CYTOTOXIC T CELL RESPONSE TO
MINOR HISTOCOMPATIBILITY ANTIGENS:
APPARENT LACK OF H-2 RESTRICTION IN KILLERS
STIMULATED BY MEMBRANE FRAGMENTS*

By LINDA M. PILARSKI and DIMITRIOS VERGIDIS‡

From the Department of Immunology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Cytotoxic T cells generated in response to minor histocompatibility antigens (minor H Ag)[1] are H-2 restricted in that they appear to be able to recognize the minor H Ag only in association with H-2K or H-2D major H Ag (1-4). This could result from recognition by one receptor of a new antigenic determinant formed by interaction of both minor and major H moieties or could be caused by independent concurrent recognition of minor H and of H-2 by two separate receptors. H-2-restricted recognition may be a fundamental property of cytotoxic T cells or alternatively, it may be a result of the selective forces operating on the T cell pool during ontogeny such that only H-2-restricted precursor T cells are able to respond to a minor H antigen challenge. This latter model implies that the potential for unrestricted recognition by cytotoxic T cells does in fact exist, but that such precursor T cells are not effectively triggered. This could be due either to the type of priming and in vitro challenge regimes, or to suppressive interactions preventing this induction and/or expression. With this latter mechanism in mind, we have attempted to manipulate the cytotoxic response specific for minor H Ag such that any putative T cell precursors whose receptor specificity is directed against a minor H Ag would be triggered. In theory, this type of approach could reveal subsets of killer cells not normally induced that express previously undetected receptor specificities.

The cytotoxic T cell response to minor H Ag shares similar inductive requirements to those of the response to major H-2 Ag (5). It is positively regulated by Lyt-1$^+$2$^-$ helper T cells and can be suppressed by inhibitory T cells. The inhibitors appear to be quite different from those that suppress responses to major H in that their Lyt phenotype is Lyt-1$^+$2$^-$ (6). An adherent type of antigen-processing cell may or may not be involved in the generation of a cytotoxic response based on evidence in the literature (7-14). The present study establishes such a requirement.

Although it exerts a strong quantitative effect on the response, we have found no evidence that would indicate an ability of the adherent cell to change the character of the cytotoxic response in terms of restriction. However, we did find that the physical state of antigen has a profound influence on the H-2 restriction properties of the killers

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generated. Whenever B10.D2 or DBA/2 minor H Ag were present as intact cells, an H-2-restricted response was generated; in contrast, when the antigen was B10.D2 or DBA/2 freeze-thaw membrane fragments, the response, although totally dependent on adherent cells, was predominantly unrestricted, although it did include a smaller subset of H-2 restricted killers. The apparently unrestricted killers could not be explained as being H-2-restricted killers whose receptor specificity is crossreactive with allogeneic H-2; they seem to recognize minor H as an independent entity. This implies that the T cell receptor repertoire does in fact include receptors for minor H, but that these T cells are not induced when the stimulating antigen is an intact cell bearing minor H. Disruption of the cell membrane to produce fragments may include disruption of the antigenic organization such that minor H Ag come into existence as independent immunogenic units.

Materials and Methods

Animals and Immunizations.   Male or female 6–12-wk BALB/cCr, B10.D2, C57B1/10 (B10), and DBA/2 mice were obtained from the University of Alberta animal breeding unit. B10.D2 and BALB/cJ mice were purchased from The Jackson Laboratory, Bar Harbor, ME. BALB.B mice were purchased from Dr. H. McDevitt, Stanford University, Palo Alto, CA. No differences in experimental results were seen using B10.D2 derived from Alberta as compared with those from The Jackson Laboratory. Responder BALB/c mice were primed by intravenous transfer of 1–2 × 10^7 irradiated B10.D2 or DBA/2 spleen cells; at 2–4-wk intervals they received one to two boosts. These primed mice were used in experiments from 1 to 6 mo after the last boost. The best responses were obtained from spleen cells taken 6 wk or later after the last boost.

In Vitro Culture.   Cell cultures were in 24-well Linbro culture plates (Linbro Chemical Co., Hamden, CT). Culture medium was minimal essential medium (MEM) (F-15; Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) with 10^{-4} M beta-mercaptoethanol, 5% fetal calf serum (FCS) (Gibco Laboratories), and 50 μM gentamycin. Each culture contained 10^7 (untreated or nylon wool-passed) primed spleen cells as responders, and either 10^7 untreated DBA/2 or B10.D2 stimulator cells, the equivalent of 10^7 nylon wool-passed stimulators, or the freeze-thaw antigen (FT-Ag) equivalent of 10^7 cells. Stimulator cells were given 1,500 rad before use. Adherent cells where added were the adherent cells derived from 10^7 unprimed BALB/c spleen cells unless otherwise indicated. Cultures were incubated at 37°C in 10% CO_2/air for 5 d.

Cell Fractionation.   Nylon wool fractionation of responder and stimulator cells was by the method of Julius et al. (15); nonadherent cells were recovered and used at numbers equivalent to the unfractionated populations to avoid enrichment of remaining cell types. Nonadherent cells were generally 15–25% of the total population loaded on the column.

Adherent cells were prepared by the method of Shiozawa et al. (16). Normal spleen cells from the mouse strain indicated in the results section were allowed to adhere for 120 min to the bottom of the Linbro well to be used for the subsequent culture. The nonadherent cells were dislodged by gentle rocking and removed by suction; the remaining adherent cells were then washed twice with medium. If these adherent cells were to be preincubated with FT-Ag, the antigen was added in medium without FCS and the mixture allowed to incubate for 90 min at 37°C. Excess antigen was not washed away unless so indicated. Plates were then irradiated with 1,500 rad, and responder cells were added to each well. If adherent cells were not preincubated with FT-Ag, then they were irradiated before addition of other cell types.

FT-Ag Preparation.   Spleen cells to be disrupted were suspended at a concentration of 10^8/ml in MEM without FCS. The suspension was frozen in liquid N_2 and thawed in a 37°C bath; this was repeated twice more. The preparation was centrifuged at 2,000 rpm to remove unlysed cells; this was verified by microscopic inspection. It was then stored in liquid N_2 until use.

Target Cells.   Spleen cells from the appropriate mouse strain were cultured in Costar 6-well plates (Costar, Data Packaging, Cambridge, MA), in 5 ml of medium plus 5% FCS plus 5 μg/ml concanavalin A (Con A) for 2–3 d before use as either labeled targets or cold competitors. Labeling with ^{51}Cr was as previously described (5). Although not normally used as targets,

thioglycollate-stimulated peritoneal exudate cells (PEC) were tested to determine if the same pattern of results could be obtained as that seen with blasts. In H-2 restriction experiments, identical results were obtained using either PEC or blast cells.

Assay. All the wells in a particular group (from 2 to 18 replicates) were pooled and resuspended in a volume of 1 ml/culture. Cytotoxicity was assayed by adding $3-5 \times 10^4$ labeled target cells to microtiter wells along with 1/10, 1/20, 1/40, and 1/80 fraction of a culture for 4 h at 37°C. Each culture dilution was assayed in triplicate as previously described (5). For competition assays, cold targets were added to the wells at ratios of 30:1 or 60:1, cold/hot.

Results

Adherent Cells Are Required for the Response to Minor H Ag. To clearly delineate the role of adherent cells in the cytotoxic response, it was necessary that the responder cell population and the source of antigen be depleted of adherent cells. To this effect, we used nylon wool-passed primed BALB/c responder cells and either nylon wool-passed DBA/2 cells or a membrane preparation thereof (FT-Ag) as our source of antigen. The experiment shown in Table I shows that whereas nylon wool responder cells generate strong cytotoxicity to untreated DBA/2 stimulators (line 1), weak or no cytotoxicity arises in cultures with nylon wool-passed DBA.2 cells or DBA/2 FT-Ag (lines 3 and 5). Co-culture of irradiated normal BALB/c adherent cells with these adherent cell-depleted responders reconstitutes the cytotoxic response to minor H Ag expressed on both nylon wool-passed DBA/2 cells and on FT-Ag preparations.

Experiments to determine the optimal conditions for the response to FT-Ag indicated that the FT-Ag preparation was effective over a 10-fold range of cell equivalents (Table II, experiment 1). The number of adherent cells required for the response was between 10^6 and 10^7 cells per culture, indicating that 1/10 the number of adherent cells originally resident in the responder population were capable of fully stimulating the response (Table II, experiment 2). No response was obtained in the absence of FT-Ag (experiment 1, line 6), and DBA/2 adherent cells were able to stimulate a strong anti-minor H response (experiment 2, line 7). These controls establish the fact that stimulation with BALB/c adherent cells incubated with FT-Ag is as effective as stimulating with an intact DBA/2 adherent cell.

Our initial assumption was that these adherent cell were "expressing" on their

TABLE I
Adherent Cells are Required for the Response of Nylon Wool-passed Responder Cells to Minor H Ag

Irradiated Stimulator Cells	Percent specific lysis		
	1:10	1:20	1:40
Unfractionated DBA/2	57	58	44
Unfractionated DBA/2 + BALB/c adherent cells	70	74	64
Nylon wool-passed DBA/2	28	27	19
Nylon wool-passed DBA/2 + BALB/c adherent cells	80	86	74
DBA/2 FT-Ag	0	0	0
DBA/2 FT-Ag + BALB/c adherent cells	84	93	75

2×10^7 equivalent nylon wool-passed DBA/2 primed BALB/c responder cells were cultured with the indicated stimulator cells with or without irradiated adherent cells derived from 10^7 BALB/c spleen cells. Assay was on day 5 on DBA/2 Con A blast cells. The 1:40 dilutions represent a killer to target ratio of 2.5:1.

TABLE II

Conditions for Stimulation of a Response by Nylon Wool-passed Responder Cells to FT-Ag

	Responder cells	Cell equivalents of FT-Ag	Percent specific lysis	
			FT-Ag	FT-Ag + adherent cells
Experiment 1:	Untreated	10×10^6	49	—
	NW-passed*	10×10^6	0	27
	NW-passed	5×10^6	0	42
	NW-passed	2×10^6	0	41
	NW-passed	1×10^6	0	25
	NW-passed	None	—	0
		Number of BALB/c adherent cells		
Experiment 2:	NW-passed	None	0	—
	NW-passed	10×10^6	—	33
	NW-passed	3×10^6	—	51
	NW-passed	1×10^6	—	42
	NW-passed	3×10^5	—	11
	NW-passed	1×10^5	—	0
	NW-passed	DBA/2 adherent cells: 58		

10^7 primed B10.D2 or DBA/2 primed BALB/c responder cells were co-cultured with the indicated adherent cell with or without FT-Ag. In experiment 1, the adherent cells from 10^7 BALB/c spleen cells were used with B10.D2 FT-Ag. In experiment 2, DBA/2 FT-Ag was used. For experiment 1, the 1:80 dilution point is recorded (K/T, 0.5:1); for experiment 2, the 1:40 dilution is recorded (K/T, 1:1). In both experiments the entire dilution series of 1:10–1:80 was run.
* Nylon wool-passed.

surface the minor H Ag determinants from the FT-Ag preparation. If this were correct, one might expect that if FT-Ag were presented to the adherent cells for "processing", and the unbound antigen removed, then the adherent cells might present antigen in an immunogenic form to responder cells analagous to the antigen presentation by adherent cells that occurs in the T cell proliferation assay (17, 18). We found that this was not the case. In the experiment of Table III, adherent cells were incubated with FT-Ag for 90 min and then washed to remove unbound antigen. Such "antigen-pulsed" cells were unable to stimulate a cytotoxic response to minor H (line 3). This experiment also tested the ability of adherent cells from primed spleen to present antigen; these were as efficient as were normal adherent cells and also required the continued presence of the FT-Ag to stimulate the response (lines 4 and 5).

Killers Stimulated by Adherent Cells Presenting FT-Ag Appear to Lack H-2 Restriction. Cytotoxic T cells specific for minor H that have been generated under "conventional" conditions are unable to kill target cells bearing the correct minor H but an incorrect major H [e.g., B10(H-2^b)]. The killers generated by stimulation with cells bearing minor H or with syngeneic adherent cells incubated with FT-Ag were typed for H-2 restriction specificity by the method of cold target competition (Table IV). If an intact DBA/2 cell was used as the stimulator, the resulting killers were always H-2 restricted as defined by the inability of cold B10 cells to compete for the

TABLE III

Continued Presence of FT-Ag is Required for the Adherent Cell-stimulated CTL Response

Adherent cell	CTL Response			
	1:10	1:20	1:40	1:80
None	0	0	0	0
Normal adherent cell; excess FT-Ag not washed away	29	24	13	14
Normal adherent cell; excess FT-Ag washed away	0	0	0	0
Adherent cells from primed mice; excess FT-Ag not washed away	32	26	23	10
Adherent cells from primed mice; excess FT-Ag washed away	0	0	0	0
Normal adherent cells; no FT-Ag added	0	0	0	0

Adherent cells were preincubated with B10.D2 FT-Ag for 90 min, and then excess antigen was washed away where indicated. 10^7 equivalent nylon wool-passed B10.D2-primed BALB/c responders were then added. Assay was on B10.D2 Con A blast cells. The washing procedure does not appear to damage the adherent cells in any way since if fully allogeneic FT-Ag is used, adherent-bound fragments are fully immunogenic for an anti-H-2^b response (data not shown).

TABLE IV

Killers Stimulated by Adherent Cells Preincubated with FT.Ag Do Not Appear to be H-2 Restricted

Antigen	BALB/c adherent cells	Percent Lysis of ^{51}cr-DBA/2 cold competitor				
		None	DBA/2	B10	BALB/c	
Unfractionated DBA/2	—	57	0	52	50	(1:10)*
NW-passed‡ DBA/2	—	28	5	33	40	(1:10)
DBA/2 adherent cells	—	80	13	68	72	(1:40)
Unfractionated DBA/2	+	74	9	62	68	(1:20)
NW-passed DBA/2	+	86	10	63	79	(1:20)
DBA/2 FT-Ag	+	75	9	6	70	(1:40)
DBA FT-Ag	§	23	4	0	23	(1:10)

10^7 nylon wool-passed DBA/2-primed BALB/c responder cells were cultured with the indicated stimulator combination for 5 d. Assay was on 3 × 10^4 DBA/2 Con A blast cells. Cold competitors were Con A blast cells used at 30:1 ratio of cold to hot. The competition was done for a 1:10 to 1:40 dilution series of killers, but only the indicated dilution is recorded above; results were consistant for the whole series. Results were obtained in four independent experiments using four different batches of primed mice. The lack of H-2 restriction seen under the conditions of line 6 has been observed in 10 independent experiments, using both DBA/2 and B10.D2 stimulated responses.

Cytotoxicity on B10 targets: line 1, 2, 0%; lines 3–7, 20–60%. Cytotoxic T cells in cultures of the above type were all found to be of the Lyt-1^+2^+ phenotype. This includes both restricted and unrestricted killers generated in response to either B10.D2 or to DBA/2. This is in contrast to previously published work by ourselves (6) and others (4), and suggests that the Ly phenotype changes depending on the environmental status of the mice at any given point in time.

* The culture dilution at which competition first became maximal is reported here. Most of the responses are on the plateau of the killing curve and are therefore an underestimate of the actual killing potential. It was felt that such responses would bias the results against observing a lack of H-2 restriction, and that competition by cold B10 would therefore be highly significant.

‡ Nylon wool-passed.

§ Untreated responder cells were used. No additional adherent cells were added and no preincubation with FT-Ag occurred.

killers (lines 1–3). Addition of syngeneic BALB/c adherent cells to the cultures (lines 4 and 5) or in interleukin 2 (data not shown) did not change the H-2 restricted character of the response.

By contrast, killers stimulated by syngeneic adherent cells presenting DBA/2 FT-Ag had a specificity that did not appear to be H-2 restricted. Both DBA/2 and B10 cold targets were very efficient competitors of the response to DBA/2 labeled targets (lines 6 and 7). This was not because adherent cells were the antigen-presenting unit, as DBA/2 adherent cells stimulated an H-2-restricted response (line 3 compared with line 6). Neither was it due to considerations involving the magnitude of the response, as the killing activity generated in response to DBA/2 adherent cells was always comparable to that stimulated by BALB/c adherent cells presenting DBA/2 FT-Ag. Increasing the numbers of cold competitors to a ratio of 60:1 did not reveal the existence of non-H2-restricted killers in the populations stimulated by DBA/2 cells (data not shown).

The non-H-2-restricted killers found in cultures stimulated by BALB/c adherent cells presenting FT-Ag did not always comprise the entire set of killers. Depending on the group of primed mice used, we frequently observed a small subset of killers specific for B10.D2 or DBA/2 that could not be competed by B10 cold competitors. When it was generated, this H-2-restricted subset always comprised a smaller proportion of the killers than the subset of unrestricted killers.

Although killers were detectable on B10 target cells under certain conditions, we found that this was not a reliable indicator of the existence or extent of non-H-2-restricted killers. In the experiment of Table IV, for example, no killing of B10 targets was seen if unfractionated or nylon wool-passed DBA/2 cells were used as stimulators, but if BALB/c adherent cells were added to these cultures, or if DBA/2 adherent cells were used as stimulators, then good killing of B10 targets was generated. These same adherent cell-supplemented cultures, however, exhibited only H-2-restricted killers on DBA/2-targets as measured by cold target competition. This phenomenon reliably occurred for either DBA/2- or B10.D2-stimulated responses; the results are most easily interpreted as indicating the existence of two nonoverlapping subsets of killers. All subsets of killers detected in these experiments bear Thy-1 antigens based on the fact that killing is completely eliminated by treatment with anti-Thy-1.2 (data not shown). Killers were also found to be of the Lyt-1^{+}2^{+} phenotype (see legend to Table IV).

The Non-H-2-restricted Killers are Specific for Minor H and Do Not Cross-React with H-2^b. The lack of H-2 restriction observed in the experiment shown in Table IV could reflect the presence of killer cells whose anti-minor H specificity is genuinely unrestricted by H-2, or it could indicate that cytotoxic cells recognizing minor H complexed with H-2^d, cross-react with H-2^b. If the response were due to cross-reaction, we would expect that the killers should recognize BALB.B (H-2^b) as well as B10 (H-2^b). If the killing were specific for minor H only, B10 but not BALB.B should be recognized. To distinguish between these two possibilities, cytotoxic T cells were generated in response to B10.D2 adherent cells (expected to be H-2 restricted) and to BALB/c adherent cells incubated with B10.D2 FT-Ag (expected to lack H-2 restriction). These killers were then absorbed on macrophage monolayers (19) of B10.D2, B10, or BALB.B genotype; the nonadherent killer cells were assayed for their ability to kill B10.D2 target cells (Fig. 1). When B10.D2 adherent cells stimulated the generation of killers, neither B10 nor BALB.B could efficiently absorb out anti-B10.D2
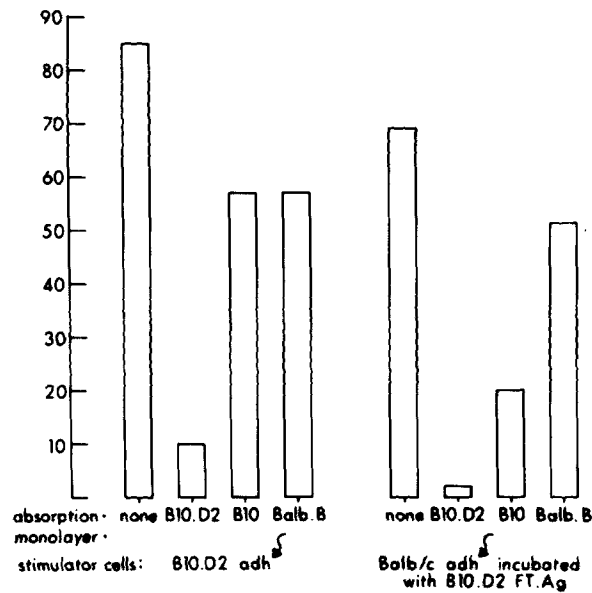
Fig. 1. Cytotoxic cells were generated by co-culture of nylon wool-passed primed BALB/c responders with either B10.D2 or BALB/c adherent cells preincubated with B10.D2 FT-Ag. The viable cells at day 5 of culture were harvested and allowed to absorb on macrophage monolayers from the indicated mouse strain. Absorbtion was for 90 min at 37°C in 25-mm^2 tissue culture flasks that had been seeded with 2×10^7 thioglycollate-activated PEC as described by Vasquez et al. (19). The nonadherent cells were removed by gentle pipetting and assayed for cytotoxicity on B10.D2 Con A blast cell targets. The nonadherent cells were used at cell numbers equivalent to the untreated control to avoid enrichment of remaining cells. A complete dilution curve of killing was done for all groups (1:10–1:80), but only the 1:40 dilution is recorded in the figure. Results were consistent for all dilutions.

TABLE V

Response to FT-Ag by Primed BALB/cJ

Stimulator cells	None	Percent specific lysis of B10.D2 targets cold competitors			
		B10.D2	B10	BALB.B	BALB/cJ
B10.D2	79	10	69	83	74
BALB/cJ and adherent cells incubated with B10.D2 FT-Ag	48	0	16	40	39

1.6×10^7 nylon wool-passed BALB/cJ responders (Qa-2^a) were cultured with B10.D2 or B10.D2 FT-Ag (Qa-2^a) for 5 d. Viable cells were pooled and assayed on ^{51}Cr-labeled B10.D2 blast cells with or without the indicated cold competitors (all of which were also Qa-2^a) at a 30:1 cold/hot ratio. Killers were assayed at 1:10, 1:20, and 1:40 of a culture, but only the results at 1:40 (line 1) or 1:10 (line 2) are included here. Essentially the same results were obtained at all culture dilutions.

killers, although good absorption occurred on B10.D2 monolayers. In contrast, over one-half of the killers generated in response to BALB/c adherent cells incubated with B10.D2 FT-Ag were absorbed out by B10 monolayers as compared with BALB.B monolayers; again, good absorption was obtained with B10.D2 monolayers.

The Unrestricted Killers Do Not Recognize Qa-2 Determinants. It is very likely that the BALB/cCr responders used here express the *Qa-2^b* allele (L. Flaherty, personal

communication). Since B10.D2 or DBA/2 are both $Qa-2^a$, the possibility existed that the unrestricted killers were in fact recognizing $Qa-2^a$ gene products. The results of the experiment shown in Fig. 1 makes this possibility unlikely, but to ensure that complications due to $Qa-2$ incompatibility did not play a role in the generation of unrestricted killers, we performed identical experiments using BALB/cJ responders $(Qa-2^a)$ (Table V). The results obtained under these $Qa-2$ syngeneic conditions were identical to those reported in Table IV. Cytotoxic T cells generated in response to B10.D2 adherent cells were H-2 restricted (line 1). In contrast, cytotoxicity generated in response to BALB/cJ adherent cells presenting B10.D2 FT-Ag were almost exclusively H-2 unrestricted (line 2). This experiment also confirms the results of the experiment shown in Fig. 1, in that B10 but not BALB.B cold targets were able to compete for the anti-B10.D2 killer cells. A small subset of H-2-restricted killers were also generated in this group of cultures (line 2, 16% lysis in the presence of B10 cold competitors).

Discussion

Several groups (9, 12–14, 20–25) have reported the participation of an adherent cell in cytotoxic responses to H-2 or to trinitrophenyl modified syngeneic cells, but the function of the cell appears to be as a secretor of soluble factors rather than as an antigen-processing and -presenting unit. In fact, very little direct evidence exists to implicate the adherent cell in antigen presentation for cytotoxic responses, although allogeneic splenic adherent cells and purified dendritic cells have been shown to be very potent stimulators of an anti-H-2 cytotoxic response (25, and unpublished observations). Weinberger et al. (26, 27) have used a system very similar to the one reported here to show that an antigen-processing cell is involved in the generation of a cytotoxic response to major H. In the cases of responses to minor H (7, 8, 10, 11) or to H-Y male antigen (28), circumstantial evidence points to the participation of a postulated antigen-processing/presenting cell in a phenomenon known as cross-priming (7). When an animal is injected with a cell bearing minor H Ag and allogeneic or semi-allogeneic H-2 antigens, cytotoxic precursors specific for an H-2 restricted response to "self minor H" are primed. Because the priming alloantigen could not have expressed the minor H in association with the host H-2 type, it can be concluded that the minor H have been taken up by a host macrophage-like cell and presented in association with the "correct" host H-2 type to cytotoxic T cell precursors (7). This antigen-presenting cell appears to be a necessary component of the priming step only when an allogeneic cell is the source of antigen. Both Forman (10), and Korngold and Sprent (11) cite evidence that can be most easily interpreted to mean that priming by a cell bearing minor H in association with syngeneic H-2 occurs directly without a requirement for processing by a macrophage intermediary. Furthermore, in none of the published work does the phenomenon of cross-priming operate at the level of the generation of cytotoxic cells by secondary challenge in vitro, although it does operate on a secondary challenge in vivo (29). Thus, the role of an antigen-processing or -presenting cell in the generation of H-2 restricted cytotoxic responses is problematical.

The concept that an antigen-processing cell allows cross-priming of the H-2-compatible anti-minor H response by an allogenic cell (7, 8) has as a necessary corollary that killers generated in a system dependent upon such a cell will be H-2 restricted. We were therefore very surprised to find that killer cells generated in

response to adherent cells presenting B10.D2 membrane fragments were in fact predominantly unrestricted in specificity, as determined by competition. These unrestricted killers could have been cross-reactive with H-2^b, in that the H-2-restricted receptors specific for H-2^b-B10 minors see a similar epitope on H-2^b, analogous to reports in a number of other systems (30–34). Alternatively, they could recognize B10 minors as an entity independent of H-2. To distinguish between these two interpretations, BALB/c cytotoxic cells generated in response to adherent cells presenting B10.D2 FT-Ag were absorbed on macrophage monolayers of B10.D2, B10, and BALB.B haplotypes. If the killers were genuinely unrestricted rather than bearing cross-reactive H-2-restricted receptors, then the cytotoxicity should have been absorbed out by B10 but not by BALB.B monolayers. This in fact was the pattern observed, indicating that the killers recognized minor H independently of major H Ag, in that they were unable to bind to the H-2^b expressed on BALB.B but could bind to the minors expressed on B10. This experiment also rules out the involvement of Qa-2 or Qa-3 as the target antigen, as both B10 and BALB.B express the same Qa allele (35–37). These conclusions are further substantiated by the experiments using BALB/cJ responder cells in which all components of the systems are syngeneic at the Qa-2 allele. Unrestricted anti-B10.D2 killers in this system were susceptible to cold target competition by B10 but not by BALB.B competitors (Table V).

The existence of unrestricted killers specific for minor H has not been reported previously. The major factor allowing us to detect them here was the use of membrane fragments as the source of antigen. It should be emphasized that in all cases where intact B10.D2 cells served as immunogen, whether or not the response was dependent upon BALB/c adherent cells, the cytotoxic cells generated were completely H-2 restricted in specificity. This indicates that the generation of unrestricted killers depends not on the processing by the adherent cell, although this may be an important part of the response, but on the nature of the antigen used (compare lines 5 and 6, Table IV). In addition, it seems possible that the multiple boost priming regime we have used is a relevant factor. Either, or both, of the following two interpretations seems reasonable.

First, it is possible that B10 minors do not exist as an immunogenic moiety on an intact cell; the only immunogenic form of minor H Ag may be a complex of minor H and H-2. If this were the case, then fragmentation of the surface membranes by freeze-thaw procedures may disrupt the antigenic complexes and reveal the B10 minor H as an immunogenic epitope dissociated from H-2. This model maintains that the adherent cell presents B10 but does not process it to form a new H-2/minor complex on the adherent cell surface. This intepretation is supported to some extent by the elegant experiments of Watt and Gooding (38) who showed that SV40/H-2 complexes on the surface of hybrid cells were able to dissociate and reassort to form previously nonexistent SV40/H-2 complexes within a very short time period in the absence of protein synthesis. Any simple formulation of this hypothesis, however, is negated by the fact that intact B10.D2 cells, because they function as targets, do in fact bear antigenic determinants recognized by the unrestricted killer cells.

A second possibility is that, under normal inductive conditions (e.g., with intact cells as the source of antigen) inhibitory cells suppress the induction of unrestricted killers but allow the generation of H-2-restricted killers. When membrane fragments are used as the source of antigen, the suppressive factors are absorbed away (via their

"Fc" portion) on macrophage membrane bits present in the FT-Ag preparation, rendering them ineffective as reported by Ptak et al. (39). Thus, both H-2-restricted and -unrestricted killers can arise in such cultures. This model derives indirect support from several observations. Unbound FT-Ag must remain in the cultures (Table III); these unbound membrane fragments may serve a purpose other than that of antigen. This interpretation is further strengthened by the fact that adherent cells that have been incubated with allo-FT-Ag and from which the unbound antigen has been washed away are very potent stimulators of a response to allogeneic major H-2 antigens (data not shown). If the above model were correct, the cytotoxic response to intact cells might be expected to become at least partially unrestricted if syngeneic (BALB/c) freeze-thaw membranes were added to the cultures as a way of absorbing the suppressive factors on an ineffective vector. Our observation that cytotoxic responses to detergent-solubilized B10.D2 antigens are also predominantly unrestricted in character does not easily accord with this suppressor model (unpublished data). Experiments are in progress to further analyze the factors responsible for the generation of unrestricted killers.

Neither model excludes the generation of restricted killers in cultures responding to the FT-Ag, and in fact both H-2-restricted and -unrestricted killers were usually generated in a given experiment, although occasionally only unrestricted killers arose; these were almost always the larger subset of a mixed response. This suggests that both minor H as an independent entity and minor H as a complex with $H-2^d$ were present in the FT-Ag membrane preparation. Alternatively, the BALB/c adherent cell may process minor H in such a way that some of the minor H is reassociated with $H-2^d$. In experiments using B10 membrane fragments instead of B10.D2, both unrestricted killers and a subset of $H-2^d$-restricted killers (as defined by competition) were generated, providing strong evidence that processing can occur, because B10 FT-Ag cannot include antigens consisting of B10 minors complexed with $H-2^d$ (data not shown).

A final possibility is that the apparently unrestricted killers are in fact restricted by the product of an inappropriate H-2 allele (i.e., $H-2^b$). This might occur if the use of the membrane fragments as antigen were to change immunodominance patterns, such that a normally undetectable subset of killers that see similar determinants on complexes of both $H-2^b$ and $H-2^d$ minors is selectively amplified. This type of mechanism has been suggested by Wettstein and Frelinger (4) based on the Ly phenotypes of killers generated against different minor H-bearing stimulator cells. Experiments are in progress to determine whether or not $H-2^b$ gene products are a necessary component of the target antigen recognized by killers generated in the response of BALB/c to B10.D2 fragments.

Summary

The secondary cytotoxic T cell response of BALB/c to B10.D2 or DBA/2 minor histocompatibility antigens in vitro requires the participation of an adherent cell. Nylon wool-passed spleen cells were only able to respond to nonadherent intact stimulator cells, or to membrane fragments derived from those cells, if a syngeneic adherent cell were present in the cultures. When the H-2 restriction properties of cytotoxic cells generated in response to various types of stimulation were analyzed, it was found that responses to B10.D2 or DBA/2 intact cells were always H-2 restricted.

Responses to syngeneic adherent cells presenting B10.D2 or DBA.2 freeze-thaw antigen were either entirely or predominantly lacking in H-2 restriction as defined by efficient competition by B10 (H-2^b) cold target cells. These unrestricted killers appeared to recognize minor histocompatibility as an independent determinant rather than as an H-2^d/minors moiety cross-reaction with H-2^b, because they were not absorbed by BALB.B (H-2^b) macrophage monolayers, but were absorbed by B10 monolayers. Similarly, B10 but not BALB.B cold targets were able to compete for the anti-B10.D2 killers. These experiments eliminate the possibility that the lack of restriction was due to an H-2^b restricted receptor cross-reactive with H-2^b. Possible models to explain these findings are discussed.

References

1. Bevan, M. J. 1975. The major histocompatibility complex determines susceptibility to cytotoxic T cells directed against minor histocompatibility antigens. *J. Exp. Med.* **142:**1349.

2. Matzinger, P., and G. Mirkwood. 1978. In a fully H-2 incompatible chimera, T cells of donor origin can respond to minor histocompatibility antigen in association with either donor or host H-2 type. *J. Exp. Med.* **148:**84.

3. Wettstein, P. J., and J. A. Frelinger. 1977. H-2 effects on cell-cell interactions in the response to single non-H-2 alloantigens. II. H-2D region contrtol of H-7.1 specific stimulator function in mixed lymphocyte culture and susceptibility to lysis by H-7.1 specific cytotoxic cells. *J. Exp. Med.* **146:**1356.

4. Wettstein, P. J., and J. A. Frelinger. 1981. T lymphocyte responses to non-H-2 histocompatibility antigen. I. Role of Ly-1$^+$2$^+$ T cells as cytotoxic effectors and requirement for Ly-1$^+$2$^+$ T cells for optimal generation of cytotoxic effectors. *J. Immunol.* **127:**43.

5. Pilarski, L. M. 1981. Regulation of the cytotoxic T cell response to minor histocompatibility antigens. *Transplantation (Baltimore).* **32:**188.

6. Pilarski, L. M., and I. F. C. McKenzie. 1981. Lyt antigens on the T cells specific for minor histocompatibility antigens: suppression by an Lyt 1$^+$2$^-$ regulator. *Transplantation (Baltimore).* **32:**5.

7. Bevan, M. J. 1976. Minor H antigens introduced on H-2 different stimulating cells crossreact at the cytotoxic T cell level during *in vivo* priming. *J. Immunol.* **117:**2233.

8. 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.

9. Pettinelli, C. B., A.-M. Schmitt-Verhulst, and G. M. Shearer. 1979. Cell types required for H-2-restricted cytotoxic responses generated by trinitrobenzene sufonate modified syngeneic cells or trinitrophenyl-conjugated proteins. *J. Immunol.* **122:**847.

10. Forman, J. F., and J. W. Streilein. 1979. T cells recognize minor histocompatibility antigen on H-2 allogeneic cells. *J. Exp. Med.* **150:**1001.

11. Korngold, R., and J. Sprent. 1980. 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. *J. Exp. Med.* **151:**314.

12. Hurme, M., B. E. Bang, and M. Sihvola. 1980. Generation of H-2-restricted cytotoxic T

cells by ultraviolet light-treated trinitrophenyl-modified syngeneic cells: increased require-
ment for adherent cells. *J. Immunol.* **125**:2484.

13. Lee, K.-C., J. Kay, and M. Wong. 1979. Separation of functionally distinct subpopulations
of *Corynebarcterium parvum*-activated macrophages with predominantly stimulatory or sup-
pressive effect on the cell-mediated cytotoxic T cell response. *Cell. Immunol.* **42**:28.

14. Yamashita, U., T. Hamaoka. 1979. The requirement of Ia-positive accessory cells for the
induction of hapten-reactive cytotoxic T lymphocytes *in vitro. J. Immunol.* **123**:2637.

15. Julius, M. H., E. Simpson, L. A. Herzenberg. 1973. A rapid method for the isolation of
functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3**:645.

16. Shiozawa, C., B. M. Longenecker, and E. Diener. 1980. *In vitro* cooperation of antigen-
specific T cell-derived helper factor, B cells, and adherent cell or their secretory product in
a primary IgM response to chicken MHC antigens. *J. Immunol.* **125**:68.

17. Schwartz, R. H., A. Yano, and W. E. Paul. 1978. Interaction between antigen presenting
cells and primed T lymphocytes. *Immunol. Rev.* **40**:153.

18. Rosenthal, A. S. 1978. Determinant selection and macrophage function in genetic control
of the immune response. *Immunol. Rev.* **40**:136.

19. Vasquez, A., A. Senik, and C. Meuport-Sautes. 1980. Multiple CTL subsets generated by
H-2.L locus products stimulation: evidence for an antigenic determinant private to H-2.L.
J. Immunol. **125**:74

20. Miller, C. L., and R. T. Mischell. 1976. Differential regulatory effects of accessory cells of
the generation of cell-mediated immune reactions. *J. Immunol.* **114**:692.

21. Lutz, C. T., and F. W. Fitch. 1979. Accessory cell requirements for the generation of
cytolytic T lymphocytes. *J. Immunol.* **122**:2598.

22. Schilling, R. M., R.A. Phillips, and R. G. Miller. 1976. Requirement for non-T cells in the
generation of cytotoxic T lymphocytes *in vitro.* I. Use of nude mice as source of non-T cells.
J. Exp. Med. **144**:241.

23. Hodes, R. J., B. S. Handwerger, and W. D. Terry. 1974. Synergy between subpopulations
of moue spleen cells in the *in vitro* generation of cell-mediated cytotoxicity. *J. Exp. Med.*
140:1646.

24. Nussenzweig, M. C., R. M. Steinman, B. Gutchinov, and Z. A. Cohn. 1980. Dendritic cells
are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes. *J.
Exp. Med.* **152**:1070.

25. Steinman, R. M., M. D. Witmer, M. C. Nussenzweig, L. L. Chen, G. Kaplan, and Z. A.
Cohn. 1980. Dendritic cells, an important new cell type in MHC-linked functions. *In*
Macrophage Regulation of Immunity. E. Unanue and A. Rosenthal, editors. Academic
Press, Inc., New York. 297–305.

26. Weinberger, D., S. H. Herrmann, M. F. Mescher, B. Benacerraf, and S. Burakoff. 1980.
Cellular interactions in the generation of cytolytic T lymphocyte responses: role of Ia-
positive splenic adherent cell in presentation of H-2 antigen. *Proc. Natl. Acad. Sci. U. S. A.*
77:6091.

27. Weinberger, O., S. Herrmann, M. F. Mescher, B. Benacerraf, and S. J. Burakoff. 1981.
Antigen-presenting cell function in induction of helper T cells for cytotoxic T-lymphocyte
responses: evidence for antigen processing. *Proc. Natl. Acad. Sci. U. S. A.* **78**:1796.

28. Simpson, E., and R. D. Gordon. 1977. Responsiveness to HY antigen, Ir gene complemen-
tation and target cell specificity. *Immunol. Rev.* **35**:59.

29. Matzinger, P., and M. J. Bevan. 1977. Induction of H-2-restricted cytotoxic T cells: *in vitro*
induction has the appearance of being unrestricted. *Cell. Immunol.* **33**:92.

30. Bevan, M. J. 1977. Killer cells reactive to altered-self antigens can also be alloreactive. *Proc.
Natl. Acad. Sci. U. S. A.* **74**:2094.

31. Finberg, R., S. Burakoff, H. Cantor, and B. Benacerraf. 1978. Biological significance of

alloreactivity: T cells stimulated by Sendai virus-coated syngeneic cells specifically lyse allogeneic target cells. *Proc. Natl. Acad. Sci. U. S. A.* **75:**5145.

32. Braciale, T. J., M. E. Andrew, and V. L. Braciale. 1981. Simultaneous expression of H-2-restricted and alloreactive recognition by a cloned line of influenza virus-specific cytotoxic T lymphocytes. *J. Exp. Med.* **153:**1371.

33. von Boehmer, H., H. Hengartner, W. Nabholz, W. Lernhardt, M. H. Schreier, and W. Haas. 1979. Fine specificity of a continuously growing killer cell line specific for H-Y antigen. *Eur. J. Immunol.* **9:**592.

34. Pfizenmaier, K., H. Stockinger, M. Rollinghoff, and H. Wagner. 1980. Herpes-simplex-virus-specific, H-2D^k-restricted T lymphocytes bear receptors for H-2D^d alloantigen. *Immunogenetics.* **11:**169.

35. Flaherty, L., D. Zimmerman, and T. H. Hansen. 1978. Further serological analysis of the Qa antigens: analysis of an anti-H-2.28 serum. *Immunogenetics.* **6:**245.

36. 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.

37. Flaherty, L. 1976. The *Tla* region of the mouse: identification of a new serologically defined locus, Qa-2. *Immunogenetics.* **3:**533.

38. Watt, T. S., and L. R. Gooding. 1980. Formation of SV40 specific and H-2 restricted target cell antigen by somatic cell fusion. *Nature (Lond.).* **283:**74.

39. Ptak, W., K. F. Naidorf, and R. K. Gershon. 1977. Interference with the transmission of T cell-derived messages by macrophage membranes. *J. Immunol.* **119:**444.