

SELECTION OF CYTOTOXIC T-CELL PRECURSORS
SPECIFIC FOR MINOR
HISTOCOMPATIBILITY DETERMINANTS

I. Negative Selection Across H-2 Barriers Induced with
Disrupted Cells but Not with Glutaraldehyde-
treated Cells: Evidence for Antigen Processing*

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It is well accepted that cytotoxic lymphocytes specific for viruses (1), haptens (2), and minor histocompatibility antigens (HA)¹ (3, 4) recognize antigen in an H-2-restricted fashion during the effector phase. Precisely how the precursors of these cells respond to antigen during the induction phase, however, is poorly understood. In the case of skin graft rejection (5-9) and delayed-type hypersensitivity (10) to minor HA, mice can be primed with antigen presented on H-2-incompatible cells. Likewise, H-2 heterozygous mice primed with minor HA on cells of one parental strain develop cytolytic activity for target cells of both parental haplotypes (11, 12). This effect of cross-priming suggests either that certain aspects of the response to minor HA are not H-2 restricted or, conversely, that the antigens are processed by host cells and then presented in association with host H-2 determinants in a restricted fashion (12).

To attempt to clarify this question and, in particular, to gain information on the early stages of the cytotoxic T-cell response, we have studied the requirements for inducing negative and positive selection to minor HA in vivo. Previous work with this selection model has established that within 1 d of T-cell confrontation with such antigens as heterologous erythrocytes (13, 14) or foreign H-2 determinants (13-16) the responding T cells leave the circulation (undergo negative selection) and become selectively sequestered in the lymphoid tissues. Here, the cells proliferate extensively before reentering the circulation in expanded numbers after 2-3 d (positive selection). In the case of T-helper cells for heterologous erythrocytes, T-cell selection appears not to involve a response to free antigen but rather to antigen that has been processed by macrophages or related cells (17). The present studies suggest that, at least in certain situations, a similar requirement for antigen processing applies to the induction of cytotoxic lymphocytes specific for minor HA.

* Supported by grants AI-10961, AI-15393, and CA-15822 from the U. S. Public Health Service.

¹ Abbreviations used in this paper: CML, cell-mediated lympholysis; HA, histocompatibility antigen(s); LU, lytic units; MR, maximum release of radioactivity; SR, spontaneous release; TDL, thoracic duct lymphocytes.

Materials and Methods

Mice. CBA/J($H-2^k$), B10.BR/SgSn($H-2^k$), C57BL/10J($H-2^b$)(B10), B10.D2($H-2^d$) B10.A($H-2^a$), and (BALB/c[$H-2^d$] \times A/J[$H-2^a$])F₁ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. B10.K($H-2^k$), B10.AQR($H-2^{y1}$) (fourth backcross generation), B10.T(6R)($H-2^{y2}$), and B10.S(9R)($H-2^{t4}$) mice were a gift from W. L. Elkins, University of Pennsylvania (Philadelphia, Pa.). B10.OL($H-2^{o1}$) mice were provided by C. David, Mayo Clinic, Rochester, Minn. B10.Q($H-2^q$) mice were given by D. Gasser, University of Pennsylvania (Philadelphia, Pa.), and B10.A(4R)($H-2^{h4}$) and B10.HTT($H-2^{t3}$) mice were donated by P. Doherty, Wistar Institute, Philadelphia, Pa. (B10.A \times B10.OL)F₁ mice were bred in our colony.

Media. RPMI-1640 (Microbiological Associates, Walkersville, Md.) supplemented with 10% fetal calf serum, 10^{-5} M 2-mercaptoethanol, and 40 μ g/ml Garamycin (Schering Corp., Kenilworth, N.J.) was used in all experiments.

Preparation of Cells. Spleen cells were obtained by shearing the organs with a tissue grinder (Pyrex, Arthur H. Thomas Co., Philadelphia, Pa.) in cold medium. Clumps were removed by passage over cotton filters, and the suspended cells were washed twice by centrifugation. Cell viability was assessed by dye exclusion.

Injection. All cell suspensions were given intravenously in volumes of 1 ml unless stated otherwise.

Selection to minor HA. The technique used was essentially similar to that described previously for inducing selection to H-2 determinants (16). In brief, CBA mice were injected intravenously with large doses of spleen cells taken from normal syngeneic or minor HA-different allogeneic mice. To study negative selection, thoracic duct cannulae were inserted in the recipients \approx 18 h later and thoracic duct lymphocytes (TDL) were collected under sterile conditions between 22 and 40 h after spleen cell injection; other groups of mice were used to provide spleen and lymph node cells, these cells being harvested at 40 h after spleen-cell injection. For positive selection, the injected mice were cannulated at day 4 or 5 postinjection and TDL were collected overnight.

Priming to Minor HA In Vivo. All host mice used for selection were immunized intraperitoneally 3–6 wk before with 2×10^7 viable B10.BR spleen cells.

Irradiation of Cells. Cells were exposed to 2,000 rad of ^{137}Cs γ -irradiation at a dose of 102 rad/min and then incubated at 37°C for 3–4 h (16). The cells were then washed twice before injection. The viability of the injected cells was 70–80%.

Disruption of Cells. Spleen cells were resuspended to 2×10^8 /ml in round-bottom plastic tubes and subjected to high frequency sonication (Branson Sonifier [Branson Sonic Power Co., Danbury, Conn.], 6 A, 30 s) at 4°C. No intact cells remained when the sonicates were viewed microscopically.

Treatment of Cells with Glutaraldehyde. Spleen cells were first resuspended in low ionic strength buffer and passaged over cotton filters to remove dead cells (18). In a modification of the method of Bubbers and Henney (19), cells were then washed once and resuspended at room temperature in 10 ml 0.002% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) diluted in buffered saline (pH 7.1). Fixation was stopped after 30 s by addition of 30 ml 0.15 M glycine (diluted in buffered saline). The cells were then washed twice in cold 0.15 M glycine and resuspended to a final concentration of 120×10^6 /ml for injection. Viability of the cells at the time of injection was \geq 96%.

Generation of Cytotoxic Lymphocytes In Vitro. Aliquots of $1-2 \times 10^7$ TDL were washed twice and placed in plastic tissue culture flasks (Corning Glass Works, Science Products Div., Corning, N.Y.) in 20 ml of medium and stimulated with 2×10^7 irradiated (1,200 rad) B10.BR, B10.K, or third-party H-2-different spleen cells. Cultures were incubated for 6 d at 37°C in 8% CO₂. Cells were then harvested, washed twice, counted, and resuspended to appropriate concentrations for assaying cell-mediated lympholysis (CML).

Target Cells for CML. To prepare target cells, 4×10^8 lymph node cells were stimulated for 2–3 d with 2 μ g/ml of concanavalin A (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) in 2-ml Linbro plates (Linbro Chemical Co., Hamden, Conn.). Blasts were labeled during the final 18 h with 20 μ Ci of ^{51}Cr -sodium chromate (New England Nuclear, Boston, Mass.). After labeling, the cells were washed three times and resuspended to 2×10^5 /ml.

CML Assay. In a modification of the method of Brunner et al. (20), 100 μ l of serially diluted effector cells together with 10^4 ^{51}Cr -labeled target cells in 50 μ l were placed in triplicate in V-bottom microtiter plates (Linbro Chemical Co.), centrifuged gently (400 rpm for 4 min), and then incubated for 5 h at 37°C. After centrifugation, 100 μ l of the supernates were removed to measure radioactivity in an Intertechnique γ -spectrometer. Maximum release of radioactivity (MR) and spontaneous release (SR) were determined with respect to radioactive counts released after incubation with centrimide-saline and medium, respectively; SR ranged from 15 to 35% of MR. The percent specific lysis was calculated by the formula: (experimental release – SR/MR – SR) \times 100; CML obtained on the syngenic CBA targets was invariably <7%.

Calculation of Lytic Units of CML Activity. To compare the relative levels of CML in the experimental groups vs. the control group, lytic units (LU) of CML activity were calculated by a modification of the method of Cerottini and Brunner (21). LU were defined arbitrarily in terms of the number of effector cells required to exert 25% CML (LU 25%). LU 25% were calculated from dose-response linear regression lines plotted with respect to CML observed with three different killer:target ratios; correlation coefficients for linearity were generally >0.95. The lytic activity of each experimental group of effector cells compared with the control group was calculated by the formula: (LU 25% control/LU 25% experimental) \times 100.

Results

Experimental Design. The basic aim was to examine the requirements for inducing CBA(*H-2^b*) T cells to exert cytotoxic responses against the multiple minor HA on *H-2*-compatible B10.BR target cells (CBA and B10.BR differ at a minimum of six minor HA loci [22]). To study selection to these antigens, CBA mice were injected intravenously with large doses of spleen cells tape from mice of the B10 congenic lines, e.g., B10.BR, B10(*H-2^b*), or B10.D2(*H-2^d*); these strains all express the same minor HA foreign to CBA but have differing *H-2* haplotypes. TDL, spleen, or lymph node cells were harvested from the recipients at day 1 (negative selection) or day 5 (positive selection) posttransfer and cultured in vitro for 6 d with *H-2*-compatible B10.BR or B10.K stimulator cells; these stimulators were used irrespective of the *H-2* haplotype of the cells used to produce selection. CML (^{51}Cr release) was then tested on a variety of target cells.

Preliminary studies established that the effector cells in the CBA anti-B10.BR CML responses were T (Thy-1.2-positive) cells (data not shown). Because primary CML responses to minor HA in vitro are very low, the CBA TDL donors were primed with B10.BR spleen cells 3–6 wk before inducing selection. At this time, TDL from the primed donors were not directly cytotoxic even at high (300:1) killer:target cell ratios (data not shown) and hence were probably typical circulating memory cells.

To facilitate comparison of levels of cytotoxic activity, the data in most of the tables are shown first in terms of specific ^{51}Cr release observed with different killer:target cell ratios, and second as the percent lytic activity established with respect to the control cells, i.e., cells from CBA mice preinjected with syngenic spleen cells (Materials and Methods).

Specificity of the CML Response of CBA T Cells Cultured with B10.BR Stimulators: Genetic Mapping Studies. Before studying selection it was first necessary to prove that the CBA anti-B10.BR CML response was indeed *H-2* restricted, i.e., as reported for other anti-minor HA responses (3, 4).

The cytolytic activity of unselected CBA TDL (TDL from recipients of syngenic spleen cells) cultured in vitro with B10.BR stimulators is shown in the left-hand column of Table I. As expected, CML was high with the stimulating population

TABLE I
Genetic Mapping of CML Activity of CBA TDL Stimulated In Vitro with B10.BR Spleen: Effect of Negatively Selecting Responder Cells Against Qa-1^a Determinants on (BALB/c × A/J)F₁ (CAF₁) Spleen Cells

⁵¹ Cr-labeled target cells	H-2 haplotype								Qa-1 allele*	CML activity of CBA TDL cultured in vitro with B10.BR spleen: ‡ Percent specific lysis with killer:target cell ratios of 25:1/12:1/2:1	
	K	I-A	I-B	I-J	I-E	I-C	S	D		CBA-CBA TDL	CBA-CAF ₁ TDL
CBA	k	k	k	k	k	k	k	k	b	6/5/0	0/0/0
B10.BR	k	k	k	k	k	k	k	k	a	80/81/57	64/57/28
B10.K	k	k	k	k	k	k	k	k	b	58/50/24	41/38/18
B10	b	b	b	b	b	b	b	b	b	7/0/0	0/0/0
B10.D2	d	d	d	d	d	d	d	d	b	0/0/0	0/0/0
B10.Q	q	q	q	q	q	q	q	q	?	0/0/0	0/0/0
B10.A	k	k	k	k	k	d	d	d	a	69/57/20	44/33/12
B10.OL	d	d	d	d	d	d	k	k	?	36/38/14	36/34/12
B10.A(4R)	k	k	b	b	b	b	b	b	b	30/31/4	38/22/8
B10.T(6R)	q	q	q	q	q	q	q	d	a	56/43/16	9/6/0
B10.S(9R)	s	s	s	k	k	d	d	d	a	49/51/19	9/0/0
B10.HTT	s	s	s	s	k	k	k	d	b	0/0/0	0/0/0
B10 ₄ .AQR§	q	k	k	k	k	d	d	d	a	40/31/0	0/0/0

* Information from Klein et al. (23) and L. Flaherty (Personal communication.). Although there is some uncertainty in the literature on the identity of the *T* region-linked antigens that evoke H-2-unrestricted CML (24-28), the prevailing opinion at present is that the determinants are probably encoded by genes closely linked to the *Qa-1* locus adjacent to the *Tla* locus (L. Flaherty, Personal communication.).

‡ TDL from CBA mice injected intravenously 1 d before with 1×10^8 irradiated CBA spleen (CBA-CBA TDL) or (BALB/c × A/J)F₁ (*Qa-1^b* × *Qa-1^a*) spleen (CBA-CAF₁ TDL). TDL donors primed 4 wk before with B10.BR spleen.

§ Although these mice were only of the fourth backcross generation, they contain >94% of the B10 background genome.

(B10.BR) and the closely related strain, B10.K, but was not detectable with three H-2-different target cells that expressed the minor HA of B10.BR, i.e., B10, B10.D2, and B10.Q(*H-2^q*). Although these data implied that CML was H-2 restricted, good killing was surprisingly observed with B10.T(6R) (*qqqqqqd*) targets, i.e., cells lacking the *H-2^k* alleles of the CBA responders. Moreover, CML was also apparent against two strains possessing *H-2^k* alleles only in the *I* region, i.e., B10.S(9R) (*ssskkddd*) and B10₄.AQR (*qkkkkddd*) (Table I, footnote). It is evident from Table I that these three strains, together with the stimulating population B10.BR, all differ with respect to the responder cells at the *Qa-1* locus: CBA mice are *Qa-1^b*, whereas the other four strains are *Qa-1^a*. On this point several groups have shown that determinants encoded by genes mapping to the right of the *H-2* complex in or near the *Qa-1* locus (Table I, footnote) lead to high cytotoxic responses that do not show H-2 restriction (24-28).

The effect of depleting CBA T cells of reactivity to *Qa-1^a* determinants is shown in the right-hand column of Table I. To induce negative selection to these determinants, TDL were collected from CBA mice that had been injected 1 d previously with large doses (10^8) of irradiated (BALB/c × A/J)F₁ spleen cells, i.e., cells that express the foreign *Qa-1^a* determinants of B10.BR but lacking the majority of the minor HA of

TABLE II

Negative and Positive Selection of CBA Response to B10.BR Minor H Determinants: CML-Precursor Reactivity of Lymphoid Cells from CBA Mice Injected with Irradiated B10.BR Spleen cells 1 or 5 d Before

Irradiated spleen cells given to donors of CBA lymphoid cells	Dose of spleen cells injected*	CBA lymphoid cells tested (time after spleen cell injection)	CML activity of CBA lymphoid cells cultured in vitro with B10.K stimulators	
			Percent specific lysis on B10.BR targets with killer:target cell ratios of 30:1/15:1/3:1	Percent lytic activity compared with control cells‡
CBA	2×10^8	TDL (day 1)	62/55/17	—
B10.BR	2×10^8	TDL (day 1)	19/0/0	2§
B10.BR	1×10^8	TDL (day 1)	0/0/0	<1§
B10.BR	0.5×10^8	TDL (day 1)	65/58/19	111
CBA	2×10^8	spleen (day 1)	60/39/8	—
B10.BR	2×10^8	spleen (day 1)	81/65/23	212
CBA	2×10^8	lymph node (day 1)	86/72/46	—
B10.BR	2×10^8	lymph node (day 1)	80/73/42	84
CBA	2×10^8	TDL (day 5)	84/69/34	—
B10.BR	2×10^8	TDL (day 5)	87/85/49	288

* Spleen cells exposed to 2,000 rad and then cultured for 4 h in vitro before injection; dose refers to number of viable cells injected.

‡ CML activity of cells from mice injected with B10.BR spleen compared with control mice given syngeneic spleen (Materials and Methods).

§ Culture of these cells with third-party (BALB/c \times B6)F₁ (*H-2^d* \times *H-2^b*) stimulators led to high CML against B10 (*H-2^b*) and B10.D2(*H-2^d*) target cells, i.e., >40% lysis at killer:target cell ratios of 15:1.

this strain. It is evident that this procedure virtually abolished the anomalous lysis against B10.T(6R), B10.S(9R) and B10₄.AQR target cells but only marginally affected lysis of other targets. However, the point to be emphasized is that the specificity of CML was now in accord with the results of other workers (3, 4), i.e., CML to the minor HA of the stimulator strain was restricted to target cells that were compatible with the responder strain at either the *K*- or *D*-end of the *H-2* complex. There was no lysis of target cells that were *H-2* compatible only in the *I* region.

The data in Table I imply that CML to Qa-1^a determinants would be avoided if B10.K (*Qa-1^b*) cells rather than B10.BR cells were used as stimulators. This was indeed the case, i.e., in other experiments (data not shown), the CML specificity of CBA TDL stimulated with B10.K cells was virtually identical to that seen in Table I with CBA-CAF₁ TDL cultured with B10.BR cells. Hence to prevent the complication of generating anti-Qa-1^a activity,² most of the experiments to be considered below employed B10.K cells as stimulators and/or target cells. B10.K mice were in short supply and hence could not be used in all experiments. All of the experiments in the tables below were repeated at least once and normally 2–3 times.

Negative Selection to Minor HA Induced with Irradiated H-2-Compatible Spleen Cells. To induce selection, CBA mice were injected intravenously with large doses of CBA or

² It is worth mentioning that the anti-Qa-1 CML apparent in Table I tended to be variable in magnitude and on occasions was virtually undetectable (Table III, footnote).

TABLE III
*Negative Selection of CBA Response to B10.BR Minor H Determinants
 Induced by Injection of Irradiated B10.BR, B10, or B10.D2 Spleen Cells*

Irradiated spleen cells (2×10^8) given to donors of CBA TDL 1 d before*	H-2 haplotype of injected spleen cells	CML activity of CBA TDL cultured in vitro with B10.BR or SJL stimulators		
		Stimulation with B10.BR		Stimulation with SJL
		Percent specific lysis on B10.BR targets with killer:target cell ratios of 20:1/5:1/1:1‡	Percent lytic activity compared with control cells§	Percent specific lysis on SJL target with killer:target cell ratios of 20:1/5:1/1:1‡
CBA	H-2 ^k	52/33/5	—	ND
B10.BR	H-2 ^k	4/0/0	<1	58/38/9
B10	H-2 ^b	0/0/0¶	<1	67/36/14
B10.D2	H-2 ^d	2/0/0¶	<1	63/40/13

* Spleen cells were irradiated and cultured in vitro before injection as in Table II.

‡ In other experiments, CML tested on B10.K target cells gave similar results.

§ See Table II.

|| Not done.

¶ According to the data presented in Table I, selection against B10 or B10.D2 cells (both *Qa-1^b*) should have led to only a partial reduction in the response to B10.BR targets, the residual activity being directed to *Qa-1^k* determinants. The fact that there was near-total unresponsiveness towards B10.BR targets in the experiments illustrated (and also in one other experiment) is thus surprising. However, it should be mentioned that in other experiments injection of B10.D2 cells did lead to the expected incomplete reduction in the response to B10.BR (*Qa-1^k*) targets but complete unresponsiveness to B10.K (*Qa-1^b*) targets. We attribute this variability in the degree of unresponsiveness to B10.BR targets to the fact that, at times, anti-*Qa-1* killing forms only a minor component of the CBA/J anti-B10.BR response.² It is to be emphasized that in all three experiments in which B10.K targets were used to monitor selection (data not shown), prior injection of CBA/J mice with B10.BR, B10, or B10.D2 cells all led to almost total unresponsiveness to B10.K targets (<1% lytic activity).

B10.BR spleen cells; these cells were heavily irradiated before injection to prevent recirculation and entry into the central lymph (16). Lymphoid cells were harvested from the recipients 1 or 5 d later, stimulated in vitro with B10.K cells, and then tested for their capacity to exert CML against B10.BR targets (Experimental Design).

The results of a typical experiment are shown in Table II. It can be seen that TDL from CBA mice given B10.BR spleen cells in large doses 1 d before were unable to generate CML against B10.BR targets. Selection was specific because stimulation of the TDL with third-party H-2-different cells gave high levels of lysis (Table II, footnote; and later tables). The extent of selection against B10.BR was dose dependent: 2×10^8 and 10^8 cells usually caused near-complete unresponsiveness, whereas selection was minimal with smaller cell doses.

In contrast to the unresponsiveness of TDL, spleen cells taken at 1 d after B10.BR spleen cell injection contained normal or enriched numbers of B10.BR-reactive cells

TABLE IV

Negative and Positive Selection of CBA Response to B10.BR Minor H Determinants Induced by Injection of Sonicated B10.BR, B10, or B10.D2 Spleen Cells

Sonicates of spleen cells (2×10^8) given to donors of CBA lymphoid cells*	CBA lymphoid cells tested (time after spleen cell injection)	CML activity of CBA TDL cultured in vitro with B10.BR or SJL stimulators		
		Stimulation with B10.BR		Stimulation with SJL
		Percent specific lysis on B10.BR targets with killer:target cell ratios of 25:1/12:1/2:1	Percent lytic activity compared with control cells‡	Percent specific lysis on SJL targets with killer:target cell ratios of 25:1/12:1/2:1
CBA	TDL (day 1)	78/74/46	—	ND§
B10.BR	TDL (day 1)	7/9/0	<1	68/56/12
B10	TDL (day 1)	19/10/4	<1	68/52/10
B10.D2	TDL (day 1)	0/0/0	<1	58/58/10
CBA	spleen (day 1)	78/65/42	—	ND
B10.BR	spleen (day 1)	86/67/48	140	ND
B10	spleen (day 1)	61/48/26	32	ND
B10.D2	spleen (day 1)	70/70/33	58	ND
B10.BR	TDL (day 5)	74/61/37	42	ND

* See Materials and Methods for preparation of sonicates; sonicates given intravenously. To remove reactivity to Qa-1^a determinants, the CBA lymphoid donors were also injected with irradiated CAF₁ spleen cells, i.e., as in Table I.

‡ See Table II.

§ Not done.

(Table II). With lymph node cells the response was either unchanged or moderately reduced. When TDL were harvested at 5 d posttransfer rather than at day 1, anti-B10.BR responses were usually enhanced.

Collectively, these data imply that, as with other antigens, T cells that encounter minor HA in vivo rapidly become sequestered in the lymphoid tissues, particularly in the spleen, and then reenter the circulation several days later after clonal expansion.

Negative Selection with Irradiated H-2-Incompatible Spleen Cells. Further studies showed that the H-2 haplotype of the minor HA-bearing spleen cells given to the donors of CBA TDL had no apparent effect on the degree of selection to B10.BR minor HA determinants. Thus, irrespective of whether the injected spleen cells were H-2 compatible (B10.BR) or H-2 different (B10 or B10.D2) with respect to the responding T cells, reactivity to B10.BR targets (Table III) or B10.K targets (data not shown) was virtually undetectable; again, responsiveness to third-party antigens was unimpaired (Table III).

Negative Selection with Disrupted Spleen Cells. Because heavily irradiated cells die within a few hours of injection (29), the above data raised the possibility that antigen selection was initiated not by intact cells but by cell fragments. According to this possibility, disrupting the spleen cells before injection would not impair selection. Indeed, as shown in Table IV the injection of extensively sonicated B10.BR, B10, or B10.D2 spleen cells all led, 1 d later, to complete unresponsiveness of TDL against B10.BR targets. As with injection of intact irradiated cells, spleen cells taken at 1 d

TABLE V
Negative Selection of CBA Responses to B10.K Minor H Determinants Induced by Injection of Glutaraldehyde-treated Spleen Cells: Selection Induced with B10.BR Spleen but not with B10, B10.D2, or B10.Q Spleen

Glutaraldehyde-treated spleen cells (1.2×10^6) given to donors of CBA TDL 1 d before*	CML activity of CBA TDL cultured in vitro with B10.BR or (BALB/c \times B6)F ₁ stimulators			
	Stimulation with B10.BR		Stimulation with (BALB/c \times B6)F ₁ ($H-2^d \times H-2^b$)	
	Percent specific lysis on B10.K targets with killer:target cell ratios of 20:1/10:1/2:1	Percent lytic activity compared with control cells‡	Percent specific lysis on B10($H-2^b$) targets with killer:target cell ratios of 20:1/10:1/2:1	Percent specific lysis on B10.D2($H-2^d$) targets with killer:target cell ratios of 20:1/10:1/2:1
CBA	74/72/34	—	ND§	ND
B10.BR	10/5/0	<1	71/57/21	83/64/27
B10	67/62/31	84	ND	ND
B10.D2	58/52/22	49	67/56/16	6/3/0
B10.Q	77/63/39	127	ND	ND

* See Materials and Methods for glutaraldehyde treatment.

‡ See Table II.

§ Not done.

after injection of sonicated cells gave high responses and the reactivity of TDL returned toward normal levels by day 5 posttransfer.

Negative Selection with Glutaraldehyde-treated Spleen Cells. The simplest interpretation of the above data is that, at least in H-2-incompatible situations, T-cell selection depended upon the minor HA on the injected spleen cells being processed in some way by host cells, e.g., by macrophages or related cells. If so, mild fixation of the injected spleen cells might impair antigen processing and thereby block selection. To examine this point the injected spleen cells were treated lightly with glutaraldehyde (0.002% for 30 s [Materials and Methods]). This treatment had no immediate effect on cell viability as assessed by dye exclusion, and ⁵¹Cr-labeling studies indicated that the cells homed normally to the spleen after intravenous injection (data not shown).

The effects of glutaraldehyde-treating the selecting cells is shown in Table V. In the case of injecting H-2-compatible B10.BR cells, negative selection was as marked with glutaraldehyde-treated cells as with intact irradiated cells (compare with Table II). With H-2-different B10, B10.D2, or B10.Q cells, by contrast, glutaraldehyde treatment almost completely abolished selection, i.e., TDL from mice preinjected with these cells generated near-normal levels of CML against either B10.K targets (Table V) or B10.BR targets (data not shown).

Although H-2-different glutaraldehyde-treated cells failed to induce negative selection to minor HA, injection of these cells did lead to effective selection against the H-2 determinants on the cells. For example, when CBA TDL from recipients of glutaraldehyde-treated B10.D2($H-2^d$) cells were stimulated in vitro with (BALB/c \times B6)F₁($H-2^d \times H-2^b$) cells, CML was low or absent against B10.D2 targets but was high against the third-party B10($H-2^b$) targets (Table V).

TABLE VI

Negative Selection to Minor H Determinants Induced by Injecting CBA Mice with Glutaraldehyde-treated Spleen Cells from Mice of the B10 Recombinant Strains

Glutaraldehyde-treated spleen cells (1.2×10^6) given to donors of CBA TDL 1 d before*	CML activity of CBA TDL cultured in vitro with B10.K stimulators. Killer:target cell ratios of 25:1/12:1/2:1					
	Percent specific lysis on B10.K targets	Percent lytic activity	Percent specific lysis on B10.A targets	Percent lytic activity	Percent specific lysis on B10.OL targets	Percent lytic activity
CBA (kkkkkkkk)	49/41/22		49/42/17	—	34/28/13	—
B10.OL (ddddddkk)	31/27/7	22	41/28/9	46	8/3/0	<1
B10.A (kkkkkddd)	0/0/0	<1	0/0/0	<1	0/0/0	<1
(B10.A \times B10.OL)F ₁	9/1/0	<1	9/7/0	<1	0/0/0	<1
B10 ₄ .AQR (qkkkkddd)	0/0/0	<1	0/0/0	<1	0/0/0	<1

* See Table V.

Negative Selection with Glutaraldehyde-treated Cells from H-2 Recombinant Mice. To attempt to map the apparent H-2 restriction of selection observed with glutaraldehyde-treated cells, selection was studied in CBA mice given glutaraldehyde-treated cells from mice of the B10 recombinant strains. When B10.OL (ddddddkk) cells were used for selection, culture of the recipient CBA TDL with B10.K stimulators led to effective lysis against B10.A (kkkkkddd) targets but not against B10.OL targets (Table VI). These data implied that selection affected T cells specific for determinants mapping in the D-end of the H-2 complex but did not involve K-end-specific T cells. Reciprocal findings were expected when B10.A cells were used for selection. Curiously, however, this was not the case: Selection with B10.A cells led to unresponsiveness not only against B10.A targets but also against B10.OL targets, i.e., as for selection with (B10.A \times B10.OL)F₁ cells. Identical findings applied to selection with B10₄.AQR (qkkkkddd) cells, i.e., cells that were H-2 compatible with the host only in the I region. Three other experiments gave comparable findings. In two of these experiments, as a specificity control, the cells selected against B10.A and B10₄.AQR cells were tested for their capacity to generate CML against third-party H-2^b target cells. High levels of lysis were obtained (data not shown).

Discussion

Most of the evidence on cross-priming to minor HA has come from studies on the long-term effects of immunizing mice with H-2-different cells. Our findings that negative selection to minor HA can be induced with irradiated or disrupted H-2-different cells implies that cross-priming can occur very rapidly, i.e., within 1 d. As with other examples of cross-priming, these data per se might either reflect processing of antigen by host cells (thus enabling the antigen to become associated with self H-2 determinants) or alternatively signify a lack of H-2 restriction during T-cell induction. The experiments on the effects of using glutaraldehyde-treated cells for selection were designed to discriminate between these two possibilities.

The rationale here was that if cross-priming resulted from antigen processing, treatment of the antigen-bearing cells with a fixative such as glutaraldehyde might interfere with this phenomenon and thereby impair the capacity of the cells to induce

selection across H-2 barriers. Indeed, testing this prediction showed that negative selection to minor HA induced by H-2-different cells was low or undetectable when the cells were treated briefly with low concentrations of glutaraldehyde before injection. Significantly, glutaraldehyde treatment did not affect selection in H-2-compatible situations, i.e., in contrast to irradiated or sonicated cells, selection with glutaraldehyde-treated cells was H-2 restricted.

In interpreting these data it should first be stressed that the precise mechanism of action of glutaraldehyde treatment in the selection assay is unknown. Because glutaraldehyde cross-links proteins (30), our working hypothesis is that treatment with this reagent retards destruction of the antigen-bearing cells and/or interferes with antigen processing by host cells, e.g., by somehow preventing moieties of antigen from forming an immunogenic association with host H-2 determinants. Although this is largely speculation it is difficult to offer an alternative explanation that fits the data. The fact that glutaraldehyde-treated cells remained immunogenic in H-2-compatible situations makes it unlikely that the antigenicity of the minor HA on the selecting cells was impaired. Likewise, the H-2 determinants on the cells seemed to remain intact because effective selection was observed against the H-2 determinants on glutaraldehyde-treated cells (Table V). Indeed there appears to be general agreement in the literature that glutaraldehyde treatment does not denature cell surface components (19, 31, 32), at least under the mild conditions used in this study. It may also be mentioned that in our hands glutaraldehyde treatment does not prevent long-term cross-priming, i.e., CBA mice injected several weeks before with glutaraldehyde-treated B10.D2 spleen cells give typical secondary responses to B10.K cells *in vitro* (R. Korngold and J. Sprent. Unpublished data.).

Whatever the exact consequences of treating cells with glutaraldehyde, the fact that selection to minor HA was H-2 restricted would seem to exclude the possibility that selection reflected an H-2-unrestricted response to antigen.³ Can one conclude therefore that cytotoxic T-cell induction depends upon some form of antigen processing? In the case of cross-priming (presentation of antigen on H-2-different cells), the data on the immunogenicity of irradiated and disrupted cells vs. glutaraldehyde-treated cells in the selection assay are strongly in favor of this viewpoint. The precise mechanism of induction to minor HA, however, is far from clear. Because the specificity of the effector cells was directed to K- and D-end H-2 determinants (Table I), it is tempting to extrapolate from the induction of I region-restricted T cells involved in T-B collaboration and argue that cytotoxic precursor cells are triggered by contact with antigen that becomes associated with K/D determinants on macrophages or related cells. Although appealing from the point of view of symmetry, this notion has to be viewed with caution for the following reasons: (a) The recent evidence that I region-restricted T-helper cells control responses to minor HA (33-36) adds a new dimension to the problem of cytotoxic T-cell induction and implies that, in addition to K/D determinants, the antigen-presenting cells might also have to express Ia determinants. (b) There is no direct evidence that macrophages or accessory cells play other than a nonspecific role in cytotoxic T-cell induction *in vitro* (37). For in

³ Although primed mice were used in these experiments, recent studies in which unprimed T cells were primed to antigen on adoptive transfer after selection to glutaraldehyde-treated cells have given similar findings.

vivo responses, the only direct evidence that a specific class of antigen-presenting cells is required for CML induction is the finding of Zinkernagel et al. (38) that H-2-compatible cells of bone marrow origin are needed for generating CML to virus-infected cells; whether these cells belong to the macrophage lineage is unknown. (c) If typical macrophages do control antigen presentation, it remains to be explained why cross-priming is easily demonstrable in vivo but not in vitro (12). For these reasons the case that cytotoxic T-cell induction is simply a reflection of T-macrophage interaction and closely resembles the induction of T cells involved in T-B collaboration is far from being proved.

The conclusion that antigen processing is a prerequisite for inducing responses to minor HA across H-2 barriers does not necessarily imply that processing is mandatory in H-2-compatible situations. Indeed the argument that glutaraldehyde treatment impairs antigen processing rests on the assumption that the selection to minor HA observed with H-2-compatible glutaraldehyde-treated cells reflects a response to the injected cells per se. This latter finding was predictable because virtually all of the injected spleen cells presumably expressed the requisite combination of minor HA plus self K/D determinants. Nevertheless it is conceivable that, even in this situation, the capacity to present antigen is unique to a particular class of cells. This question is currently under investigation.

Finally, comment should be made on the paradoxical effects of inducing selection with glutaraldehyde-treated cells from mice of the B10 recombinant strains. Because there is general agreement that H-2K- and H-2D-restricted CML is mediated by distinct subgroups of T cells, the expectation was that these subgroups could be separated by inducing selection with minor HA-bearing cells that were compatible with the host at only one end of the H-2 complex. The results of selecting CBA(H-2^k) mice against glutaraldehyde-treated B10.OL (ddddddd) cells were consistent with this prediction, i.e., selection with these cells abolished CML to B10.OL target cells but only minimally impaired the response to B10.A (kkkkddd) targets. Reciprocal selection with B10.A cells, by contrast, led to unresponsiveness against both B10.A and B10.OL targets. In interpreting these data it may be relevant that in the case of B10.A cells, the cells used for selection shared Ia antigens with the host. Hence, negative selection with B10.A cells might remove not only K-end-restricted cytotoxic precursors but also I region-restricted T-helper cells critical for facilitating differentiation in vitro of the unselected D-end-restricted cells (36). Accordingly, selection with cells that were H-2 compatible with the host only in the I region would be expected to have a similar effect. This was indeed found to be the case, i.e., selection of CBA mice with glutaraldehyde-treated B10₄.AQR (qkkkkddd) cells led to marked unresponsiveness against both B10.A and B10.OL targets (Table VI).

Which particular H-2I subregion(s) must be matched to obtain selection is currently under investigation. Preliminary studies have shown unexpectedly that selection of the CBA anti-B10.K response can be induced with either B10.A(4R) (kkbbbb) cells or B10.HTT (ssskkkd) cells, i.e., cells that are H-2I compatible with the host only at the I-A and I-E/C subregions, respectively. Such findings are not easily interpreted. Proving the participation of I region-restricted T-helper cells in this system, however, will require demonstrating that addition of these cells in culture can reconstitute the cytotoxic activity of the supposedly T-helper cell-deficient selected T cells. Such studies are in progress.

Summary

Intravenous injection of CBA mice with H-2-compatible irradiated B10.BR spleen cells led to a sequence of negative and positive selection of the host T-cell response against the multiple foreign minor histocompatibility antigens (HA) on the injected cells. By 1 d posttransfer, thoracic duct lymphocytes (TDL) of the host had lost the capacity to differentiate *in vitro* into cytotoxic cells specific for the injected minor HA; spleen and lymph node cells, by contrast, gave normal or enriched responses at this time. By 5 d posttransfer, TDL were hyperresponsive to the injected antigens. Selection with disrupted (sonicated) cells gave similar findings.

With injection of either irradiated or disrupted spleen cells, the H-2 haplotype of the minor HA-bearing cells had no apparent effect on the magnitude of selection. By contrast, treatment of spleen cells with glutaraldehyde before injection led to H-2 restriction of selection, i.e., negative selection of the CBA response to B10.BR was marked with injection of glutaraldehyde-treated H-2-compatible B10.BR cells but was minimal with H-2-different B10 or B10.D2 cells. These data are taken to imply that, at least in H-2-incompatible situations, the minor HA-bearing cells must be processed by host cells, i.e., to allow the antigens to become associated with self H-2 determinants.

Circumstantial evidence from studies on the specificity of selection induced with glutaraldehyde-treated cells from mice of the B10 recombinant strains suggested that I region-restricted T cells may control the induction of H-2K, D-restricted cytotoxic precursor cells.

The skillful typing assistance of Mrs. K. King and Mrs. T. McKearn is gratefully acknowledged. We are most grateful to Doctors David, Doherty, Elkins, and Gasser for their gifts of various mouse strains, and to Doctors Elkins, Gasser, McKearn, Silvers, and Wettstein for reading the manuscript.

Received for publication 17 September 1979 and in revised form 15 November 1979.

References

1. Doherty, P. C., R. V. Blanden, and R. M. Zinkernagel. 1976. Specificity of virus-immune effector T cells for H-2K or H-2D compatible interactions: implications for H-antigen diversity. *Transplant. Rev.* **29**:89.
2. Shearer, G. M., T. G. Rehn, and A. M. Schmitt-Verhulst. 1976. Role of the murine major histocompatibility complex in the specificity of *in vitro* T cell mediated lympholysis against chemically-modified autologous lymphocytes. *Transplant. Rev.* **29**:222.
3. Bevan, M. J. 1975. The major histocompatibility complex determines susceptibility to cytotoxic T cells directed against minor histocompatibility antigens. *J. Exp. Med.* **142**:1349.
4. Gordon, R. D., E. Simpson, and L. E. Samelson. 1975. *In vitro* cell-mediated immune responses to the male specific (H-Y) antigen in mice. *J. Exp. Med.* **142**:1108.
5. Snell, G. D., N. Wheeler, and M. Aaron. 1957. A new method of typing inbred strains of mice for histocompatibility antigen. *Transplant. Bull.* **4**:18.
6. Gasser, D. L., and W. K. Silvers. 1972. Genetics and immunology of sex-linked antigens. *Adv. Immunol.* **15**:215.
7. Gordon, R. D., B. J. Mathieson, L. E. Samelson, E. A. Boyse, and E. Simpson. 1976. The effect of allogeneic presensitization on H-Y graft survival and *in vitro* cell-mediated responses to H-Y antigen. *J. Exp. Med.* **144**:810.
8. Wettstein, P. T., G. Haughton, and J. A. Frelinger. 1977. Immune response to histocom-

- patibility antigens: H-2 control of *in vivo* and *in vitro* effector:target interactions. In *Immune System: Genetics and Regulation*. E. E. Sercarz, L. A. Herzenberg, and C. F. Fox, editors. Academic Press, Inc., New York. 623.
9. Murasko, D. M. 1978. Apparent lack of H-2 restriction of allograft rejection. *J. Immunol.* **121**:958.
 10. Smith, F. I., and J. F. A. P. Miller. 1979. Delayed-type hypersensitivity to allogeneic cells in mice. III. Sensitivity to cell surface antigens coded by the major histocompatibility complex and by other genes. *J. Exp. Med.* **150**:965.
 11. Bevan, M. J. 1976. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med.* **143**:1283.
 12. Matzinger, P., and M. T. Bevan. 1977. Induction of H-2 restricted cytotoxic T cells: *in vivo* induction has the appearance of being unrestricted. *Cell. Immunol.* **32**:92.
 13. Sprent, J., J. F. A. P. Miller, and G. F. Mitchell. 1971. Antigen-induced selective recruitment of circulating lymphocytes. *Cell. Immunol.* **2**:171.
 14. Rowley, D. A., J. L. Gowans, R. C. Atkins, W. L. Ford, and M. E. Smith. 1972. The specific selection of recirculating lymphocytes by antigen in normal and preimmunized rats. *J. Exp. Med.* **136**:499.
 15. Ford, W. L., and R. C. Atkins. 1971. Specific unresponsiveness of recirculating lymphocytes after exposure to histocompatibility antigens in F₁ hybrid rats. *Nat. New Biol.* **243**:178.
 16. Sprent, J., and J. F. A. P. Miller. 1976. Effect of recent antigen priming on adoptive immune responses. III. Antigen-induced selective recruitment of subsets of recirculating lymphocytes reactive to H-2 determinants. *J. Exp. Med.* **143**:585.
 17. Sprent, J. 1978. Role of the H-2 complex in induction of T helper cells in vivo. I. Antigen-specific selection of donor T cells to sheep erythrocytes in irradiated mice dependent upon sharing of H-2 determinants between donor and host. *J. Exp. Med.* **148**:478.
 18. von Boehmer, H., and K. Shortman. 1973. The separation of different cell classes from lymphoid organs. IX. A simple and rapid method for removal of damaged cells from lymphoid cell suspensions. *J. Immunol.* **2**:293.
 19. Bubbers, J. E., and C. S. Henney. 1975. Studies on the synthetic capacity and antigenic expression of glutaraldehyde-fixed target cells. *J. Immunol.* **114**:1126.
 20. Brunner, K. T., J. Mauel, J. C. Cerottini, and B. Chapuis. 1968. Quantitative assay of the lytic action of immune lymphoid cells on ⁵¹Cr-labelled allogeneic target cells *in vitro*: inhibition by isoantibody and by drugs. *Immunology.* **14**:181.
 21. Cerottini, J. C., and R. T. Brunner. 1974. Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. *Adv. Immunol.* **18**:67.
 22. Korngold, R., and J. Sprent. 1978. Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. *J. Exp. Med.* **148**:1687.
 23. Klein, J., L. Flaherty, J. L. VandeBerg, and D. C. Shreffler. 1978. H-2 haplotype, genes, regions, and antigens: first listing. *Immunogenetics.* **6**:489.
 24. Stanton, T. H., and E. A. Boyse. 1976. A new serologically defined locus, Qa-1 in the T1a-region of the mouse. *Immunogenetics.* **3**:525.
 25. Klein, J., and C. Chiang. 1978. A new locus (H-2T) at the D end of the H-2 complex. *Immunogenetics.* **6**:235.
 26. Forman, J., and L. Flaherty. 1978. Identification of a new CML target antigen controlled by a gene associated with the Qa-2 locus. *Immunogenetics.* **6**:227.
 27. Fisher Lindahl, K. 1979. Unrestricted killer cells recognize an antigen controlled by a gene linked to T1a. *Immunogenetics.* **8**:71.
 28. Wernet, D., and J. Klein. 1979. Unrestricted cell-mediated lympholysis to antigens linked to the T1a locus in the mouse. *Immunogenetics.* **8**:361.

29. Anderson, R. E., and N. L. Warner. 1976. Ionizing radiation and the immune response. *Adv. Immunol.* **24**:215.
30. Avrameas, S., and T. Ternynck. 1969. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. *Immunochemistry.* **6**:53.
31. Frost, P., and C. V. Sanderson. 1975. Tumor immunoprophylaxis in mice using glutaraldehyde-treated syngeneic tumor cells. *Cancer Res.* **35**:2646.
32. Lightbody, J. J., and Y. M. Kong. 1976. Mitogen-stimulated glutaraldehyde-fixed spleen cells: ability to stimulate in the mixed lymphocyte reaction and generate effector cells in cell mediated lympholysis. *J. Immunol.* **117**:1336.
33. Simpson, E., and R. D. Gordon. 1977. Responsiveness to HY antigen. Ir gene complementation and target cell specificity. *Immunol. Rev.* **35**:59.
34. Gordon, R. D., and E. Simpson. 1977. Immune response gene control of cytotoxic T cell responses to HY. *Transplant. Proc.* **9**:885.
35. von Boehmer, H., W. Haas, and N. K. Jerne. 1978. Major histocompatibility complex-linked immune-responsiveness is acquired by lymphocytes of low-responder mice differentiating in thymus of high-responder mice. *Proc. Natl. Acad. Sci. U. S. A.* **75**:2439.
36. von Boehmer, H., and W. Haas. 1979. Distinct Ir genes for helper and T killer cells in the cytotoxic response to H-Y antigen. *J. Exp. Med.* **150**:1134.
37. Pettinelli, C. B., A. Schmitt-Verhulst, and G. M. Shearer. 1979. Cell types required for H-2-restricted cytotoxic responses generated by trinitrobenzene sulfonate-modified syngeneic cells or trinitrophenyl-conjugated proteins. *J. Immunol.* **122**:847.
38. Zinkernagel, R. M., G. N. Callahan, A. Althage, S. Cooper, J. W. Streilein, and J. Klein. 1978. The lymphoreticular system in triggering virus plus self-specific cytotoxic T cells. Evidence for T help. *J. Exp. Med.* **147**:897.