RECOGNITIVE SPECIFICITY OF HUMAN CYTOTOXIC T LYMPHOCYTES

I. Antigen-Specific Inhibition of Human Cell-Mediated Lympholysis*

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Cellular destruction by in vitro sensitized lymphocytes is mediated by cytotoxic T lymphocytes (CTLs) in the cell-mediated lympholysis (CML) assay (1–7). Target cells that bear major histocompatibility complex (MHC) antigens in common with the sensitizing cell are lysed. Studies of MHC recombinants have demonstrated that the strongest of these antigens recognized by CTLs are genetically controlled by the regions of the MHC that control the serologically defined (SD) antigens, and may in fact be the SD antigens themselves (8–13).

We here describe a sensitive human CML method that frequently detects significant cytotoxicity against "third-party" target cells unrelated to the stimulating cell donor when the lysis of autologous lymphocytes by the same CTLs does not differ from zero. This "cross-killing" has been detected even when the stimulating cells and third-party cells have no shared or cross-reacting SD antigens by serological criteria (14).

The specificity of this cross-killing has been examined by using a CML-blocking technique in which unlabeled target cells added to the test culture competitively inhibit lysis of *3Cr-labeled targets (15–17). These experiments demonstrate that cross-killing of third-party target cells by human CTLs requires the recognition of specific antigenic determinants that were present on the initial stimulating cells.

Materials and Methods

Lymphocytes. Human peripheral blood lymphocytes were obtained from healthy nonimmunized human donors and purified by Ficoll-Hypaque gradient flotation.

Cell-Mediated Lympholysis (CML)

IN VITRO SENSITIZATION. 12 × 10⁶ mitomycin-C-treated "stimulating" cells and 9 × 10⁶ untreated "responding" cells are cultured together in upright 50-ml tissue culture flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) containing 10–20 ml of "final medium," which consists of RPMI-1640 containing 25 mM HEPES buffer (Grand Island Biological Co., Grand Island, N. Y.)

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1 Abbreviations used in this paper: CML, cell-mediated lympholysis; CTLs, cytotoxic T lymphocytes; MHC, major histocompatibility complex; MLC, mixed lymphocyte cultures; SD, serologically defined.
supplemented with 4 mM L-glutamine (GIBCO) 1.2% Penicillin-Streptomycin (Grand Island Biological Co.), and 20% heat-inactivated human serum. These flasks are incubated for 6 days in a 37°C, 95% air, and 5% CO₂ humid atmosphere, after which all cells are harvested and resuspended in final medium after washing once. All cell populations obtained by this method are referred to as CTLs even when no cytotoxic activity is demonstrated.

TARGETS AND BLOCKERS. At the same time that sensitization cultures are established, 5-30 × 10⁶ lymphocytes are cultured in upright 50-ml tissue culture flasks containing 10-20 ml final medium. On day 6, 5-10 × 10⁶ of these cells are labeled with ²⁵²Cr as described previously (7), and resuspended at 1 × 10⁵ radiolabeled cells/ml. The remaining unlabeled target cells are resuspended at varying concentrations and will be referred to as "blockers."

STANDARD ⁵¹Cr-RELEASE ASSAY. Cytotoxicity assays are performed in 10 × 75 mm glass test tubes (Kimble Div., American Hospital Supply Corp., Evanston, Ill.) by adding 0.1 ml of ²⁵²Cr-labeled target cells (1 × 10⁴ cells) to 0.2 ml of CTL suspensions of varying concentrations. These are centrifuged at 150 g for 5 min and then incubated in a 37°C, 5% CO₂ humid atmosphere for 4 h. Iced Medium 199 (1.7 ml) is then added to stop the reaction and resuspend all cells. Tubes are spun at 600 g for 10 min, and the ²⁵²Cr containing supernates are decanted into glass tubes and counted for ²⁵²Cr content in a gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.). ²⁵²Cr-release data (mean counts per minute of triplicates ± SD) are expressed as percent cytotoxicity:

\[
\text{% cytotoxicity} = \frac{(\text{exp.} - \text{SR})}{(\text{max.} - \text{SR})} \times 100,\]

where (SR) is the ²⁵²Cr released spontaneously during 4 h by 1 × 10⁴ labeled target cells alone; max. is the ²⁵²Cr released by 1 × 10⁴ target cells lysed by detergent; and exp. is the ²⁵²Cr released by targets in the presence of CTLs. In CML studies of 53 different target cell preparations, the ²⁵²Cr released by detergent has averaged 87.2% of the total ²⁵²Cr incorporated into the target cells, while the spontaneous release value has averaged 11.1% of the total.

This CML method sensitively detects cytotoxic activity directed at target cells to which responding lymphocytes have been sensitized. Cytotoxicity by two CTLs per target averaged 29.5 ± 13.5%, and in every combination of unrelated individuals tested, the killing by one CTL per target has been significantly greater than zero. In contrast, the "autokilling" observed by specifically sensitized CTLs tested at ratios of 10 or more per autologous target cell has averaged −0.1 ± 1.2%.

CML-BLOCKING STUDIES. Specific CML blocking is performed by adding 0.2 ml of "blocker" cell suspension to each tube before the addition of CTLs. Analysis of ²⁵²Cr data obtained from these blocking experiments will be discussed in the Results section.

Proliferative Studies. Mixed lymphocyte cultures (MLC) are performed by the method of Hartzman et al. (18) in final medium (described above).

HL-A SD Typing. Serological detection of HL-A SD specificities was kindly performed by Dr. Rudolf Wank using a modified two-stage microcytotoxicity assay (19). Testing was performed with at least 120 separate alloantisera which detect eleven alleles of the LA locus and 14 alleles of the Four locus. Due to lack of antisera, alleles of the third SD locus (AJ) have not been identified for these studies (20).

Results and Discussion

Magnitude of "Cross-Killing." Extensive human CML studies have correlated the magnitude of cytotoxicity on third-party target cells unrelated to the initial MLC-stimulating cells with the number of shared serologically detected (SD) antigens (8, 12). If individuals B and C are picked at random, or share no SD antigens by serological criteria, previous reports have found no or low levels of significant cross-killing of C targets by CTLs from an ABₘ culture (4, 9, 10). Using the present CML method, the cross-killing has often been remarkably high, even when the specific sensitizing and third-party cells have no shared or cross-reacting SD antigens (14).

In the experiment presented in Table I, CTLs mediate significant cytotoxicity
CTLs from the indicated MLC as well as unstimulated cells from Z, were tested on target cells from individuals W, X, Y, and Z. No cross-reacting or shared antigens were detected serologically among individuals W, X, and Y. Typing results are as follows: W, (1, 8); X, (2, 12); Y, (10, 7, 27); and Z, (10, 11, W15, W16). Percent cytotoxicity by 25 x 10° CTLs on 1 x 10° target cells are shown. Potency values in parentheses represent the number of specifically sensitized cells from Z that cause the same percent cytotoxicity as 25 x 10° of the CTLs being examined. Potency values were interpolated from a log-linear graph of several specific CTL concentrations vs. percent cytotoxicity.

### Table I

| CTLs | Target | W | X | Y | Z |
|------|--------|---|---|---|---|
| ZWₘ |       | 62.5 ± 2.8 (28) | 55.9 ± 3.2 (10) | 38.5 ± 2.7 (2) | 1.7 ± 3.9 |
| ZXₘ |       | 48.5 ± 4.3 (10) | 67.6 ± 6.2 (25) | 29.3 ± 3.5 (1) | 0.3 ± 3.1 |
| ZYₘ |       | 40.7 ± 3.8 (6)  | 28.6 ± 2.3 (2)  | 68.9 ± 3.4 (25) | -1.6 ± 3.2 |
| Z—  |       | 0.4 ± 1.7       | -3.1 ± 2.3      | -1.7 ± 2.5      | —          |

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on third-party targets bearing no serologically detectable cross-reactivities with the sensitizing cell donor. Each of the cross-killing combinations demonstrate that "cross-reactivities" not detectable by current serological methods can be identified by in vitro-sensitized CTLs; the biological significance of these cross-reactivities remains unclear. For example, the destruction of X targets by CTLs from the ZWₘ(ZWₘ/*X) mixed culture does not prove the existence of antigenic determinants shared by X and W that are recognized by Z, even though this relationship is often assumed. Several other specific and nonspecific mechanisms of CML cross-killing are possible; these have been tested experimentally using the CML-blocking method.

**Antigen-Specific Blocking of CML.** The amount of cytotoxicity observed using this and most other CML assays is roughly proportional to the log of the CTL to target (K/T) ratio. This ratio is usually diminished by decreasing the number of CTLs while the number of ⁵¹Cr-labeled targets is fixed. If the number of CTLs and target cells were fixed and the K/T ratio was decreased by the addition of unlabeled target cells, one would also predict a decrease in the percent cytotoxicity. This is the rationale behind the CML-blocking method (15–17).

In the absence of CTLs, the blockers alone cause little or no variation from the SR value. This allows all ⁵¹Cr release data to be calculated with the SR corresponding to 0% killing, even in the presence of blockers. In Table II, any inhibition of CML caused by the autologous L blockers must be due to steric effects (i.e., nonspecific physical interference with the interaction between CTLs and labeled targets). In every combination tested the specific Q blockers inhibited the cytotoxicity to a greater degree than the L blockers, thereby demonstrating antigen-specific blocking.

Two separate approaches toward the quantitation of blocking data are presented. The percent "specific blocking", given in column A, indicates what fraction of the cytotoxicity obtained in the presence of autologous L blockers is
### Table II
Quantitation of CML Blocking

| Blockers x 10^4 per tube (LQ or Q) | Released ^51Cr ± SD | Cytotoxicity | A Specific blocking* | B CTL† potency | C Specific potency inhibition§ |
|-----------------------------------|---------------------|--------------|----------------------|----------------|-------------------------------|
|                                   | cpm                 | %            | %                    |                |                               |
| 0                                 | 1,656 ± 64          | 65.2         | 50                   |                |                               |
| 5 L                               | 1,568 ± 129         | 60.8         | 37                   | 65             |                               |
| Q                                 | 1,325 ± 77          | 48.8         | 20                   | 13             |                               |
| 50 L                              | 1,383 ± 170         | 51.7         | 17                   |                |                               |
| Q                                 | 899 ± 24            | 27.7         | 46                   | 85             |                               |
| 100 L                             | 1,360 ± 28          | 50.5         | 16                   |                |                               |
| Q                                 | 682 ± 28            | 17.0         | 66                   | 92             |                               |
| 200 L                             | 1,337 ± 91          | 49.4         | 14                   |                |                               |
| Q                                 | 477 ± 25            | 6.9          | 86                   | 0.7            | 95                            |

* ^51Cr released by 1 x 10^4 Q targets incubated with 50 x 10^4 CTLs from an LQ or Q culture was measured in the presence of varying numbers of L or Q blockers. ^51Cr data is expressed as the mean cpm ± SD of triplicate cultures. The percent cytotoxicity is based on the following spontaneous release (SR) and maximum release (max.) values (mean ^51Cr cpm of triplicates ± SD): SR, 337 ± 33; max., 2,361 ± 70.

Percent specific blocking = 100 x (CML units with autologous blockers - CML units with allogeneic blockers)/CML units with autologous blockers, where "autologous blockers" are from the responding cell donor.

† CTL potency is determined by interpolating the cytotoxicity obtained in the presence of blockers to the plot of cytotoxicity vs. log (K/T) obtained with varying numbers of unblocked CTLs (not shown). The potency of any CML combination is the number of unblocked CTLs which would cause the same percent cytotoxicity as the blocked combination.

§ Percent specific potency inhibition = 100 x (potency of CTLs with autologous blockers - potency of CTLs with allogeneic blockers)/(Potency of CTLs with autologous blockers).

∥ 100 x 10^4 L blockers were not tested. This ^51Cr-release value was interpolated logarithmically from values obtained for 50 x 10^4 and 200 x 10^4 L blockers.

### Blocking Analysis of Cross-Killing Specificity

Monolayer adsorption studies of CTLs have proven the existence of distinct antigen-reactive cytotoxic cell populations (22–24). This finding, together with the absence of nonspecific
autokilling in this CML system, suggests that all cytotoxicity, including cross-

killing, may be mediated by antigen-specific subpopulations of CTLs. There are

at least two separate mechanisms by which CTLs reactive to antigens on third-
party target cells could be generated in a CML-sensitization culture.

**Nonspecific Alloreactive Activation.** Activation of A responder cells by

Bm-stimulating cells has been shown to induce most of the CTL precursors from

individual A that recognize Bm to differentiate into CTLs specifically cytotoxic

for B. In addition, this activation mechanism may nonspecifically induce differ-

entiation of a small fraction of other CTL precursor populations that are reactive

to CML antigens not present on B; some CTLs able to recognize antigens present

on C targets but not on B, may be activated and therefore cause cross-killing on

C targets.

**Specific Antigen-Dependent Activation.** Activation of A responder cells

by Bm stimulators may induce only the A cells that are B reactive to become
cytotoxic, leaving "dormant" all cytotoxic precursors not able to recognize anti-
gens on B target cells. The observed killing of C targets by CTLs from an ABm

MLC would result from recognition of antigenic determinants present on C that

are also present on B.

Although previous CML reports have assumed that CML cross-killing demon-

strates shared antigens, both of the above possibilities must be considered; the

mechanism of "nonspecific alloreactive activation" may be particularly rele-

vant to those cases of significant CML cross-killing in the absence of shared or

cross-reacting serologically detectible specificities (25, 26).

These two mechanisms have been tested by blocking studies of CML cross-
killing; in all cases the results have supported the second mechanism. One such

experiment using lymphocytes obtained from three randomly selected unrelated

donors is presented in Fig. 1. The specific cytotoxicity mediated by CTLs from an

ABm culture on B targets is inhibited from 28.1 to 9.5% cytotoxicity by the

addition of B blockers, while the same CTLs on B targets are inhibited to a

lesser extent by third-party blockers from individual C, 17.5% cytotoxicity. In

contrast, the cross-killing on C targets is inhibited to the same degree by B and

C blockers. This suggests that all the antigens ABm recognizes on the C targets

are present on B blockers and were present on the initial Bm-stimulating cells. If

any of the cross-killing on C targets had been due to nonspecific alloreactive

activation there should have been CTLs from the ABm culture reactive only to C

and thereby subject to blocking by C but not B. In such an experiment reciprocal

specificity studies are crucial to prove that the observed result is actually due to

antigen recognition rather than differential steric effects; as seen in Fig. 1, the

equivalent result is obtained in the reciprocal combinations.

**Selective Blocking of Cytotoxic Subpopulations.** Experiments using the

above protocol prove that cross-killing of ABm/C is not mediated by a subpopula-
tion of nonspecifically activated CTLs reactive only to C and not B. However

these same experiments by themselves cannot prove that cross-killing of C

targets results from the recognition of antigens on the C cells that were also

recognized on Bm in the initial ABm sensitization culture. It could still be argued

that the observed inhibition of ABm/C by B blockers has no relation to the

recognition of shared target antigens. The unlabeled B target cells may block
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Fig. 1. Control of CML blocking by human MHC. CTLs from ABm and ACm sensitization cultures are tested on B and C target cells in the presence of A, B, or C blocking cells. Individuals A, B, and C are randomly selected unrelated donors. Optimal blocking discrimination in this experiment was observed with $5 \times 10^4$ CTLs and $30 \times 10^4$ blocking cells; parallel results were also obtained using $5 \times 10^4$ CTLs with $5 \times 10^4$ blockers, and $30 \times 10^4$ CTLs with $30 \times 10^4$ blockers (not shown). Control values for each target: B: SR, 198 ± 27; max., 1,328 ± 92 and C: SR, 239 ± 20; max., 1,619 ± 21.

ABm/*C via some mechanism related to the presence of Bm as the specific stimulator, and independent of target antigens present on C. This possibility is ruled out by experiments in which reactivity to multiple antigens can be selectively blocked; CTLs simultaneously stimulated by Em and Fm (Table III) are effectively blocked only by E blockers on E targets, and only by F blockers on F targets.

In total, these experiments demonstrate that CTLs are generated in vitro via the mechanism of "specific antigen-dependent activation," and that all CML cross-killing may result from recognition of shared antigens. These data also suggest that stimulation with several antigenic determinants, whether on two different allogeneic cells or possibly just on one stimulating cell, induces the differentiation of different cytotoxic populations for each determinant: these individual populations may specifically kill or be blocked only by cells bearing that single antigenic determinant.

CML Blocking is Controlled by the MHC. In the above studies, blocking of specific killing (i.e., ABm/*B) by unrelated third-party blocking populations has never been as effective as that caused by the specific blocker, suggesting the absence of at least some specific target antigens from the third-party cells. However a population of third-party cells bearing all the antigens recognized on the specific target would be expected to block as well as the specific blocker. Because CML reactivity is directed primarily against antigens controlled by the MHC, it is likely that cells from any individual sharing the same MHC recognized on the stimulating cells should block equivalently to the specific blockers.

In Fig. 2, individuals J and K are HL-A LD and SD identical siblings, H is a
Targets mediated by 25 \times 10^4 CTLs from DE_m, DF_m, and DE_mF_m cultures was assayed on 1 \times 10^4 E and F targets in the presence of 50 \times 10^4 D, E, or F blockers.

* Percent specific blocking, see Table II legend. Control values for each target: E: SR, 229 ± 6; max., 1,724 ± 48; F: SR = 294 ± 9; max. = 2,447 ± 40.

Cytotoxicity mediated by 25 \times 10^4 CTLs from DE_m, DF_m, and DE_mF_m cultures was assayed on 1 \times 10^4 E and F targets in the presence of 50 \times 10^4 D, E, or F blockers.

| Targets | CTLs | Cytotoxicity with 50 \times 10^4 blocking cells per tube | Specific blocking |
|---------|------|--------------------------------------------------------|------------------|
|         |      | D blockers | E blockers | F blockers | E blockers | F blockers |
| E       | DE_m | 36.0 ± 2.8 | 13.9 ± 1.1 | 34.7 ± 2.9 | 61        | 4         |
|         | DF_m | 20.5 ± 1.5 | 0.7 ± 0.8  | 4.2 ± 2.0  | 97        | 80        |
|         | DE_mF_m | 34.0 ± 5.0 | 7.3 ± 1.8  | 35.6 ± 2.7 | 79        | <0        |
|         | None  | -0.9 ± 0.9 | -           | -1.7 ± 0.8 |           |           |
| F       | DE_m | 23.8 ± 0.7 | 10.0 ± 3.8 | 7.0 ± 1.7  | 58        | 71        |
|         | DF_m | 39.3 ± 2.4 | 40.0 ± 6.7 | 16.1 ± 2.2 | <0        | 59        |
|         | DE_mF_m | 31.9 ± 2.3 | 33.4 ± 1.8 | 10.8 ± 2.0 | <0        | 66        |
|         | None  | 1.0 ± 1.8  | 0.1 ± 1.7  |           |           |           |

Cytotoxicity mediated by 25 \times 10^4 CTLs from DE_m, DF_m, and DE_mF_m cultures was assayed on 1 \times 10^4 E and F targets in the presence of 50 \times 10^4 D, E, or F blockers.

* Percent specific blocking, see Table II legend. Control values for each target: E: SR, 229 ± 6; max., 1,724 ± 48; F: SR = 294 ± 9; max. = 2,447 ± 40.

haplo-identical sibling differing by a maternal MHC, and M is their mother. In this experiment, some CTLs are derived from cultures in which responder and stimulator are haplo-identical so that any kinship member with the same stimulating MHC should block. This is observed on both H and J targets; KH_m/*H is blocked similarly by H and M, while HJ_m/*J is blocked by J, K, and M. On both of these targets, blockers from unrelated individual U cause no significant blocking compared to the blockers autologous to the CTLs. These U cells, however, were the only effective block of HU_m/*U.

Of importance is the significant killing of U targets by CTLs from the HJ_m culture. Individual U has no SD antigens that cross-react with the 1-17 haplo-type recognized by sibling H on cells from J, K, or M. The cross-killing of U targets by these CTLs is inhibited significantly by blockers from K, M, and U (P < 0.005). The amount of blocking by family members K and M is not significantly different from the blocking by U (P > 0.05). This last combination demonstrates three separate properties of CML cross-killing. First, cross-killing can be observed even when "full-house" typing reveals no cross-reacting SD determinants. Second, as shown in Fig. 1 and Table III, cross-killing results from the recognition of antigens shared by the stimulating cell and the third-party target cells. Third, these shared but serologically undetected antigens are controlled by the MHC.

Several lines of evidence have mapped the genetic control of CML detected antigens to the SD regions of the MHC, and have suggested that the serologically-detected and CML-detected antigens are genetically identical (8-13). If this is the case, detection of shared antigens by blocking of CML cross-killing and not by serological testing suggests that CTLs and antisera recognize different submolecular structures of the SD-gene products, or they recognize the same
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Fig. 2. CML cross-killing detects shared antigens. Family Bey members J and K are MHC-identical siblings, H is haplo-identical to J and K, M is their mother, and U is an unrelated individual. HL-A SD specificities for each are: H, 2-14 and 11-5; J, 2-14 and 1-17; K, 2-14 and 1-17; M, 11-5 and 1-17; and U, 2, 12, 13, W29. Individual U shares antigen 2 with sibs H, J, and K, but specificities 12, 13, and W29 do not cross-react serologically with any of the antigen specificities in the family. All results shown on J targets were paralleled on targets from MHC identical sib K. Similarly, CTLs from an HKm culture demonstrated CML specificity comparable to HJm-derived CTLs. CML reactions were also performed in all combinations using 5 x 10⁴ CTLs per tube; while the percent cytotoxicity was less than that shown for 25 x 10⁴ CTLs per tube, the specificity of cytotoxicity and blocking was identical. The percent cytotoxicity mediated on all four targets (H, J, K, and U), by each of the five blocker populations in the absence of any CTLs ranged from -2.3 to 3.9, and did not differ significantly from 0. Control values for each target: H: SR, 213 ± 28; max., 2,083 ± 48; J: SR, 143 ± 39; max., 1,411 ± 6; and U: SR, 244 ± 40; max., 1,989 ± 68.

structures differently. In either case the CTL antigen receptor demonstrates different recognitive specificity than the antigen-binding site of the immunoglobulin (27).

Alternatively, the antigens recognized by CTLs might not be controlled by the SD loci, but instead by different closely linked loci within the SD regions. Under these conditions, the impressive correlation of SD antigen sharing with the magnitude of the CML reaction would be attributed to linkage disequilibrium. This would demonstrate a far greater recognitive dichotomy between immunoglobulins directed at SD antigens and in vitro sensitized CTLs directed at still some other target molecule.

Summary

The specificity of antigen recognition by in vitro sensitized human cytotoxic T lymphocytes (CTLs) has been studied using a sensitive cell-mediated lysis (CML) assay. Frequently, high levels of cytotoxicity are observed on third-party targets unrelated to sensitizing or responding cells; however, no cytotoxic-
ity differing significantly from zero has been observed on targets autologous to the responding CTLs. This "cross-killing" of third-party target cells has been observed when stimulating and third-party cells bear no cross-reacting serologically defined (SD) antigens, thought to be the target antigens recognized by CTLs. CML-blocking studies, using unlabeled normal human lymphocytes to inhibit $^{32}$P release from radiolabeled target cells, have shown that cross-killing, even in the absence of shared SD determinants, results from CTLs recognizing antigens shared by the third-party targets and the initial stimulating population. Furthermore, these antigens have been mapped to the major histocompatibility complex (MHC). The ability of human CTLs to specifically recognize MHC-controlled antigens not detected serologically suggests that SD antigens may be recognized differently by alloantisera and CTLs, or that MHC antigens other than SD may be the targets of CTLs in CML.

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