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Immunology

Killer cells reactive to altered-self antigens can also be alloreactive

(cytotoxic lymphocytes/major histocompatibility antigens/minor histocompatibility antigens)

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ABSTRACT **Murine cytotoxic thymus-derived lymphocytes immunized against cells bearing foreign minor histocompatibility antigens are specific for the immunizing minor antigens and for their own major H-2 antigens; they do not lyse target cells that bear the correct minor antigens plus a different *H-2* haplotype. These are referred to as "altered-self" or "self-plus-X" killer cells. Alloreactive killer cells are those which respond to allogeneic cells expressing a foreign (non-self) *H-2* haplotype. In this study, cytotoxic lymphocytes were immunized against minor histocompatibility differences *in vivo* and *in vitro*. These effector cells killed the immunizing altered-self target very well and showed about 1% cross-reactive lysis of an allogeneic target differing from themselves only at *H-2*. These cross-reactive clones were then selected for by repeated *in vitro* stimulation with the cells bearing foreign H-2 such that an effector population was obtained which lysed both the altered-self and the alloreactive target with the same efficiency. Cold target competition experiments established that the same killer cell could lyse either target; however, it was not determined if a killer cell uses the same receptor to respond to altered-self antigens as it does to respond to foreign H-2 antigens.**

A very large fraction of thymus-derived (T) lymphocytes is committed to respond to any allogeneic cell that differs genetically from the responder at the major histocompatibility complex (MHC, the H-2 complex in mice, refs. 1–4). Between 1 and 10% of T cells respond to any foreign MHC haplotype and, because this region is polymorphic, it is likely that most T cells are alloreactive to one or another of the non-self *H-2* haplotypes. A large fraction of cytotoxic T lymphocytes (CTL), a subfraction of T cells, can be induced to kill cells bearing any foreign allele of $H\text{-}2^k$ or *H-2D* (5–7). In the mouse, then, *H-2* differences are the strongest barriers to tissue transplantation; but it is probably not the normal physiological function of T cells to respond to allogeneic cells.

The most important recent discovery relating the MHC to the physiological function of T cells is that of *H-2* restriction. It was found that CTL of $H\text{-}2^a$ haplotype immunized against autologous cells that had been altered chemically or by virus infection (X) could lyse X-altered-H-2^a targets but not X-altered-H-2^b or X-altered-H-2^c targets (8, 9). The cytotoxic response to minor H antigens [i.e., when responder and stimulator cells bear the same *H-2* but differ in non-*H-2* coded minor *H* loci (10)] is similarly *H-2* restricted (11, 12). *H-2* coded structures of the target are therefore involved in the lytic interaction even when the CTL and target carry the same *H-2* genes—i.e., in the response to "altered-self" antigens.

According to the altered-self hypothesis (8, 9, 13) the explanation of *H-2* restriction is that the CTL has one receptor which binds two components of the target cell, X-plus-H-2. The dual-recognition hypothesis (deriving from previous work on interactions between T cells and thymus-independent lymphocytes and between T cells and macrophages) postulates that $H\text{-}2^k$ and D serve as self-markers for CTL, which therefore has two separate receptors, one for self H-2 and one for X (14, 15).

In understanding the function of T cells and their antigen binding requirements, it is important that we determine the relationship between reactions to altered-self targets and to targets that bear foreign H-2 antigens (alloreactions). We need to determine: (*i*) whether alloreactive T cells can also respond to altered-self antigens, and (*ii*) if they do, whether the receptor systems used are the same in both cases. With regard to the first point, although it has been established that most of the effector and precursor CTL involved in anti-H-2 reactions are Ly-1^-, -2^+, -3^+, (16, 17), there is recent evidence that suggests that precursor or effector CTL for altered-self reactions are Ly-1^+. The precursor CTL for TNP-altered-self may be Ly-1^+, -2^+, -3^+ (18), the effector CTL in response to a syngeneic tumor may be Ly-1^+, -2^+, -3^+ (19), and effector CTL to virus-infected cells after primary *in vivo* immunization may also be Ly-1^+ (20). This implies that either alloreactive T cells and altered-self reactive T cells belong to two separate subsets or that the antigenic stimulus delivered to T cells by these antigens is different. On the other hand, Heber-Katz and Wilson (21) presented evidence that alloreactive cells include those T cells responsive to conventional antigens. They positively selected T cells responsive to one MHC haplotype in mixed lymphocyte culture, parked them in animals that lacked T cells, and later demonstrated that the parked cells contained helper T cell activity for the antibody response to sheep erythrocytes. In this work there could be no definitive proof that the same T cell could respond to both antigens because the assay systems for the responses were so different. The evidence rested on the completeness of the negative selection against bystander T cells in the mixed lymphocyte culture.

Here I report studies that show conclusively that an altered-self reactive CTL can also respond to and lyse cells that differ at *H-2*. F_1 (Balb/c × Balb.B) ($H\text{-}2^{d/b}$) CTL reactive to the minor H differences of B10.D2($H\text{-}2^d$) were positively selected for by *in vivo* and *in vitro* immunization with B10.D2. These effectors showed a small degree of cross-reactive lysis of Balb.K($H\text{-}2^k$) targets. The cells lysing Balb.K were further selected for by repeated stimulation in long-term mixed lymphocyte cultures with Balb.K. A highly selected CTL population capable of lysing B10.D2 and Balb.K targets almost equally was eventually obtained. It was established that the same killer cell could lyse either target by showing that the rate of lysis of labeled B10.D2 targets was specifically inhibited by addition of an excess of unlabeled Balb.K targets (cold target competition

Abbreviations: T lymphocyte, thymus-derived lymphocyte; MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; X, foreign minor antigen.

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experiments). Similarly, lysis of labeled Balb.K targets was inhibited in the presence of B10.D2 targets. How this finding may be related to the number of receptors on T cells as proposed by the altered-self and dual-recognition hypotheses is discussed.

MATERIALS AND METHODS

Mice. Balb.K($H\text{-}2^k$) and F_1(Balb/c × Balb.B) ($H\text{-}2^{d/b}$) mice were bred at The Salk Institute. Balb.K and Balb.B stocks were originally obtained from F. Lilly. C57BL/10Sn (B10, $H\text{-}2^b$), B10.D/2nSn($H\text{-}2^d$), and B10.BR/SgSn($H\text{-}2^k$) mice were purchased from the Jackson Laboratory, Bar Harbor, ME.

***In Vivo* Immunization.** Young adult female F_1(Balb/c × Balb.B) mice were injected intraperitoneally with 2×10^7 viable B10.D2 spleen cells suspended in Hanks' balanced salt solution.

Mixed Lymphocyte Cultures. The culture medium was RPMI 1640 medium (Microbiological Associates, Bethesda, MD) supplemented with 50 μM 2-mercaptoethanol and 5% (vol/vol) fetal calf serum. For the first *in vitro* immunization, responder spleen cells at 4×10^6 viable cells per ml were stimulated with an equal number of irradiated (1000 rads from a ^{60}Co source) stimulator cells in 35-mm tissue culture dishes (Falcon Plastics, Bioquest, Oxnard, CA). Dishes were placed in a box, gassed with a mixture of 10% CO_2/7% O_2/83% N_2, and placed on a rocker platform at 37°. After 5 days, some dishes were used to assay for cytotoxic activity and the remainder were harvested by pipetting and the cells were placed in tissue culture flasks (Falcon, no. 3012), 10–25 ml per flask. The flasks were loosely stoppered and incubated upright in a 10% CO_2/air incubator at 37°. When the cells had settled, about half of the supernatant medium was replaced with fresh medium.

For subsequent *in vitro* stimulations the cells were removed from flasks and centrifuged, and the viable cells were counted. They were restimulated with irradiated cells in dishes on a rocking platform again, but this time at a density of 1 to 2×10^6 cells per ml. Secondary stimulation was for 4 days. Then some cells were used in cytotoxicity assays and the remainder were placed in new flasks.

^{51}Cr-Release Cytotoxicity Assay. This was performed exactly as described previously (12). Target cells were spleen cells cultured for 2 days with lipopolysaccharide (thymus-independent cell blasts) or concanavalin A (T cell blasts). Targets were labeled with sodium [^{51}Cr]-chromate (Amersham/Searle, Arlington Heights, IL) and washed twice before use. Serial 3-fold dilutions of the killer cells were titrated against 3 to 4×10^4 ^{51}Cr-labeled targets for 3–4 hr in 1 ml of medium in 35-mm petri dishes on a rocking platform. At the end of the assay the contents of the dishes were transferred to tubes and centrifuged, and 0.5 ml of the supernatant was removed for counting. Percent specific lysis was calculated as follows:

$$\frac{\text{cpm released in presence of killers} - \text{cpm released spontaneously}}{\text{total cpm originally in targets} - \text{cpm released spontaneously}} \times 100.$$

RESULTS

Long-Term Mixed Lymphocyte Culture. The CTL response of F_1(Balb/c × Balb.B) ($H\text{-}2^{d/b}$) mice to the minor H antigens of B10.D2($H\text{-}2^d$) is *H-2* restricted and the effectors do not lyse B10($H\text{-}2^b$) targets (12). They do crossreact to a slight extent on B10.BR($H\text{-}2^k$) targets; this is not due to non-$H\text{-}2^d$ restricted recognition of B10 minors because the same degree of crossreaction is observed on Balb.K($H\text{-}2^k$) targets. The F_1 responder and Balb.K mice are congenics and differ only at the *H-2* region. The crossreactive lysis of B10.BR and Balb.K targets is mediated by the same fraction of effector cells as demonstrated in cold target competition experiments (unpublished data).

Spleen cells from F_1 mice that had been primed *in vivo* with B10.D2 cells were stimulated in mixed lymphocyte culture for 5 days with B10.D2. On day 5, the CTL lysed B10.D2 targets efficiently, caused no lysis of syngeneic targets, and showed a slight crossreaction (probably about 1%) on Balb.K targets (Fig. 1). The cells were parked in flasks until day 14 when they were restimulated with either Balb.K or B10.D2 cells and then assayed for cytotoxicity on day 18. CTL reboosted with B10.D2 showed the same pattern of lysis of B10.D2 and Balb.K targets as on day 5 although this time lysis of B10.D2 was about 3-fold more efficient on a per cell basis (on day 5, 40% lysis of B10.D2 at a ratio of 3.3:1; on day 18, 40% lysis at 1:1). CTL boosted on day 14 with the alloreactive Balb.K stimulus were about 13-fold less active in lysing B10.D2 (40% lysis at 13:1) than the cells boosted with B10.D2 on day 14. Therefore, the recall of cytotoxic activity is antigen-specific to a large degree. These Balb.K-boosted CTL lysed Balb.K targets quite effectively, approximately one-seventh as well as they lysed B10.D2.

The cells that had been boosted with Balb.K on day 14 were parked once more in flasks until day 25 when they were cultured with irradiated Balb.K again or with no irradiated cells. Cytotoxicity assays were performed on day 29. The unstimulated cells had little activity against any target but those boosted with irradiated Balb.K cells lysed B10.D2 and Balb.K targets almost equally effectively (less than a 2-fold difference in the rates of lysis).

The data so far have shown that recall of CTL effector function in these long-term cultures is largely antigen-specific. The cold target competition experiments presented below show that most of the CTL clones selected by stimulation with Balb.K can lyse both Balb.K and the original altered-self B10.D2 target. These two points together indicate that the crossreactive clones can also be *induced* to become effectors with either antigen.

Cold Target Competition Experiments. The first experiment with these long-term cultured F_1 cells was done on day 29. Lysis of labeled B10.D2 and Balb.K blasts by the same highly selected population of CTL was studied in the presence of an excess of unlabeled F_1(Balb/c × Balb.B), B10.D2, or Balb.K blasts (Fig. 2). The ratio between unlabeled and labeled blasts in the assay was 75:1. Lysis of either target was inhibited about 3-fold by F_1 blasts which are syngeneic to the CTL. This syngeneic inhibition is always seen when relatively large numbers of unlabeled cells are added (12, 22). Syngeneic inhibition is taken here as "nonspecific" and is the base line from which specific blocking is considered. Fig. 2 *left* shows that unlabeled alloreactive Balb.K blasts specifically inhibited the lysis of the altered-self target, ^{51}Cr-labeled B10.D2, 3-fold; unlabeled B10.D2 inhibited 20-fold. With ^{51}Cr-labeled Balb.K as indicator, unlabeled B10.D2 caused 4-fold inhibition and unlabeled Balb.K caused 11-fold inhibition (Fig. 2 *right*).

A second cold target competition experiment was done with anti-B10.D2 CTL that had been boosted with irradiated Balb.K on day 25, parked in culture flasks until day 36, and then boosted once more with Balb.K. The assay was done on day 40 of culture. Lysis of the original altered-self target, B10.D2, was not significantly different from lysis of $H\text{-}2^k$ targets (either B10.BR or Balb.K) at this time. B10.D2 and B10.BR blasts were used as labeled indicator cells in this experiment, with a 60-fold excess of unlabeled B10($H\text{-}2^b$), B10.D2, and B10.BR as blockers.

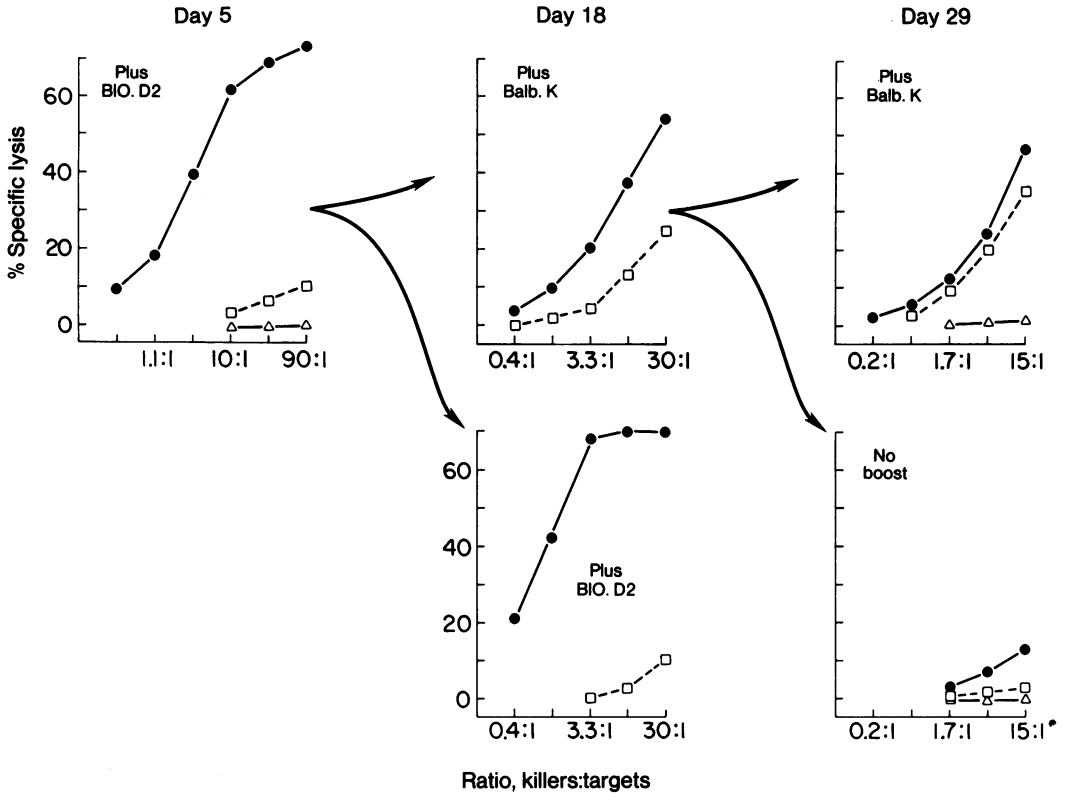

FIG. 1. Cytotoxic activity of responding F_1(Balb/c × Balb.B) ($H\text{-}2^{d/b}$) spleen cells in long-term mixed lymphocyte culture. Spleen cells were removed from F_1 mice injected 18 days previously with 2×10^7 B10.D2($H\text{-}2^d$) cells and cultured on day 0 with irradiated B10.D2 spleen cells. Some of the cells were assayed for cytotoxicity on day 5, and the remainder were parked in culture flasks. Cells were recultured with irradiated Balb.K($H\text{-}2^k$) or B10.D2($H\text{-}2^d$) cells on day 14 and assayed on day 18. On day 25 of culture, the cells stimulated with Balb.K on day 14 were recultured with irradiated Balb.K or with no stimulating cells and assayed on day 29. Viable cell recovery from days 0–5 was 68%; from days 14–18 it was 130% after boosting with Balb.K and 213% after boosting with B10.D2; from days 25–29 it was 164% after boosting with Balb.K and 71% with no added stimulus. Targets used were ^{51}Cr-labeled blasts of F_1(Balb/c × Balb.B) (△), B10.D2 (●), and Balb.K (□). Spontaneous release of ^{51}Cr varied from 10.8 to 21.1%.

Lysis of labeled B10.D2 targets was specifically inhibited 7-fold by unlabeled B10.BR, and 27-fold by B10.D2 (Fig. 3 *left*). Lysis of labeled B10.BR targets was inhibited 4-fold by B10.D2, and 20-fold by B10.BR (Fig. 3 *right*).

This highly selected population of CTL, immunized first *in vivo* and on day 0 of culture with B10.D2 and on days 14, 25, and 36 with Balb.K, appeared to consist largely of clones that could lyse B10.D2 and Balb.K. The cold target competition experiments indicate that lysis of the original altered-self target, B10.D2, is inhibited 75–87% by an excess of Balb.K or B10.BR, and lysis of the alloreactive $H\text{-}2^k$ targets is specifically inhibited 80% by B10.D2.

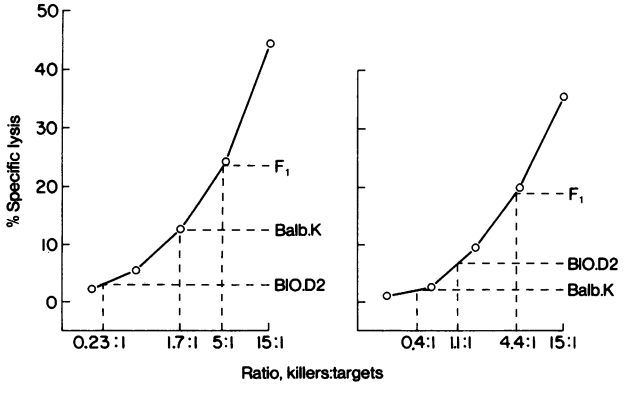

FIG. 2. Crossinhibition of lysis of ^{51}Cr-labeled B10.D2($H\text{-}2^d$) (*Left*) and Balb.K($H\text{-}2^k$) (*Right*) targets by either cold target. Solid lines show lysis of 3×10^4 target cells by serial 3-fold dilutions of the F_1(Balb/c × Balb.B)($H\text{-}2^{d/b}$) CTL population from day 29 of long-term mixed lymphocyte culture (Fig. 1) in the absence of unlabeled inhibitor cells. Horizontal broken lines indicate the lysis observed in the presence of 2.2×10^6 unlabeled blasts as marked, at the killer: target ratio of 15:1. The inhibited level of lysis is extrapolated from the standard uninhibited curve to give the equivalent killer:target ratio. Spontaneous release of ^{51}Cr was 10.8% and 19.5%.

In order to show that such cross-inhibition does not normally occur with F_1 anti-B10.D2 and F_1 anti-Balb.K CTL, the following control was performed. Both populations of CTL were generated separately—F_1 anti-B10.D2 by *in vivo* priming followed by 5 days of mixed lymphocyte culture, and F_1 anti-Balb.K by a 5-day primary mixed lymphocyte culture immunization. The effectors were mixed to give approximately equal lysis of B10.D2 and Balb.K targets. Inhibition of lysis of labeled B10.D2 and Balb.K targets was then studied with a 65-fold excess of unlabeled F_1(Balb/c × Balb.B), B10.D2, or Balb.K blasts. Table 1 shows that under these conditions, when separate effectors were lysing either target, no crossinhibition greater than syngeneic inhibition could be detected. For example, lysis of ^{51}Cr-labeled B10.D2 was inhibited well by unlabeled B10.D2, but unlabeled Balb.K showed no more inhibition than was caused by unlabeled F_1 blasts.

DISCUSSION

The cold target competition experiments reported here establish that one F_1(Balb/c × Balb.B)($H\text{-}2^{d/b}$) effector CTL can lyse either an altered-self target cell, in this case B10.D2($H\text{-}2^d$), or a target cell that differs from itself at *H-2*, in this case Balb.K ($H\text{-}2^k$). The data also establish something of the nature of CTL

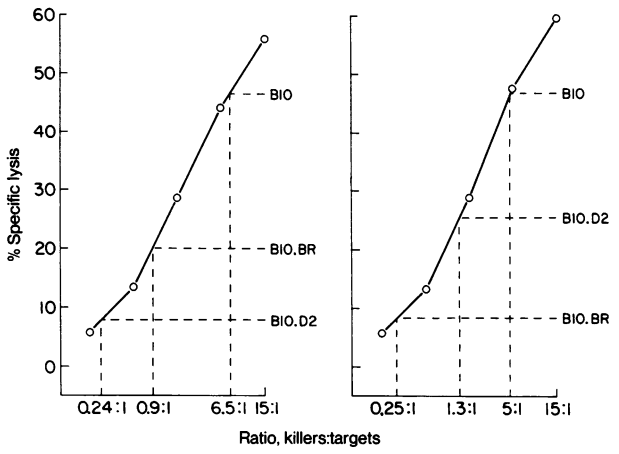

FIG. 3. Cold target competition experiment with F_1(Balb/c × Balb.B) ($H\text{-}2^{d/b}$) CTL selected to lyse both altered-self B10.D2 ($H\text{-}2^{d}$) ^{51}Cr-labeled targets (*Left*) and alloreactive ($H\text{-}2^{k}$) ^{51}Cr-labeled targets, Balb.K and B10.BR (*Right*). CTL were immunized originally against B10.D2 and then against Balb.K on days 14, 25, and 36 of long-term mixed lymphocyte culture. On day 40, serial 3-fold dilutions were assayed for lysis of 3.5×10^4 ^{51}Cr-labeled blasts of B10.D2 (*Left*) and B10.BR (*Right*) (solid lines). At the 15:1 killer: target ratio, lysis was inhibited by the addition of 2.1×10^6 unlabeled blasts of B10($H\text{-}2^{b}$), B10.D2, or B10.BR (horizontal broken lines). Spontaneous release of ^{51}Cr was 10.5% and 11.1%. Labeled Balb.K targets were lysed as well as B10.BR; B10 targets were lysed very poorly.

crossreactivity. Killers immunized against cells bearing foreign *H-2* often crossreact quite strongly on third-party cells bearing a different *H-2* type (23, 24), and anti-minor H CTL may also crossreact detectably on *H-2* dissimilar cells (Fig. 1; ref. 25). Theoretically, crossreactive lysis could have occurred if 100% of the clones lysed the third-party targets with 1% efficiency (compared to the lysis of the immunizing targets). Alternatively, crossreactive lysis could have been due to only 1% of the clones lysing both targets efficiently, while 99% of them lysed only the specific target. Because the crossreaction can be selected for by immunization with the third-party cells, until lysis of both targets becomes approximately equal, the latter explanation is probably correct.

The selection procedure which I placed on these cytotoxic T cells in culture is probably analogous to the experiments with thymus-independent cells performed by Richards *et al.* (26). They immunized rabbits with hapten A and showed by isoelectric focusing that a small, specific fraction of the serum antibody to A could also bind hapten B. Secondary immunization with B induced these crossreactive clones without inducing the noncrossreactive anti-A clones.

The first immunization of F_1(Balb/c × Balb.B) cells in culture with B10.D2 selects for (positive selection) the survival of anti-B10.D2 lymphocytes and selects against (negative selection) cells that do not react with B10.D2. Cells selected against include most of the F_1 cells which would normally respond to the $H\text{-}2^{k}$ coded differences of Balb.K. Secondary immunization with Balb.K is therefore selective for the clones that react with B10.D2 and Balb.K. The cold target competition experiments (Figs. 2 and 3) showed that cross-inhibition of lysis (e.g., of ^{51}Cr-labeled Balb.K by unlabeled B10.D2) was not quite as effective as homologous inhibition. This is probably because selection against anti-Balb.K-only cells in the early immunizations with B10.D2 was not complete. Thus, a small number of cells that could lyse Balb.K but not B10.D2 were left at day 14 when the first immunization with Balb.K was done. Because of the incompleteness of negative selection, the cold target competition experiments had to be performed to show that most of the CTL lysed both targets.

Table 1. Cold target competition experiment with artificially mixed population of altered-self and alloreactive CTL*

Ratio, CTL: ^{51}Cr-blasts	Unlabeled blasts†	% Specific lysis ^{51}Cr-B10.D2	% Specific lysis ^{51}Cr-Balb.K
28:1	None	52.8	58.5
9.3:1	None	37.6	40.2
3.1:1	None	17.5	19.0
1:1	None	6.5	7.6
0.3:1	None	1.9	3.0
28:1	F_1	41.8	43.0
28:1	B10.D2	1.2	42.1
28:1	Balb.K	40.1	3.9

* F_1(Balb/c × Balb.B) anti-B10.D2 and anti-Balb.K effector CTL were mixed in 1.3:1 ratio to give almost equal rates of lysis of either target.

† A 65-fold excess of unlabeled blasts over ^{51}Cr-labeled blasts was used. F_1 = F_1(Balb/c × Balb.B).

Recall of cytotoxic effector function in these cultures appeared to be quite antigen-specific. Thus, on day 14, when most of the memory CTL were specific for B10.D2 only, addition of irradiated B10.D2 cells recalled 13-fold more anti-B10.D2 CTL activity compared to addition of irradiated Balb.K cells (Fig. 1). If a helper T cell response is also required to re-induce CTL and if recall of helper activity was antigen-specific in these cultures, to explain the fact that Balb.K can specifically reinduce cells that were first induced by B10.D2 one might postulate that Balb.K and B10.D2 share helper determinants as well as cytotoxic determinants. Since it is not established that helper T cells were completely selected out or that there was not sufficient helper activity remaining in the cultures even in the absence of added B10.D2 antigen, this postulate can only be made tentatively. Furthermore, it is not established that helper T cells are required to induce CTL.

I do not know how the results presented here can be reconciled with the findings that alloreactive CTL and altered-self reactive CTL may differ in their Ly phenotypes (18–20). I do not believe that there are two separate, nonoverlapping populations of CTL. Differences in Ly phenotype might be correlated with the stage of T cell development which in turn may depend on the receptor specificity as defined by "closeness to self antigens." Foreign H-2 antigens might select cells from one end of this spectrum of receptors while the self-plus-X antigens so far studied select from the other end.

These results show that one killer cell can respond to foreign cells that are antigenic due to differences either at minor H loci or at major H loci. There are several possible explanations for this at the level of surface receptors on T cells. First, in analogy with what has been suggested for immunoglobulin, one CTL receptor molecule might have separate antigen binding sites for different determinants expressed on the two antigens (26). Second, one T cell may have more than one species of antigen-binding receptor on its surface. Because so many lymphocytes respond to major H antigens, thereby threatening to overoccupy the T cell repertoire with alloreactivity, many investigators have proposed this model (1, 2). Third, the dual-recognition model would say that H-2 antigens differ qualitatively from all other cell-surface antigens. To respond to minor H antigens requires, in addition to the antigen-specific anti-X

receptor binding of the minor antigen, that the anti-self H-2 receptor also binds to the target. In the case of a foreign H-2 antigen, reaction of one receptor with foreign H-2 is sufficient. In this model, H-2 molecules of the stimulating or target cell are given special intrinsic properties such as being the only site at which CTL killing signals can be delivered. Contrary to this notion is the finding that target cells that lack H-2 can still be lysed by CTL when agglutinated with phytohemagglutinin or concanavalin A (27, 28). If the killing acceptor site is not part of H-2 but only normally associated on the membrane with H-2, then this objection could be overruled (R. Langman, personal communication).

Finally, T cells may have one receptor, and the combining site that binds self H-2^d-plus-foreign B10 minor antigen is the same as that binding foreign H-2^k-plus-self Balb minors. That is, if H-2 influences the immunogenicity of all other membrane components (as it does in altered-self reactions), then allo-reactivity to cells bearing foreign H-2 might not be to H-2 seen in isolation but to H-2 seen in combination with many other surface components (29). This model, based on the altered-self interpretation of *H-2* restriction, was proposed to explain the high frequency of T cells responsive to cells bearing any foreign *H-2* haplotype. According to this, the special stimulatory property of H-2 is due to the specificity range of T cell receptors which is somehow designed to bind antigens only when they are associated with H-2.

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