmodified Con A blast targets as well as syngeneic (bm1) TNP-modified Con A blast targets at day 8 of in vitro incubation. Only microcultures with (*a*) >10% specific lysis of at least one of the three tested targets, or (*b*) >60% probability of clonality were included in either panel. Of the 23 microcultures thus selected, 6 (○) (26%) showed an allorestricted pattern of lysis. Frequencies: anti-b, 1/756 (95% CL 1/559 to 1/1165; $\chi^2 = 2.913$; $P = 0.77$); anti-b^{TNP}, 1/572 (95% CL, 1/432 to 1/847; $\chi^2 = 2.083$; $P = 0.69$); anti-bm1^{TNP}, 1/960 (95% 1/701 to 1/1,522; $\chi^2 = 3.084$; $P = 0.38$).

cells from bm1 or B6 mice to generate CTL populations, the lytic specificity of which were analyzed at the clonal level.

In populations of splenic T cells from normal B6 mice, virus-infected mutant bm1 stimulator cells induced 1/70 to 1/300 CLP to clonally expand in vitro and to generate CTL populations that lysed virus-infected H-2K^{bm1} targets (Table II). Estimated frequencies for CLP of CTL populations that lysed the noninfected H-2K^{bm1} target were (as in the TNP system) two- to fourfold lower. In contrast to the TNP system, a primary in vitro CTL response to virus-infected syngeneic (B6) targets was not inducible by stimulating splenic T cells with virus-infected allogeneic H-2K^{bm1} spleen cells under these limiting dilution conditions.

The LDA of the reverse bm1–anti-B6^{HSV} MLR revealed comparable data (Table II), although the estimated frequencies for CLP of clones that lysed virus-infected or noninfected allogeneic B6 targets were again consistently lower.

The specificity of the lytic responses was assessed by a readout that included the stimulating allogeneic target, either virus-infected or noninfected, and the virus-infected syngeneic target. Two of the three patterns of specific lysis in Figs. 1 and 2 (i.e., the alloreactive and the allorestricted patterns) are discernible in the data shown in Figs. 3 and 4, and are listed in Table III. This analysis again included CTL populations that showed >10% lysis of at least one of the three tested targets, and displayed >60% probability of clonality according to limiting dilution statistics. In the B6–anti-bm1^{HSV} MLR (Fig. 3), 46% (12/26) of the selected microcultures showed an allorestricted antiviral lytic reactivity. In the reverse bm1–anti-B6^{HSV} MLR (Fig. 4), 31% (15/47) of the selected microcultures expressed an allorestricted antiviral pattern of lysis. LDA of these MLR, therefore, reproducibly (Table III) revealed that at least one-third to one-half of all lytic patterns were classifiable as allorestricted.

TABLE II

Large Fraction of Splenic CLP Is Induced to Clonal Development by Stimulation with HSV-infected Cells Bearing Allogeneic (Mutant or Wild-type) H-2K^b Molecules

MLR*		Target‡	Estimated CLP frequency§		
Responder	Stimulator		Exp. 1	Exp. 2	Exp. 3
B6	bm1^{HSV}	bm1^{HSV}	1/256	1/72	1/292
		bm1	1/488	1/171	1/1,138
		B6^{HSV}	NT‖	NT	NT
bm1	B6^{HSV}	B6^{HSV}	1/1,231	1/320	1/528
		B6	1/1,519	1/686	1/1,312
		bm1^{HSV}	NT	NT	NT

* Limiting numbers of nylon wool–nonadherent splenic responder cells from normal B6 and mutant bm1 mice were cocultured with irradiated HSV-infected, allo-H-2K^b-bearing stimulator cells in the presence of conditioned medium plus recombinant IL-2 for 7–13 d (see Materials and Methods).

‡ The lytic response was read out against allogeneic HSV-infected/non-infected and syngeneic HSV-infected LPS blast targets.

§ Frequencies of CLP that gave rise to a clone cytolytic for the target indicated. In all experiments, split-well analysis was done by dividing the culture volume in three equal aliquots. Cultures were always assayed sequentially two to three times at 2–4 d intervals; only the highest calculated CLP frequencies are included in the table.

‖ Lytic response did not titrate.

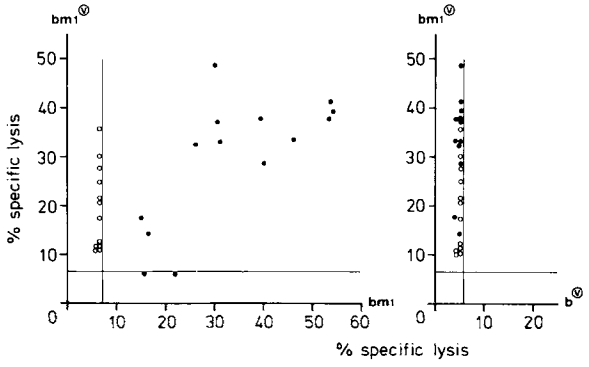

FIGURE 3. Limiting numbers of normal B6 responder cells stimulated with HSV-infected mutant bm1 spleen cells generate allorestricted CTL populations. Nylon wool–nonadherent B6 spleen cells were cocultured with HSV-infected irradiated bm1 stimulator cells under limiting dilution conditions. Individual microcultures were read out against allogeneic (bm1) HSV-infected (bm1^v) and noninfected (bm1) LPS blast targets as well as syngeneic (B6) HSV-infected (b^v) LPS blast targets at day 10 of in vitro incubation. Only micocultures with (*a*) >60% probability of clonality, (*b*) >10% specific lysis against at least one of the three tested targets were included in the analysis. Of this selected group, 12 (○) of 26 (46%) microcultures showed an allorestricted pattern of lysis. Frequencies: anti-bm1, 1/488 (95% CL, 1/361 to 1/749; $\chi^2 = 0.41$; $P = 0.93$); anti-bm1^{HSV}, 1/256 (95% CL 1/194 to 1/376; $\chi^2 = 1.33$; $P = 0.63$); anti-b^v response did not titrate.

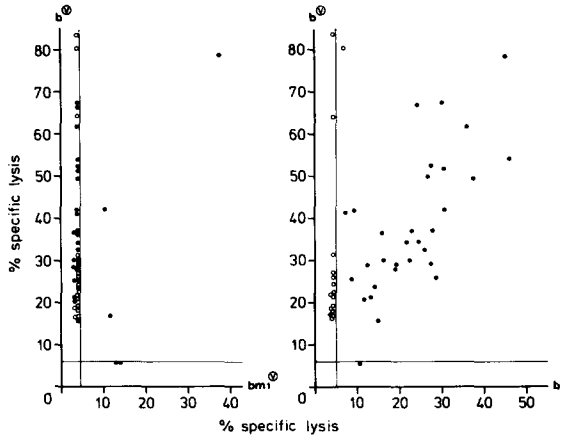

FIGURE 4. Limiting numbers of mutant bm1 responder cells stimulated with HSV-infected normal B6 spleen cells generate allorestricted CTL populations. Nylon wool–nonadherent bm1 spleen cells were cocultured with HSV-infected irradiated B6 stimulator cells under limiting dilution conditions. Individual microcultures were read out against allogeneic (B6) HSV-infected (b^v) and noninfected (b) LPS blast targets as well as syngeneic HSV-infected ($bm1^v$) LPS blast targets at day 8 of in vitro incubation. Only microcultures with (*a*) >60% probability of clonality, or (*b*) >10% lysis of at least one of the three tested targets were included in this analysis. Of this selected group, 15 (○) of 47 (31%) microcultures showed an allorestricted pattern of lysis. Frequencies: anti-B6 (b), 1/686 (95% CL, 1/528 to 1/979; χ^2 = 2.13; P = 068); anti-$B6^{HSV}$ (b^v), 1/320 (95% CL, 1/243 to 1/470; χ^2= 0.61; P = 0.62); anti-$bm1^{HSV}$ ($bm1^v$) response did not titrate.

TABLE III

Distribution of Allorestricted and Alloreactive Pattern of Specific Lysis Among CTL Populations Generated in Response to HSV-infected (Mutant or Wild-type) Allo-H-2K^b-bearing Stimulator Cells

MLR*		Exp.‡	Target§	Pattern of specific lysis¶ (%)			
				Allo-reactive	Virus-specific#	Allo-restricted	Non-specific
Responder	Stimulator		Syn^{HSV}	−	+	−	+
			Allo	+	−	−	+
			$Allo^{HSV}$	+	+	+	+
B6	$bm1^{HSV}$	1		14/21 (66)	—	7/21 (34)	—
		2		13/22 (59)	—	9/22 (41)	—
		3		14/20 (60)	—	6/20 (40)	—
bm1	$B6^{HSV}$	1		10/25 (40)	—	15/25 (60)	—
		2		3/8 (50)	—	4/8 (50)	—
		3		12/25 (48)	—	13/25 (52)	—

* See legend to Table II.

‡ Three representative experiments for each of the two types of MLR are shown.

§ The cytolytic response was read out against allogeneic HSV-infected/noninfected and syngeneic HSV-infected LPS blast targets.

¶ Only cultures showing >10% specific lysis of at least one of the three tested targets, and displaying >60% probability of clonality, were included in the analysis. Data are listed as number of cultures showing the respective pattern of lysis per total number of selected lytic cultures in the group (percent).

Presumably self-restricted, anti-HSV cytotoxic response.

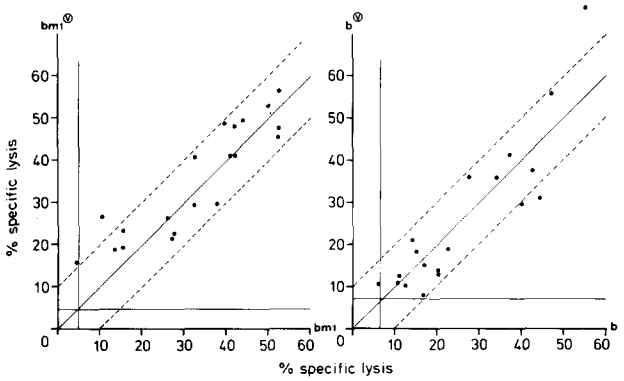

FIGURE 5. Noninfected and HSV-infected LPS blast targets are equally susceptible to CTL-mediated lysis. The B6–anti-bm1^{HSV} MLR and the bm1–anti-B6^{HSV} MLR were analyzed under limiting dilution conditions as described in Fig. 3 and 4. Only microcultures with <5% probability of clonality were plotted.

Virus-infected Targets Do Not Show an Enhanced Susceptibility to CTL-mediated Lysis. We have reported previously (11) that no allorestricted TNP-specific lytic patterns were detectable in microcultures into which high numbers (>10^3) of responding T cells were seeded at the initiation of culture, thereby causing the large majority of the generated CTL populations to be multiclonal in origin. These multiclonal CTL populations lysed unmodified and the respective TNP-modified allogeneic targets equally well (11). Controls in the present experiments indicated that virus-infected and noninfected LPS blast targets were equally susceptible to lysis by multiclonal CTL populations (Fig. 5). Consequently, we can exclude an enhanced susceptibility to lysis of virus-infected targets as a possible explanation of allorestricted lytic patterns.

Discussion

Under appropriate in vitro conditions, we have clonally expanded CTL populations out of normal peripheral T cell populations with intact alloreactivity by stimulation with cells bearing (haptenic or viral) foreign antigens and an allogeneic H-2K^b molecule. The estimated frequencies of CLP for CTL clones that lysed the antigen-bearing (i.e., hapten-modified or virus-infected) allogeneic target cell ranged from 1/70 to 1/1,200 (in two different strain combinations and two different antigen systems) in nylon wool–nonadherent mouse spleen cells; the estimated frequencies of CLP for CTL clones that lysed the respective allogeneic target cells not expressing antigen was always two- to fourfold lower. The specificity analysis of the generated CTL populations (selected for a high probability of clonality and high lysis values) demonstrated that one-fourth to one-half of these CTL clones lysed only the stimulating antigen-bearing allogeneic target but not the respective allogeneic target, in the absence of antigen, i.e., displayed an allorestricted lytic pattern. The data indicate that an allogeneic (wild-type or mutant) H-2K^b molecule can restrict the recognition of viral or haptenic determinants of a large number of CTL populations developing from CLP in the peripheral T cell pool of normal mice. The data extend our previous report (11) and are complementary to the report[2] of very high frequencies of

CLP for allorestricted antiviral CTL populations detected in a human limiting dilution system, using normal peripheral blood T cells as responders.

It was imperative to establish optimal culture conditions in the described limiting dilution system. Preliminary experiments under suboptimal culture conditions that resulted in considerably lower detectable CLP frequencies did not reveal the presence of allorestricted lytic patterns in the virus system. In similar observations made in the human limiting dilution system, it was only through the definition of a set of critical culture parameters that we observed specific allorestricted antiviral CTL responses with very high CLP frequencies.

Our results appear to indicate that a large fraction of the T cell repertoire will never be usable by the immune system of a particular individual. This unexpected implication forces us to raise basic questions regarding: (*a*) the nature of the developmental process involved in shaping the T cell receptor repertoire; (*b*) the nature of restricted recognition of T cells; and (*c*) the nature of the in vitro readout system used here. This last question is obviously a central issue, because it defines possible ways to answer the preceding ones. Although LDA is able to define the functional phenotype and/or receptor specificity of freshly isolated lymphocytes at the clonal level, the 'input' to the system (in terms of the receptor specificity of a given precursor lymphocyte to be expanded) is never controlled. It is therefore uncertain whether the measured output was present at the initiation of the system (and was only quantitatively expanded to detectable levels), or if it was generated during in vitro incubation. Conceptually, the latter assumption could explain our unexpected experimental results.

The developmental constraints that shape the T cell repertoire remain elusive. Our data certainly do not support the notion of an exclusive haplotype-specific influence of thymic, class I H-2 determinants on the generation of H-2 restriction specificities of developing CTL populations (reviewed in 25). Evidence against such an idea has been presented already (26–30) in experimental systems similar to those used originally to define the influence of the thymus on the T cell repertoire, i.e., allogeneic radiation bone marrow chimeras and restricted virus- or TNP-specific CTL responses. Thus, the mode of action (positive vs. negative selection) as well as the site (thymic vs. extrathymic) of the influences that determine class I restriction specificities of the T cell repertoire of an individual remain obscure. Recent reports (31–33) seem to indicate that only class II (not class I) H-2 restriction specificities are "learned" under the influence of the thymus. It will therefore be of interest to search for antigen-specific, allo-class II–restricted T helper cell responses under the limiting dilution conditions used here.

Although the antigen and the restricting allogeneic class I H-2 molecule were experimentally controlled in these studies, the true specificity of this CTL response is difficult to establish. Hapten modification of stimulator/target cells has offered a unique chance to analyze primary self-restricted, antigen-specific CTL responses in vitro. This has been difficult in other antigen systems. The difficulties arising in interpreting restricted anti-TNP T cell responses were mentioned in the introduction. We therefore included an alternative antigen system in the analysis, the CTL response to determinants of HSV. The generated CTL populations discriminated between two virus-infected targets that differed

only in a mutant H-2K^b molecule; this confirms a previous report (34) that HSV-infected B6 targets and HSV-infected bm1 targets do not show crossreactive lysis. This strongly argues for the exact specificity of these CTL populations, and certainly excludes natural killer cells as the cytolytic effectors involved. Natural killer cells have been shown to mediate the in vivo resistance to HSV infections in mice (35). Our data also weaken the objections of "altered self" proponents (36, 37) that, in an A-type mouse, there is always a self-restricted (A + X) receptor phenotype for any crossreactive, allorestricted (B + Y) receptor phenotype (A and B being two independent MHC haplotypes, and X and Y being two non-crossreactive antigenic determinants) (38, 39). But even the exact specificity of allorestricted T cells cannot formally rule out the above-cited altered-self interpretation. This argument extends to the question of alloreactivity: is alloreactivity a frequent type of crossreactivity of self-restricted antigen-specific T cells (40), or is the strong anti-MHC reactivity of T cells somehow linked to the role of MHC molecules as constraints on the expressed T cell repertoire? No answer is yet available to this central enigma of the T cell system.

It has been widely speculated that anti-MHC reactivity might be an early receptor phenotype expressed by developing T cells (41). This assumes that anti-MHC reactivity is the point of origin of the T cell repertoire development which, during normal ontogeny, would be an anti-self-MHC–specific T cell reactivity (42, 43). But since self-MHC determinants have no obvious privileged position among all MHC allodeterminants expressed by the respective species (44), T cell repertoire development might also be inducible by allo-MHC determinants, under certain conditions (e.g., in the in vitro system used here). These conditions might mimic certain aspects of the physiological generation of the T cell repertoire. We envisage the possibility that the T cell receptor repertoire diversifies somatically in the course of clonal T cell expansion, after being triggered by recognition of allo-MHC molecules. Somatic diversification of the repertoire seems now well established for B cells at the cellular (45) and molecular (46–48) level. The first reports of T cell diversification at the cellular (24) and molecular (49) level suggest that this might also be the case for T cells. The mechanisms by which restriction specificities are learned in this process remain to be defined. We feel that the in vitro induction of allorestricted clonal CTL populations may enable us to shed some light on this process.

Summary

Cytotoxic T lymphocyte (CTL) responses of splenic T cells from C57BL/6 (B6) mice and mutant H-2K^{bm1} (bm1) mice to haptenic (trinitrophenyl [TNP]) and herpes simplex virus (HSV) determinants in the context of an allogeneic (wild-type or mutant) H-2K^b molecule were analyzed in a modified limiting dilution system. In the B6–anti-bm1^{TNP} mixed leukocyte reaction (MLR), estimated frequencies for precursors of CTL clones that lysed bm1^{TNP} targets ranged from 1/120 to 1/400; in the bm1–anti-B6^{TNP} MLR, estimated frequencies of precursors of CTL clones that lysed B6^{TNP} targets ranged from 1/500 to 1/1,300. Estimated frequencies for precursors of CTL clones that lysed the respective unmodified and TNP-modified allogeneic targets were two- to three-fold lower. Lytic specificity patterns determined by split-well analysis showed

that at least 20–30% of the generated CTL populations (selected for a high probability of clonality) in both MLR displayed allorestricted lysis of TNP-modified concanavalin A blast targets. In the B6–anti-bm1^{HSV} MLR, estimated frequencies for precursors of CTL clones that lysed bm1^{HSV} targets ranged from 1/70 to 1/300; in the bm1–anti-B6^{HSV} MLR, estimated frequencies for precursors of CTL clones that lysed B6^{HSV} targets ranged from 1/300 to 1/1,200. Again, estimated frequencies for precursors of CTL clones that lysed the respective noninfected and virus-infected allogeneic targets were two- to fourfold lower. Of the CTL populations selected for a high probability of clonality at least 30–60% displayed allorestricted lysis of virus-infected lipopolysaccharide blast targets in both MLR.

It is concluded that a large fraction of clonally developing CTL populations stimulated with TNP-modified or HSV–infected allo-H-2K^b-bearing cells displayed an allorestricted pattern of recognition. It was further evident that the estimated frequencies of splenic precursors that generated allorestricted CTL clones was two- to threefold higher than the estimated frequencies of precursors that gave rise to the respective alloreactive CTL populations.

We thank Astrid Bellan, Christiane Steeg, Michaela Schliefer, and Anja Otterbein for expert technical assistance, and Dr. R. G. Miller, Ontario Cancer Institute, for his interest and helpful suggestions.

Received for publication 6 March 1985.

References

1. Cohn, M. 1983. The T-cell receptor mediating restrictive recognition of antigen. *Cell.* 33:657.
2. Wilson, D. B., K. Fischer-Lindahl, D. H. Wilson, and J. Sprent. 1977. The generation of killer cells to trinitrophenyl-modified allogeneic targets by lymphocyte populations negatively selected to strong alloantigens. *J. Exp. Med.* 146:361.
3. Doherty, P. C., and J. R. Bennink. 1979. Vaccinia-specific cytotoxic T-cell responses in the context of H-2 antigens not encountered in thymus may reflect aberrant recognition of a virus-H-2-complex. *J. Exp. Med.* 149:150.
4. Stockinger, H., K. Pfizenmaier, C. Hardt, M. Röllinghoff, and H. Wagner. 1980. H-2 restriction as a consequence of intentional priming: T cells of fully allogeneic chimeric mice as well as normal mice respond to foreign antigens in the context of H-2 determinants not encountered on thymic epithelial cells. *Proc. Natl. Acad. Sci. USA.* 77:7390.
5. Stockinger, H., R. Bartlett, K. Pfizenmaier, M. Röllinghoff, and H. Wagner. 1981. H-2 restriction as a consequence of intentional priming. Frequency analysis of alloantigen-restricted, trinitrophenyl-specific, cytotoxic T-lymphocyte precursors within thymocytes of normal mice. *J. Exp. Med.* 153:1629.
6. Nagy, Z. A., C. N. Baxevanis, N. Ishii, and J. Klein. 1981. Ia antigens as restriction molecules in Ir-gene controlled T-cell proliferation. *Immunol. Rev.* 60:59.
7. Schmitt-Verhulst, A. M., and G. M. Shearer. 1977. Specificity of CML and MLR clones responding to chemically modified syngeneic and allogeneic cells. *J. Supramol. Struct.* 6:206.
8. Janeway, C. A., P. D. Murphy, J. Kemp, and H. Wigzell. 1978. T-cells specific for hapten-modified self are precommitted for self major histocompatibility complex antigen before encounter with the hapten. *J. Exp. Med.* 147:1065.

9. Teh, H.-S. 1979. Frequency estimates of cytotoxic precursors to trinitrophenyl-modified alloantigens and determination of the degree of cross-reactivity between allodeterminants and trinitrophenyl-modified self determinants. *Immunogenetics.* 8:99.
10. Good, M. F., and G. J. V. Nossal. 1983. Clones of cytotoxic T lymphocytes reactive to haptenated allogeneic cells: precursor frequency and characteristics as determined by a split culture approach. *Proc. Natl. Acad. Sci. USA.* 80:1693.
11. Reimann, J., K. Heeg, R. G. Miller, and H. Wagner. 1985. Alloreactive cytotoxic T-cells. I. Alloreactive and allorestricted cytotoxic T-cells. *Eur. J. Immunol.* 15:387.
12. Schmitt-Verhulst, A. M., and G. M. Shearer. 1975. Bifunctional major histocompatibility-linked genetic regulation of cell-mediated lympholysis to trinitrophenyl-modified autologous lymphocytes. *J. Exp. Med.* 142:914.
13. Levy, R. B., and G. M. Shearer. 1979. Regulation of T-cell mediated cytotoxicity by the murine major histocompatibility complex. I. Preferential in vitro responses to trinitrophenyl-modified self K- and D-coded gene products in parental and F1 hybrid mouse strains. *J. Exp. Med.* 149:1379.
14. Arora, P. K., R. B. Levy, and G. M. Shearer. 1982. Preferential response patterns of cytotoxic T lymphocytes specific for fluorescein isothiocyanate (FITC)-modified autologous cells. *J. Immunol.* 128:551.
15. Levy, R. B., S. K. Dower, G. M. Shearer, and D. M. Segal. 1984. Trinitrophenyl modification of H-2^k and H-2^b spleen cells results in enhanced serological detection of K^k-line determinants. *J. Exp. Med.* 159:1464.
16. Weiss, E. H., A. Mellor, L. Golden, K. Fahrner, E. Simpson, J. Hurst, and R. A. Flavell. 1983. The structure of mutant H-2 gene suggest that the generation of polymorphism in H-2 genes may occur by gene conversion-like events. *Nature* 301:671.
17. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* 3:645.
18. Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith. 1978. T-cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120:2027.
19. Farrar, J. J., J. Fuller-Farrar, P. L. Simon, M. L. Hilfiker, B. L. Stadler, and W. L. Farrar. 1980. Thymoma production of T-cell growth factor (interleukin 2). *J. Immunol.* 125:2555.
20. Shearer, G. M. 1974. Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. *Eur. J. Immunol.* 4:527.
21. Christensen, N., M. Skinner, and J. Marbrook. 1983. The analysis of an anti-fluorescein cytotoxic response. *Eur. J. Immunol.* 13:701.
22. Taswell, C. 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J. Immunol.* 126:1614.
23. Miller, R. G. 1982. *In* Isolation, Characterization, and Utilization of T-Lymphocyte Clones. C. G. Fathman and F. W. Fitch, editors. Academic Press, New York. p. 220–231.
24. Reimann, J., and R. G. Miller. 1985. Rapid changes in specificity within single clones of cytolytic effector cells. *Cell.* 40:511.
25. Zinkernagel, R. M., and P. C. Doherty. 1979. MHC-restricted cytotoxic T-cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction specificity, function and responsiveness. *Adv. Immunol.* 27:51.
26. Matzinger, P., and G. Mirkwood. 1978. In a fully H-2 incompatible chimera, T cells

of donor origin can respond to minor histocompatibility antigens in association with either donor or host H-2 type. *J. Exp. Med.* 148:84.

27. Wagner, H., M. Röllinghoff, H. Rodt, and S. Thierfelder. 1980. T-cell mediated cytotoxic immune responsiveness of chimeric mice bearing a thymus graft fully allogeneic to the graft of lymphoid stem cells. *Eur. J. Immunol.* 10:521.
28. Aizawa, S., T. Sado, M. Muto, and E. Kubo. 1981. Immunology of H-2 fully incompatible bone marrow chimeras induced in specific-pathogen-free mice: evidence for generation of donor- and host-H-2-restricted helper and cytotoxic T cells. *J. Immunol.* 127:2426.
29. Aizawa, S., T. Sado, and E. Kubo. 1984. H-2 restriction specificity of T cells from H-2 incompatible radiation bone marrow chimeras: further evidence for the absence of crucial influence of the host/thymus environment on the generation of H-2-restricted TNP-specific T lymphocyte precursors. *Cell. Immunol.* 83:360.
30. Zinkernagel, R. M., T. Sado, A. Althage, and H. Kamisaku. 1984. Anti-viral immune response of allogeneic irradiation bone marrow chimeras: cytotoxic T cell responsiveness depends upon H-2 combination and infectious agent. *Eur. J. Immunol.* 14:14.
31. Kruisbeek, A. M., R. J. Hodes, and A. Singer. 1981. Cytotoxic T lymphocyte responses by chimeric thymocytes. Self-recognition is determined early in T cell development. *J. Exp. Med.* 153:13.
32. Bradley, S. M., A. M. Kruisbeek, and A. Singer. 1982. Cytotoxic T lymphocyte responses in allogeneic radiation bone marrow chimeras. The chimeric host strictly dictates the self repertoire of Ia-restricted T cells but not H-2K/D-restricted T cells. *J. Exp. Med.* 156:1650.
33. Kast, W. M., L. P. de Waal, and C. J. M. Melief. 1984. Thymus dictates major histocompatibility complex (MHC) specificity and immune response gene phenotype of class II MHC-restricted T cells but not of class I MHC-restricted T cells. *J. Exp. Med.* 160:1752.
34. Jennings, S. R., P. L. Rice, S. Pan, B. B. Knowles, and S. S. Tevethia. 1984. Recognition of herpes simplex virus antigens on the surface of mouse cells of the H-2^b haplotype by virus-specific cytotoxic T lymphocytes. *J. Immunol.* 132:475.
35. Habu, S., K.-I. Akamatsu, N. Tamaoki, and K. Okumura. 1984. In vivo significance of NK cell on resistance against virus (HSV-1) infections in mice. *J. Immunol.* 133:2743.
36. Matzinger, P., and M. J. Bevan. 1977. Hypothesis. Why do so many lymphocytes respond to major histocompatibility antigens? *Cell. Immunol.* 29:1.
37. Matzinger, P. 1981. A one-receptor view of T-cell behavior. *Nature (Lond.).* 292:497.
38. Hünig, T. R., and M. J. Bevan. 1984. Specificity of T-cell clones illustrates altered self hypothesis. *Nature (Lond.).* 294:460.
39. Hünig, T. R., and M. J. Bevan. 1982. Antigen recognition by cloned cytotoxic T lymphocytes follows rules predicted by the altered-self hypothesis. *J. Exp. Med.* 155:111.
40. Kaye, J., B. Jones, and C. A. Janeway. 1984. The structure and function of T cell receptor complexes. *Immunol. Rev.* 81:39.
41. Jerne, N. K. 1971. The somatic generation of immune recognition. *Eur. J. Immunol.* 1:1.
42. Reimann, J., and R. G. Miller. 1983. Differentiation from precursors in athymic nude mouse bone marrow of unusual spontaneously cytolytic cells showing anti-self-H-2 specificity and bearing T cell markers. *J. Exp. Med.* 158:1672.
43. Reimann, J., and R. G. Miller. 1983. Generation of autoreactive cytotoxic T lymphocytes under limiting dilution conditions. *J. Immunol.* 131:2128.

44. Reimann, J., and R. G. Miller. 1983. Polymorphism and MHC gene function. *Dev. Comp. Immunol.* 7:403.
45. Cunningham, A. J., and S. A. Fordham. 1974. Antibody cell daughters can produce antibody of different specificities. *Nature (Lond.).* 250:669.
46. McKean, D., K. Huppi, M. Bell, L. Staudt, W. Gerhardt, and M. Weigert. 1984. Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. *Proc. Natl. Acad. Sci. USA.* 81:3180.
47. Rudikoff, S., M. Pawlita, J. Pumphrey, and M. Heller. 1984. Somatic diversification of immunoglobulin. *Proc. Natl. Acad. Sci. USA.* 81:2162.
48. Sablitzky, F., and K. Rajewski. 1984. Molecular basis of an isogeneic antiidiotypic response. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3005.
49. Augustin, A. A., and G. K. Sim. 1984. T-cell receptors generated via mutations are specific for various major histocompatibility antigens. *Cell.* 39:5.