Correction. In the article "Role of positive charge on the amino-terminal region of the signal peptide in protein secretion across the membrane" by Sumiko Inouye, Xavier Soberon, Thomas Franceschini, Kenzo Nakamura, Keiichi Itakura, and Masayori Inouye, which appeared in number 11, June 1982, of Proc. Natl. Acad. Sci. USA (79, 3438-3441), an incorrect version of Fig. 1 was printed by mistake. The correct figure is printed below.

![Fig. 1. Nucleotide sequences of synthetic oligonucleotides. Bases that are altered from the wild-type sequences (6) are marked by a dot. Amino acid residues changed as a result of mutations are boxed.](image)

Correction. In the article "Amino acid sequence of mouse submaxillary gland renin" by Kunio S. Misono, Jin-Jyi Chang, and Tadashi Inagami, which appeared in number 16, August 1982, of Proc. Natl. Acad. Sci. USA (79, 4858-4862), the authors request that the following correction be noted. The M, of the heavy chain in the reduced form was given incorrectly as 31,036 in line 4 of the Abstract (p. 4858) and in line 4 of the Discussion (p. 4859). The correct M, of the heavy chain in the reduced form is 31,043.

Correction. In the article "Clonal analysis of human cytotoxic T lymphocytes: T4' and T8' effector T cells recognize products of different major histocompatibility complex regions" by Stefan C. Meuer, Stuart F. Schlossman, and Ellis L. Reinherz, which appeared in number 14, July 1982, of Proc. Natl. Acad. Sci. USA (79, 4395-4399), a typographic error was not caught. On p. 4395, in the fourth line from the bottom in the left-hand column, "T4/8" should have been "T5/8".

Correction. In the article "Mitochondrial DNA polymorphism in a maternal lineage of Holstein cows" by William W. Hauswirth and Philip J. Laipis, which appeared in number 15, August 1982, of Proc. Natl. Acad. Sci. USA (79, 4686-4690), the authors request that the following correction be noted. In the legend to Fig. 3 on p. 4688, lines 9 and 10 should read "... cloned fragment of H949B."
Clonal analysis of human cytotoxic T lymphocytes: T4+ and T8+ effector T cells recognize products of different major histocompatibility complex regions

(major histocompatibility complex gene products/cytotoxicity monoclonal antibodies)

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ABSTRACT Alloreactive human T lymphocytes were cloned in soft agar or by limiting dilution and subsequently propagated with interleukin 2 and alloantigen for 8 months or more. By indirect immunofluorescence every clone was reactive with anti-Ia antibodies as well as the T cell-specific antibodies anti-T3 and anti-T11 and expressed either T4 or T8 antigens. All 15 T8+ clones were highly cytotoxic for the sensitizing alloantigen. In contrast, only two of the seven T4+ clones mediated cytotoxic effector function. The specificity of T4+ and T8+ clones and subclones was analyzed on a panel of typing cells and by antibody blocking studies of major histocompatibility complex (MHC) determinants on the stimulating alloantigen. It was found that T8+ clones killed targets that shared class I MHC antigens (HLA-A,B) with the original stimulator cells whereas cytotoxic T4+ clones were directed at class II MHC antigens (Ia-related). Preincubation of the allogeneic target cell with a monoclonal antibody to a nonpolymorphic HLA a-chain determinant inhibited killing by the T8+ clones but did not affect T4+ cytotoxic function. In a reciprocal fashion, anti-Ia antibodies to common framework structures on the same target cell blocked killing by T4+ but not by T8+ clones. These results indicate that T4+ and T8+ T lymphocytes have receptors for different classes of MHC antigens and suggest that cytotoxic T4+ subpopulations might be important in human transplantation and autoimmune disorders.

The two human T cell subsets with unique regulatory and effector functions were defined with the use of heteroantisera and monoclonal antibodies (1-3). These have been termed T4+ and T5/8+ on the basis of the individual cell surface glycoproteins that they express. The T4+ subset was shown to provide inducer/helper activities for T-T, T-B, and T-macrophage interactions whereas the T5/8+ subset primarily functioned in a suppressive mode (4-7). Although both subsets of cells proliferated to alloantigen in mixed lymphocyte culture (MLC), the vast majority of cytotoxic effector function was detected in the T5/8+ population (4). Moreover, development of cytotoxicity by T8+ cells in general required interactions with T4+ cells or their soluble products (4, 8). In contrast, only a minor component of cytotoxic effector function resided within the T4+ subset and this was maximal when T4+ cells alone were sensitized in MLC (4).

Recently, various clinical phenomena that could be attributed to cytotoxic T cells have been observed in human autoimmune disorders associated with loss of T4/5/8 T cells (9). This finding suggested that the previously observed T4-mediated killing may be of clinical importance. To characterize the cytotoxic potential of T4+ T cells further and to determine their specificity, we generated cloned populations of allosensitized T cells.

MATERIALS AND METHODS

Derivation of Lymphocyte Populations. Lymphocytes were obtained by Ficoll/Hypaque density centrifugation of blood from a healthy donor. All Epstein-Barr virus-transformed B-cell lines (Laz 156, Laz 509, and B-C) were kindly provided by H. Lazarus (Sidney Farber Cancer Institute). HLA typed target cells (2-12) were provided by E. J. Yunis (Sidney Farber Cancer Institute).

Production and Characterization of Monoclonal Antibodies and Heteroantisera. Monoclonal antibodies against human T lymphocyte cell surface antigens were derived from the following clones: 2Ad2Al2 (anti-T3, directed at all mature T cells); 3Pt2H9 (anti-T11, sheep erythrocyte receptor antigen); 3Pt12B8 (anti-T12, all mature T cells); 21Thy2D3 (anti-T8a, defines human suppressor T cells); 19Thy5D7 and 12T4D11 (anti-T4a and anti-T4b, both define human inducer T cells) (3-7, 10-12). In addition, 7Pt4F12 and 7Pt3H9 were created (immunogen was anti-T8 + T cell chronic lymphocytic leukemia) (4). These antibodies were termed "anti-T8a" and "anti-T8b," and were of the IgM and IgG mouse isotypes, respectively. Their reactivity was identical to that of anti-T8 (1).

The monoclonal antibodies against human Ia-like antigens and beta2-microglobulin have been described (13). In brief, monoclonal antibody II reacts with a nonpolymorphic constant region of the bimolecular glycoprotein complex (p29,34) of the Ia antigen. I-1R2 defines a polymorphic supertypic cluster of HLA-DR antigens (HLA-DR3, -5, and -6) and I-1R1 recognizes a polymorphic supertypic specificity on certain SB loci (SB2 and SB3) (L. M. Nadler, personal communication). W6/32 anti-HLA monoclonal antibody (14) was purchased from Sera Laboratories (Sussex, Englad). Turkey heteroantisera against human Ia antigens (anti-p29,34) and beta2-microglobulin were provided by E. J. Yunis and were prepared as described (15), p23,30 is a rabbit heteroantisera reactive with human Ia antigens (16).

 interleukin 2 (IL-2). IL-2-containing supernatants were produced by stimulating whole peripheral blood mononuclear cells at a concentration of 2.5 X 10^9/ml for 2 hr with phytohemagluttinin (5 µg/ml; Wellcome, Beckenham, England) and phor- myristate acetate (5 ng/ml; Sigma) in the presence of irradiated 5000 rad (50 grays) Laz 156 (0.5 X 10^9/ml). Subsequently, the cells were washed extensively and resuspended in culture medium RPMI-1640 supplemented with 1% human AB serum. After 40 hr of incubation at 37°C in a humid atmosphere, 4395

Abbreviations: MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; CML, cell-mediated lympholysis; IL-2, interleukin 2; CTL, cytotoxic T lymphocytes.
the supernatants were harvested, passed through 0.45-μm filters, and stored at -70°C.

Cloning and Culture of Human T Lymphocytes. All clones were derived from a single individual's mononuclear cells (1 × 10^6/ml) which were stimulated with an equal concentration of the irradiated allogeneic B lymphoblastoid line Laz 156 for 5 days in final culture medium (RPMI-1640 with 20% human AB/glutamine [Microbiological Associates, Bethesda, MD]). Cloning of alloactivated cells was performed in a two-layer soft agar system as described (17) as well as by limiting dilution in Terasaki plates at 0.6 cells per well in the presence of 5% IL-2 and irradiated feeder cells as described above and maintained in culture for 6-9 months. These clones were analyzed with the panel of monoclonal antibodies in indirect immunofluorescence on an Epics V cell sorter (4).

51Cr Release Assay and Blocking Experiments with Monoclonal Antibodies and Heteroantisera. To examine the effects of monoclonal antibodies to T cell surface antigens on the cytotoxic effector function of various clones, clones were incubated for 30 min with monoclonal antibody at several dilutions or media prior to addition of 51Cr-labeled targets. In other experiments aimed at detecting target antigens recognized by the cytolytic T cell clones, 51Cr-labeled targets were preincubated with heteroantisera or monoclonal antibodies for 30 min at room temperature prior to addition of the effector cells. After addition of antibody as described above, the standard 4-hr cell-mediated lympholysis (CML) assay was performed (4).

RESULTS

To characterize individual human cytotoxic effector populations, T lymphocytes were stimulated for 5 days with the allogeneic B lymphoblastoid line Laz 156 and then cultured in soft agar or microtiter plates by limiting dilution. After 1 month of in vitro expansion with IL-2 and weekly stimulation by the alloantigen, cultures were screened for reactivity with anti-T4 and anti-T8 monoclonal antibodies by indirect immunofluorescence on an Epics V cell sorter. Of the initial 45 cultures screened, 22 showed a homogeneous phenotype in terms of anti-T4 and anti-T8 reactivities; 15 cultures expressed the T8 antigen and 7 cultures expressed the T4 antigen. All 15T8" cultures exhibited a high level of cytotoxicity against the stimulating alloantigen Laz 156. In contrast, only two of the seven T4" cultures killed Laz 156.

Fig. 1 shows representative phenotypes of individual T4" and T8" clones as defined by reactivity with a series of monoclonal antibodies and indirect immunofluorescence on an Epics V cell sorter. Virtually all cells in the T4" clone were anti-T4 reactive and anti-T8 unreactive whereas the opposite pattern was the case for cells within the T8" clone. Both T4" and T8" clones expressed the sheep erythrocyte receptor antigen T11 and were reactive with anti-T3 which defines an antigen present on mature T lymphocytes (not shown). In addition, both T4" and T8" clones were reactive with anti-II (11). Dr typing showed that the Ia antigens on these clones were of the original donor genotype and unrelated to the Ia expressed by the B lymphoblastoid line Laz 156 (data not shown). This observation is not surprising in light of the fact that it has been shown (18, 19) that human T cells synthesize and express Ia antigens after activation.

The cytotoxic capacity of individual T4" and T8" T cell clones was examined by testing their ability to lyse 51Cr-labeled Laz 156 cells. Both CT8 and CT4 were highly cytotoxic for Laz 156 (Fig. 2). Thus, even at an effector/target ratio of 0.5:1, 10-20% specific killing was observed. In contrast, HT4 was incapable of killing the alloantigens at any ratio tested.

To determine the specificity of the cytotoxicity mediated by the various T4" and T8" T cell clones, various HLA-A, -B, and -Dr type cells were used as targets (Table 1). Although both CT8 and CT8 were cytotoxic for the isologous target cell Laz 156, CT8 killed target cells 3 and 4 whereas CT8 killed target cells 2 and 11. Given the HLA phenotype of these cells, it would appear that the CT8 clone is directed at the B40 HLA antigen...
The above findings suggested that the cytotoxicity mediated by the T8* clones was directed against class I HLA allotype whereas the cytotoxicity mediated by the T4* clones was not. To investigate this point further, we examined the ability of monoclonal and heteroantibodies directed at class I and class II allotype to block cytotoxic effector function by individual T cell clones. Antibodies to nonpolymorphic Ia and HLA antigens had very different effects on T4 and T8 T cell clones (Table 3). For example, preincubation of the isologous target Laz 156 with either anti-p29,34 or anti-p23,30 anti-Ia antisera resulted in inhibition of killing by both T4* clones, CT4 and CT4II. In contrast, neither of the two anti-Ia heteroantibodies inhibited cytotoxicity by the two T8* clones, CT8 and CT8II. Unlike the anti-Ia heteroantisera, monoclonal antibodies anti-II and I-LR1 did not inhibit cytotoxicity mediated by either T4* or T8* clones (data not shown).

In a reciprocal fashion, the monoclonal antibody w6/32, which is directed at a nonpolymorphic determinant on the \( \alpha \) chain of HLA-A and -B, strongly inhibited CML by the T8* clones but not by the T4* clones. In contrast, anti-\( \beta_2 \)-microglobulin heteroantisera (Table 3) and monoclonal antibodies against \( \beta_2 \)-microglobulin (not shown) did not influence the level of cytotoxicity by T4* or T8* clones.

Given the differences in surface glycoprotein expression of the T cell clones themselves, it was also important to determine if individual anti-T4 and anti-T8 antibodies influenced killing function. To examine this point, cytotoxic T cell clones CT4, CT4II, CT8, and CT8II were preincubated with T cell-specific monoclonal antibody or medium alone prior to the CML assay. Anti-T8 and anti-T8 antibodies did not diminish the level of killing by the T4* subclones but markedly decreased cytotoxicity mediated by the two T8* clones (Table 4). In contrast, monoclonal antibody anti-T8II, had no influence on any clones tested. Anti-T4, and anti-T4 resulted in 30–40% reduction of cytotoxicity by the two T4* clones, CT4 and CT4II, but had no effect on killing by the T8* subclones. That this inhibition was not simply a function of antibody binding to the clonal effector population was evident from the finding that a monoclonal antibody directed at a glycoprotein present on both T4* and T8* clones, anti-T12, had no effect on inhibition of cytotoxicity.

**DISCUSSION**

In the present study, clones and subclones of alloreactive human T lymphocytes were established against an Epstein–Barr virus-transformed B lymphoblastoid line, Laz 156. For this purpose, we utilized both soft agar and limiting-dilution techniques. Of 22 clones maintained in long-term culture, 15 were reactive with anti-T8 whereas 7 were reactive with anti-T4. All 15 T8* clones demonstrated high levels of cytotoxicity against the sensitizing alloantigen but only 2 of 7 T4* clones mediated cytotoxic activity. Determination of the killing specificities of the T8* and T4* clones indicated that the former were directed at classic HLA-A or -B antigens whereas the latter were unrelated to HLA-A and -B. The HLA-A and -B specificity of the T8 clones was shown by examining their capacity to kill defined HLA type target cells as well as the finding that a monoclonal antibody to a nonpolymorphic region of the HLA heavy chain inhibited their cytotoxic function. In contrast, the T4* clones killed several targets that did not share HLA-A or -B determinants and their function was not inhibitable with anti-HLA heavy chain monoclonal antibody. Rather, cytotoxic function of T4* clones was blocked with anti-Ia heteroantisera. One of two T4* cytotoxic clones was directed at a single DR antigen and the second probably was directed at a novel class II antigen, perhaps within the SB locus (20). Taken together, these findings suggest that

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**Table 1.** CML by clones CT8 and CT8II

<table>
<thead>
<tr>
<th>Target cell</th>
<th>HLA type of target cells</th>
<th>Cytotoxicity of clone*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CT8</td>
<td>CT8II</td>
</tr>
<tr>
<td>Laz 156†</td>
<td>A3, A3</td>
<td>B7, B40</td>
</tr>
<tr>
<td></td>
<td>A2, A25</td>
<td>B13, Bw38</td>
</tr>
<tr>
<td></td>
<td>A2, A3</td>
<td>B7, B12</td>
</tr>
<tr>
<td></td>
<td>A24</td>
<td>B17, B40</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>B44, B40</td>
</tr>
<tr>
<td></td>
<td>A31</td>
<td>B22, B37</td>
</tr>
<tr>
<td></td>
<td>A28, A30</td>
<td>Bw33, B8</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>B18</td>
</tr>
<tr>
<td></td>
<td>A11, A31</td>
<td>B39, Bw48</td>
</tr>
<tr>
<td></td>
<td>A1, A3</td>
<td>B7, B37</td>
</tr>
<tr>
<td></td>
<td>A2, A26</td>
<td>Bw44</td>
</tr>
<tr>
<td></td>
<td>A2, A3</td>
<td>B7</td>
</tr>
<tr>
<td></td>
<td>A1, A2</td>
<td>Bw41, Bw44</td>
</tr>
</tbody>
</table>

* Cr release (% specific lysis). ND, not done.
† Original stimulator cell.
‡ Autologous Epstein–Barr virus-transformed cell line (Laz 509).
cytotoxic T4+ cells recognize class II antigens whereas T8+ cytotoxic effector cells are directed toward class I major histocompatibility complex (MHC) antigens.

Prior studies indicated that the majority of cytotoxic effector cells generated in primary MLC are contained within the T5/8+ T cell subset. This was demonstrated by showing enrichment of specific cytotoxic effectors after positive selection of T5+ T cells from an allosensitized population on a fluorescence-activated cell sorter as well as reduction of T8+ cell activity after depletion of T8+ cells with anti-T8 and complement from an allosensitized population (7, 21). Nevertheless, a small degree of cytotoxicity was mediated by the residual T4+ population. The latter was even more evident when T4+ cells were sensitized in the absence of T8+ cells in primary MLC (4). This observation raised the possibility that either the T8+ population, known to contain suppressor cells, regulated the expression of cytotoxicity by the reciprocal T4+ population or, alternatively, in the presence of T4+ cells during allosensitization, the T8+ cytotoxic subset has a selective growth advantage. In this regard, it is well known that the T4+ population makes helper factors which induce the T8 cells to proliferate and express maximal cytotoxicity (6, 8).

That T4 cells appear to recognize class II MHC determinants whereas T8 cells recognize class I MHC determinants is not without precedent. In the murine system, for example, it has been shown that allogeneic cells, which differ only at I MHC antigens from the responder cell, stimulate Lyt2+ T lymphocytes to proliferate and produce lymphokines whereas allogeneic cells which differ at class II MHC antigens induce activation of Lyt1+2− T cells (22). In addition, it has also been shown that cytotoxic T lymphocytes (CTL) can be generated against class II MHC antigens and that, under these circumstances, the phenotype of the cytotoxic effector cells is Lyt1+2−, unlike the conventional CTL which recognizes class I MHC and is Lyt2+ (23). The latter could be blocked with anti-Lyt2 alloantisera as well and this blocking was correlated with T cell specificity for class I MHC antigens (24). The present findings are therefore consistent with the above mouse findings and recent studies in the human suggesting that the major regulatory T cell subsets respond to different stimuli (19, 25, 26).

The observation that heteroantisera but not monoclonal antibodies directed at human Ia antigens inhibited cytotoxicity by the T4+ cytotoxic clones is worthy of comment. In this regard, several discrepancies between functional effects of heteroantisera and monoclonal antibodies to Ia antigens have already been noted. For example, by utilizing rabbit anti-p23,30 heteroantisera, Ia antigen was shown to be expressed on human cytotoxic effector cells (18) whereas with a nonpolymorphic anti-Ia monoclonal antibody, this was not found to be the case (21). Further support for distinctions between the monoclonal anti-Ia and rabbit anti-p23,30 heteroantisera, Ia antigen was shown to be expressed on human cytotoxic effector cells (18) whereas with a nonpolymorphic anti-Ia monoclonal antibody, this was not found to be the case (21). Further support for distinctions between the monoclonal anti-Ia and rabbit anti-p23,30 heteroantisera, Ia antigen was shown to be expressed on human cytotoxic effector cells (18) whereas with a nonpolymorphic anti-Ia monoclonal antibody, this was not found to be the case (21).

The possibility that our two heteroantisera contained unrelated inhibitory substances is unlikely. First, neither anti-Ia heteroantisera affected cytotoxic effector function of T8+ clones and second, the heteroantisera to β2-microglobulin did not influence cytotoxicity of either T4+ or T8+ subclones. The former findings also demonstrate that the Ia determinants expressed by the various T cell clones most likely are not involved in cytotoxic effector function.

Table 4. Inhibitory effect of monoclonal anti-T cell antibodies on MLC

<table>
<thead>
<tr>
<th>CT4</th>
<th>CT4H</th>
<th>CT8</th>
<th>CT8H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Anti-T8A</td>
<td>107</td>
<td>100</td>
<td>24</td>
</tr>
<tr>
<td>Anti-T8B</td>
<td>102</td>
<td>101</td>
<td>8</td>
</tr>
<tr>
<td>Anti-T8C</td>
<td>ND</td>
<td>ND</td>
<td>98</td>
</tr>
<tr>
<td>Anti-T4A</td>
<td>58</td>
<td>57</td>
<td>100</td>
</tr>
<tr>
<td>Anti-T4B</td>
<td>66</td>
<td>64</td>
<td>102</td>
</tr>
<tr>
<td>Anti-T12</td>
<td>100</td>
<td>98</td>
<td>97</td>
</tr>
</tbody>
</table>

Final dilution of all monoclonal antibodies was 1:500. (Similar effects were seen at dilutions of 1:50 to 1:5,000.) The effector-to-target ratio was 5:1.

* ND, not done.
To determine whether the antigens defined by anti-T8 antibodies were themselves important in the cytotoxic mechanism of allosensitized T lymphocytes, we previously examined their inhibitory effect on heterogeneous uncloned CTL populations (11). Our results indicated that only selected T8 epitopes were involved in one or another aspect of CML. These findings were confirmed in the present study at the clonal level because T8\(^+\) clones were markedly inhibited in their cytotoxic effector function by anti-T8\(_a\) and anti-T8\(_b\) antibodies but unaffected by anti-T8\(_c\). Although the inability of anti-T8 antibodies to inhibit CTL by T4\(^+\) clones is not surprising because the latter lack the T8 antigen, the findings support the idea that cell surface molecules, other than T8 antigens, on individual T4 clones are essential in some aspect of cytotoxic effector function. The observation that anti-T4 antibodies partially blocked CTL selectively by T4\(^+\) clones further supports this view. Whether these antigens are involved in a cognitive recognition event or, alternatively, in a cytotoxic effector mechanism is not clear at present. However, the inability of these antibodies to interfere with clonal proliferation (data not shown) to the alloantigen might imply that the latter is more likely.

The finding that the majority of cloned cytotoxic cells were T8\(^+\) and that most T4\(^+\) clones did not display killing function is consistent with previous in vitro studies in which heterogeneous populations of uncloned cells were used (4). The present study shows that one can dissect the heterogeneity of various cytotoxic effector T lymphocytes more precisely regardless of their frequency. More important, it implies that T4\(^+\) cytotoxic effector mechanism directed to class II MHC determinants could be involved in various disorders in which defects in T8\(^+\) cells have been noted. In this regard, it is of considerable interest that T4\(^+\) but not T8\(^+\) T cells proliferate in response to autologous Ia antigens in autologous mixed lymphocyte reactions (28–30). Perhaps cytotoxic T4\(^+\) cells play a role in tissue damage in autoimmune diseases and allograft rejection.

The authors thank Ms. Rebecca E. Hussey, Ms. Kathleen A. Fitzgerald, and Mr. James C. Hodgdon for their excellent technical assistance and Mr. Herbert Levine and Mr. John Daley for performing cell sorter analysis. This work was supported by National Institutes of Health Grant CA 59589 and National Multiple Sclerosis Grant RC 1359-A1. S.C.M. is a recipient of a fellowship from the Deutsche Forschungsgemeinschaft (DFG, Me 693/1-1).