

CLONAL HETEROGENEITY IN THE REQUIREMENT FOR T3, T4, AND T8 MOLECULES IN HUMAN CYTOLYTIC T LYMPHOCYTE FUNCTION

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The availability of monoclonal antibodies (mAb)¹ specific for human surface membrane antigens (1) has resulted in rapid progress toward elucidation of the nature of the molecules involved in cytolytic T lymphocyte (CTL)-target cell interactions. Since target cell lysis requires close contact between CTL and target cells, a general approach to this question has been the identification of mAb directed against CTL surface structures that inhibit cytolysis when added (in the absence of complement) to CTL-target cell mixtures (2). For example, work from several groups showed that mAb against T8/Leu-2 and T3/Leu-4 surface molecules inhibit the cytolytic activity of T cell populations activated in allogeneic mixed lymphocyte culture (MLC) (3-9). Recently, studies using CTL clones suggested that anti-T3 mAb inhibits the activity of all CTL irrespective of their T8/T4 phenotype, whereas anti-T8 and anti-T4 mAb selectively inhibit cytolysis mediated by T8⁺ and T4⁺ CTL, respectively (10). In the mouse, it has been well documented that mAb against Lyt-2/3, the murine homologue of T8 antigen, can inhibit CTL-mediated lysis (11). However, the degree of inhibition obtained with anti-Lyt-2/3 mAb appears to be dependent on the source of CTL used. For example, the CTL activity of primary allogeneic MLC populations was found to be readily inhibited by anti-Lyt-2/3 mAb, whereas alloreactive CTL populations generated in vivo or in secondary (after priming in vitro) MLC were more difficult to inhibit under the same experimental conditions. The cellular basis for this difference has been clarified by the analysis of CTL clones derived from these populations. Thus, most (but not all) CTL clones derived from primary MLC were strongly inhibited by anti-Lyt-2/3 mAb, whereas, the activity of most (but not all) CTL clones derived from in vivo primed precursors was resistant to inhibition by these antibodies (12). These studies strongly suggest that CTL are heterogeneous in their requirement for Lyt-2/3 molecules in cytolytic activity.

Recently, Malissen et al. (8) reported that B9.4 mAb (which appears to react with surface molecules bearing the Leu-2a/b and T8 antigenic determinants)

¹ *Abbreviations used in this paper:* CTL, cytolytic T lymphocyte; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; mAb, monoclonal antibodies; MLC, mixed lymphocyte culture; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; TCGF, T cell growth factor.

(13), inhibited only a minority of human CTL clones derived from an alloimmune donor. Since this finding was reminiscent of the above-mentioned results in the mouse, we thought it of interest to determine whether there is heterogeneity in the involvement of molecules such as T8, T4, and T3 in the lytic activity of human CTL. Therefore, a large number of primary MLC-derived CTL clones were examined for their susceptibility to inhibition by mAb directed against T8, T4, and T3 molecules respectively. In agreement with the results obtained with murine Lyt-2⁺ CTL, it will be shown that, although the majority of primary MLC-derived CTL clones expressing T8 antigen are inhibited by B9.4. mAb, ~15% are unaffected by this antibody. Moreover, a similar heterogeneity in the susceptibility to inhibition by the corresponding mAb has been documented for CTL clones expressing T4 molecules. Furthermore, evidence will be presented that the lytic activity of a significant proportion of CTL clones is unaffected by anti-T3 mAb. Finally, modulation experiments on CTL clones indicate that removal of surface T3 molecules is not always accompanied by the loss of specific cytolytic activity, thus suggesting that receptor structures on the CTL surface are not always functionally associated with T3 molecules.

Materials and Methods

Generation of Alloimmune CTL. Cytolytic T cells were generated in vitro in unidirectional mixed lymphocyte cultures (MLC) as previously described (14). Briefly 10⁶ peripheral blood T lymphocytes from normal donors were cultured with 10⁶ irradiated (5,000 rads) allogeneic spleen cells for 7 d in macrowells (final volume 1 ml). The culture medium was RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY). Human spleens were removed for technical reasons during abdominal surgery. Mononuclear cell suspensions derived from spleens were frozen and subsequently thawed as needed (15).

mAb. The B9.4 mAb (directed to T8 surface antigen) was kindly provided by Dr. C. Mawas and B. Malissen (Centre d'Immunologie INSERM-CNRS, Marseille-Luminy, France), the 4F2 mAb was a gift of Dr. A. S. Fauci (National Institutes of Health, Bethesda, MD). OKT4 and OKT3 mAb were purchased from Ortho-Pharmaceuticals (Raritan, NJ).

Cytolytic Assay. The techniques used have been described in previous reports (16, 17). Briefly, various numbers of alloimmune primary MLC T cells or T cell clones were incubated for 4 h with ⁵¹Cr-labeled target cells (5 × 10³/well) in a final volume of 200 μl in "V"-bottomed microwells (Greiner Nurtinger, Federal Republic of Germany). Target cells were PHA-activated spleen-derived lymphoblasts bearing the sensitizing alloantigens or K 562 cells (in some control experiments). In the inhibition experiments, before the addition of target cells, MLC T cells or T cell clones were preincubated for 30 min at 20°C with various doses of B9.4, OKT4, or OKT3 mAb. B9.4 mAb was added at a final concentration of 1 μg/microwell (5 μg/ml) unless otherwise specified. OKT4 or OKT3 mAb were added in aliquots of 10 μl of the standard solution to each 200-μl microwell, unless otherwise specified. When necessary, MLC cells after preincubation with mAb were washed three times to remove the antibody. Microplates were centrifuged at 100 g for 5 min and then incubated at 37°C. After 4 h, microplates were centrifuged again (200 g for 5 min) and 100 μl supernatant was removed and counted for 1 min in a scintillation counter. Percent specific ⁵¹Cr release was calculated as described previously (18), whereas spontaneous release was determined in the absence of effector cells and maximal release was determined in the presence of 1 N hydrochloric acid.

Cloning of MLC Cells. This technique has been described in previous reports (19). Briefly, primary MLC T cells were submitted to vigorous vortex mixing (to dissociate possible cell clumps) and then seeded under limiting conditions (0.3 cells/well) in round-

bottomed microwells containing 10^5 irradiated stimulator spleen cells. The culture medium was RPMI 1640 containing 10% FCS and 40% supernate from phytohemagglutinin (PHA)-stimulated human spleen cell cultures (as source of T cell growth factor (TCGF) (20).

Screening of Proliferating and/or Cytolytic Clones. Clones derived from primary MLC T cells were identified microscopically after 12–20 d as previously described (19). Cell aliquots (usually 50 μ l of the cell suspension) from proliferating microcultures were tested for cytolytic activity against ^{51}Cr -labeled specific target cells. In inhibition experiments, cells were preincubated with mAb for 30 min before the addition of the target cells.

Immunofluorescence Staining and Fluorescence-activated Cell Sorter (FACS) Sorting. The techniques used have been previously described (21). Briefly, samples of 2×10^6 MLC T cells in 100 μ l RPMI 1640 medium were incubated with B9.4 or OKT4 monoclonal antibodies for 30 min at 4°C. After two washings, the cells were resuspended in 200 μ l of medium containing fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG. Control samples were incubated with the FITC-coupled reagent only. Before sorting cells, the sample tubing of the FACS was flushed sequentially with detergent, sterile distilled water, and 96% ethanol. Then, the tubing was rinsed for 30 min with a solution of gentamycin (50 $\mu\text{g}/\text{ml}$) in phosphate-buffered saline (PBS). Because the fluorescence profiles of cells stained with B9.4 or OKT4 mAb were clearly biphasic (see Fig. 1), <5% of all cells had to be discarded because their fluorescence intensity coincided with the inflection point of the curve. Sorted cells were collected in sterile plastic tubes whose inside surfaces had been coated with sterile FCS. Once collected, cells were immediately diluted in culture medium.

Flow Cytofluorometric Analysis of T8, T4, and T3 Antigen Expression in Cloned CTL. The techniques used have been described in detail elsewhere (17). Briefly, aliquots of 10^5 cells from a number of CTL clones were stained with monoclonal either B9.4, OKT4, or OKT3 antibodies followed by fluorosceinated goat anti-mouse Ig. Control aliquots were stained with the fluorescent reagent alone. All samples were then analyzed on a flow cytometer (FACS II; Becton Dickinson, Mountain View, CA) gated to exclude nonviable cells. Further details of the FACS analysis are described elsewhere (21). Results are expressed as arbitrarily normalized fluorescence histograms i.e., number of cells vs. fluorescence intensity.

Modulation of Surface T3 Antigen by OKT3 mAb. Cells from clone A-11 or A-2, at $2 \times 10^6/\text{ml}$, were incubated in the presence or absence of saturating amounts of OKT3 mAb. After 24 h the cells were washed twice and analyzed for expression of T3 antigen (on a FACS II) and for specific CTL activity.

Results

Inhibitory Effect of B9.4 mAb on the Lytic Activity of Primary MLC-derived CTL Populations. In a first series of experiments, primary MLC-derived CTL populations were tested for specific cytolytic activity in the presence or absence of graded amounts of anti-T8 B9.4 mAb. As shown in Fig. 1, addition of B9.4 mAb resulted in a dose-dependent inhibition of cytolytic activity. It should be noted that the maximal level of inhibition never exceeded 85%, even in antibody excess. The inhibitory effect was found to be reversible since the lytic activity of MLC cells that had been pretreated with a saturating dose of antibody and washed before the cytolytic assay was similar to that of untreated MLC cells (data not shown). Under the same experimental conditions, 4F2 mAb, which reacts with a surface structure expressed by MLC-derived CTL and belongs to the same (γ 2a) subclass as B9.4 mAb (22), was not inhibitory, even when added in large excess (Fig. 1).

Clonal Analysis of CTL Inhibition by B9.4 mAb. To investigate the inhibitory effect of B9.4 mAb at the clonal level, we derived a large number of CTL clones

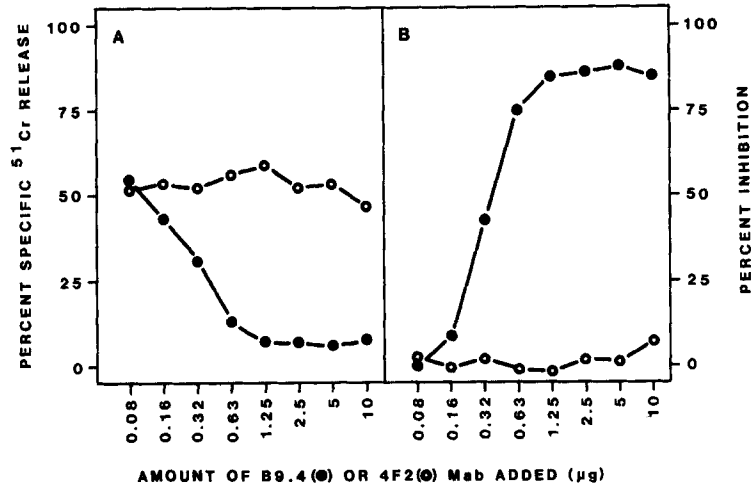


FIGURE 1. Inhibitory effect of B9.4 mAb on the CTL activity exhibited by 7 d primary MLC cells. Aliquots of 2×10^5 MLC cells were preincubated for 30 min at 20°C with increasing doses of B9.4 (●) or 4F2 (○) mAb. 5×10^5 ^{51}Cr -labeled PHA-activated blast cells bearing the sensitizing alloantigens were then added and specific cytolytic activity measured 4 h later. The results are expressed as percent specific ^{51}Cr release (panel A) and as percent inhibition (panel B).

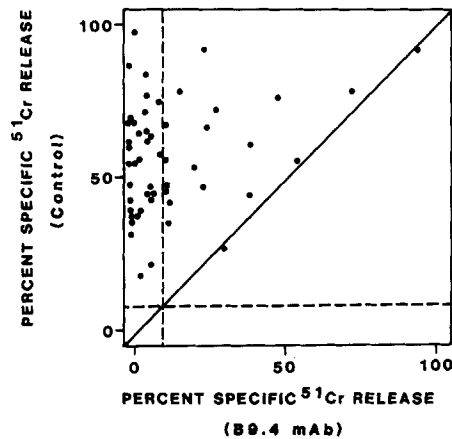


FIGURE 2. Effect of B9.4 mAb on the specific cytolytic activity of CTL clones derived from 7-d primary MLC cells. Aliquots of CTL clones were incubated for 30 min at 20°C in the presence or absence of $1 \mu\text{g}$ of B9.4 mAb. Cytolytic activity was then determined using ^{51}Cr -labeled PHA activated lymphocytes bearing the stimulating alloantigens. The dotted line represents 3 SD above the mean spontaneous ^{51}Cr release. Each point represents the control vs. the "inhibited" cytotoxicity values of a single clone. Clones resistant to B9.4-mediated inhibition would thus fall close to the 45° line.

from MLC populations by limiting dilution or micromanipulation of single cells. As described previously (17), the cloning efficiency in the microculture system we are using is 50–80%. After 14–18 d of culture, clones were tested for specific CTL activity in the presence or absence of $1 \mu\text{g}$ B9.4 mAb. The results of a representative experiments involving 52 CTL clones are shown in Fig. 2. It can

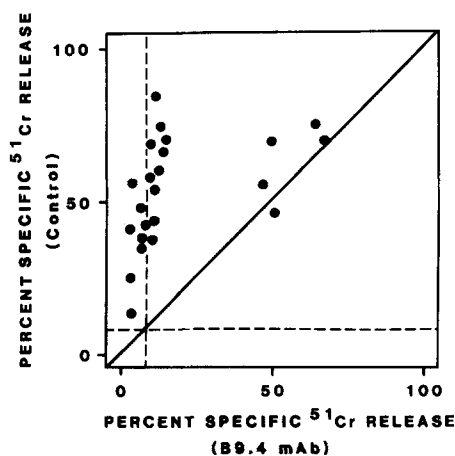


FIGURE 3. Effect of B9.4 mAb on the specific cytolytic activity of T8⁺ CTL clones derived from the T8⁺ cell fraction of 7-d primary MLC populations. The experimental design is similar to that detailed in the legend to Fig. 2. Note that 5 of the 23 T8⁺ CTL clones analyzed were not inhibited by addition of B9.4 mAb.

be seen that CTL clones were heterogeneous in their susceptibility to inhibition of cytolysis by B9.4 mAb. While the majority of clones was either completely or strongly inhibited, seven clones were either unaffected or minimally inhibited. The lack of inhibition by B9.4 mAb could possibly be explained by the lack of corresponding antigen on the cell surface of these clones. In an attempt to clarify this point, we repeated this experiment using clones derived from MLC T8⁺ populations that had been positively selected by FACS sorting of B9.4-stained cells. The CTL clones thus obtained were analyzed for susceptibility to inhibition by B9.4 mAb. The results of a representative experiment are shown in Fig. 3. It can be seen that even within CTL clones selected on the basis of T8 antigen expression, a considerable degree of heterogeneity was observed. Of the 23 clones tested, 5 were unaffected in their activity by B9.4 mAb. In three different experiments, 13–22% of T8⁺ CTL clones belonged to the latter category. To further document this finding seven uninhibited clones were selected, expanded, and re-tested for lytic activity in the presence of increasing antibody concentrations. None of the seven clones showed any significant (>5%) inhibition even at doses of B9.4 as high as 5 μ g. In a control experiment, 15 T8⁻ CTL clones were selected and analyzed for their susceptibility to inhibition by B9.4 mAb. As expected, none of them was affected even in the presence of an excess (5 μ g) of B9.4 mAb (data not shown).

Quantitative Analysis of T8 Antigen Expression on T8⁺ CTL Clones. The difference in susceptibility of T8⁺ CTL clones to inhibition by B9.4 mAb could be explained by a difference in the amount of T8 antigen expressed on the cell surface. This possibility was examined by assessing the level of T8 antigen on several inhibited and uninhibited clones. To this end, cell aliquots from each clone were incubated first with B9.4 mAb and then with a goat fluoresceinated antibody anti-mouse immunoglobulin, and then analyzed by flow microfluorometry. In parallel, the lytic activity of each clone was tested in the presence or

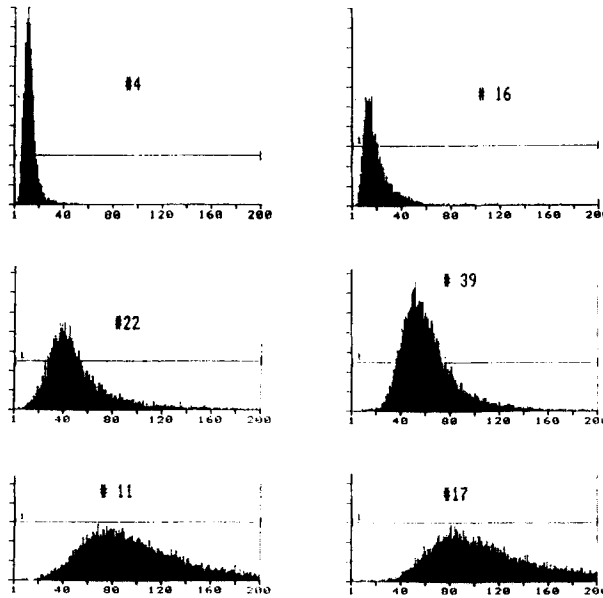


FIGURE 4. Flow cytometric analysis of T8 antigen expression of T8⁺ CTL clones susceptible or resistant to inhibition by B9.4 mAb. Aliquots of six representative clones were incubated with B9.4 mAb for 30 min at 4°C, washed and stained with fluoresceinated goat anti-mouse Ig. Samples were analyzed in a FACS II. Note that the lytic activity of clones 4, 11, and 22 was completely inhibited by B9.4 mAb, whereas clones 16, 17, and 39 were unaffected by the antibody.

absence of B9.4 mAb. In Fig. 4 are shown the fluorescence profiles of six representative CTL clones: the lytic activity of three of them (clones 4, 22, and 11) was completely blocked by B9.4 mAb, whereas the other three (clones 16, 39, and 17) were insensitive to the antibody. Although some quantitative variations in the expression of T8 antigen were observed among these clones, no correlation could be found between the amount of T8 antigen, the magnitude of cytolytic activity and the degree of inhibition of cytotoxicity by B9.4 mAb.

Clonal Analysis of CTL Inhibition by Anti-T4 mAb. A similar approach was used to examine the inhibitory effect of anti-T4 mAb at the clonal level. T4⁺ cells were isolated from MLC populations by sorting using the FACS, and cloned by limiting dilution. Out of 212 clones obtained under these conditions, 14 were found to exhibit specific cytolytic activity against the stimulating allogeneic cells. These CTL clones were tested for expression of T4 antigen by indirect immunofluorescence on the FACS. As expected, all of them displayed the T4⁺ phenotype (data not shown). Analysis of the susceptibility of these T4⁺ clones to inhibition by anti-T4 mAb was performed as described above for T8⁺ CTL clones. As shown in Fig. 5, there was a striking heterogeneity in the susceptibility of these T4⁺ CTL clones to inhibition of cytotoxicity by anti-T4 mAb. Indeed, only 6 of the 14 clones tested were affected by anti-T4 mAb. Further analysis indicated that seven of the eight uninhibited CTL clones were not affected even at a dose of anti-T4 mAb 10-fold higher than that sufficient for complete inhibition of susceptible clones. Moreover, no correlation was observed between the amount

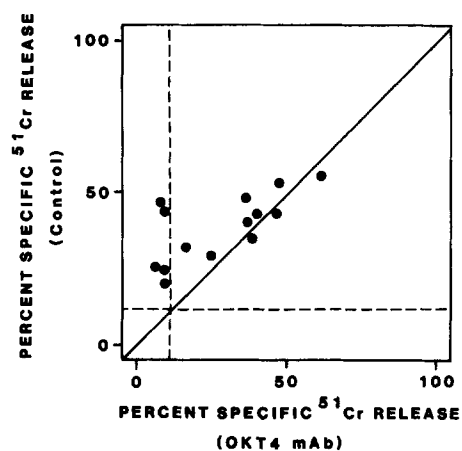


FIGURE 5. Effect of OKT4 mAb on the specific cytolytic activity of T4⁺ CTL clones derived from the T4⁺ cell fraction of 7-d primary MLC populations. The experimental design is similar to that described in the legend to Fig. 2. Note that 8 of the 14 T4⁺ CTL clones tested were not (or minimally) inhibited by addition of OKT4 mAb.

of T4 antigen expressed on the cell surface of a given clone and its susceptibility to inhibition by anti-T4 mAb (data not shown).

Clonal Analysis of CTL Inhibition by Anti-T3 mAb. It has been recently suggested that the T3 surface molecule (which is expressed on CTL irrespective of their subset distribution) is closely linked to the T cell receptor structure (23, 24). In view of the heterogeneity of inhibition observed with anti-T8 and anti-T4 mAb, it was of considerable interest to determine whether all CTL clones could be inhibited by anti-T3 mAb. To this end, the same sets of T8⁺ and T4⁺ CTL clones that had been tested above were analyzed for expression of T3 antigen as well as susceptibility to inhibition by anti-T3 mAb. In accordance with previous reports, all the clones analyzed expressed the T3 antigen as assessed by microfluorometry (data not shown). The inhibitory activity of anti-T3 mAb on T8⁺ CTL clones is shown in Fig. 6. It can be seen that only 15 of the 23 CTL clones tested were clearly inhibited by anti-T3 mAb. Moreover, the number of clones that were not inhibited by anti-T3 mAb exceeded that of clones not inhibited by B9.4 mAb (eight vs. five). It is of interest that four of the five clones unaffected by B9.4 mAb were also resistant to anti-T3 mAb. Moreover, three clones were only inhibited by B9.4 mAb, whereas clones that were only inhibited by anti-T3 were relatively rare (1 out of 23 in the experiment reported in Fig. 6). Clones resistant to the standard amount of anti-T3 mAb were still unaffected by doses 10-fold higher of mAb (data not shown). The inhibitory effect of anti-T3 mAb on 14 T4⁺ CTL clones is shown in Fig. 7. Again, both inhibited and not inhibited clones were clearly observed. While several clones were found to be resistant to both anti-T3 and anti-T4 mAbs, eight clones appeared to be sensitive only to anti-T3 mediated inhibition. It is noteworthy that the proportions of T4⁺ and T8⁺ CTL clones inhibited by anti-T3 mAb were comparable (72% and 65%, respectively).

Differential Effect of T3 Modulation on the Lytic Activity of CTL Clones. It is well documented that anti-T3 mAb can induce modulation of T3 molecules (10, 26).

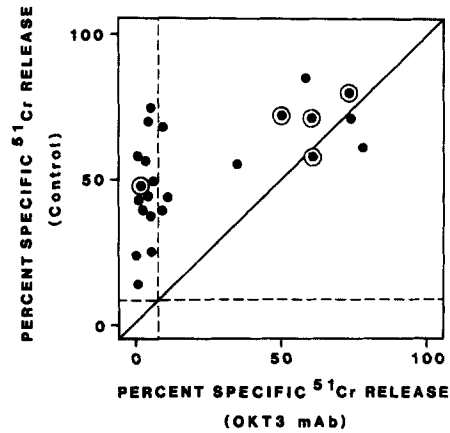


FIGURE 6. Effect of OKT3 mAb on the specific cytolytic activity of T8⁺ CTL clones. The set of clones analyzed is the same as that displayed in Fig. 3. Note that 8 of the 23 T8⁺ CTL clones were resistant to inhibition by OKT3 mAb; the 5 clones marked with an open circle are those resistant to B9.4 inhibition (cf. Fig. 3).

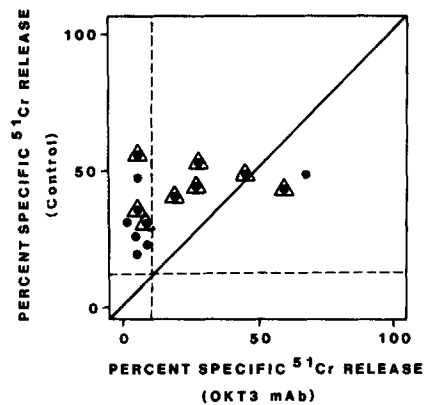


FIGURE 7. Effect of the OKT3 mAb on the specific cytolytic activity of T4⁺ CTL clones derived from primary MLC populations. The set of clones analyzed is the same as that displayed in Fig. 5. Note that 8 of the 14 clones were totally inhibited by OKT3 mAb, 3 were partially inhibited, and 3 were unaffected. The 8 clones marked with an open triangle are those resistant to OKT4 inhibition (see Fig. 5).

Moreover, studies using CTL clones showed that modulation of T3 molecules was accompanied selectively by a loss of surface structures defined by anti-clonotypic mAb, and resulted in a marked inhibition of cytolytic activity (24). Given the fact that the clones used in these studies were susceptible to inhibition by anti-T3 mAb present during the cytolytic assay, we therefore investigated whether a similar effect could be observed with CTL clones that were resistant to inhibition by anti-T3 mAb. To this end, we selected two CTL clones, namely the clone A-11 which was resistant and the clone A-2 which, on the contrary, was highly susceptible to inhibition by anti-T3 mAb (Fig. 8). Both clones expressed the T3⁺, T8⁺, T4⁻ surface phenotype and were unable to lyse K 562 target cells. Incubation with anti-T3 mAb during 24 h at 37°C resulted in the

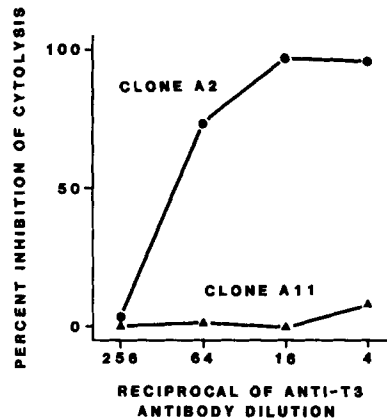


FIGURE 8. Differential inhibition of cytolytic activity of clones A2 and A11 by OKT3 mAb. Aliquots of 10^5 cells from each clone were incubated for 20 min at 20°C with the indicated dilution of the standard solution of OKT3 mAb, and subsequently tested at 0.5:1 (clone A-11) and 4:1 (clone A-2) effector/target cell ratios, respectively. Data are expressed as percent inhibition of cytolysis to facilitate comparison. Control cytolysis in the absence of the inhibition was 49% and 48% for clones A-11 and A-2, respectively.

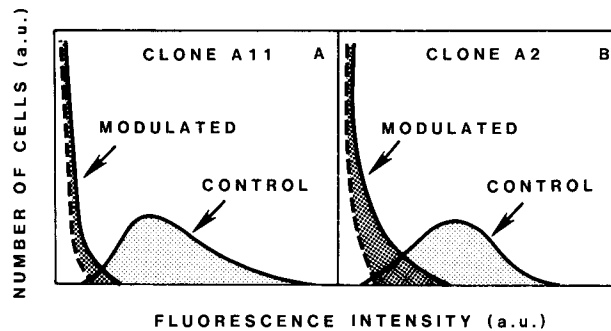


FIGURE 9. Modulation of T3 surface antigen by anti-T3 treatment of clones A-11 and A-2. Cells were incubated with saturating amounts of OKT3 mAb for 24 h at 37°C . After extensive washing, the presence of OKT3 on the cell surface was assessed by indirect immunofluorescence using the FACS. The fluorescent histogram of modulated cells is compared to a control histogram obtained with unmodulated cells. The dotted line represents the background fluorescence given by the goat anti-mouse fluoresceinated Ig.

loss of T3 molecules on the surface of both clones (Fig. 9). However, when the modulated clones were tested in parallel for specific CTL activity, clone A-2 was found to exhibit little activity, whereas the lytic activity of clone A-11 was only minimally affected (<10% inhibition) by T3 modulation (Fig. 10).

Discussion

In the present study, the involvement of surface structures recognized by the B9.4, OKT4, and OKT3 mAb in the activity of human CTL was analyzed at the clonal level. The results clearly demonstrate that CTL are clonally heterogeneous in their susceptibility to inhibition by all three mAb. Since the expression of T8 and T4 molecules on the CTL surface is mutually exclusive (10), it is obvious

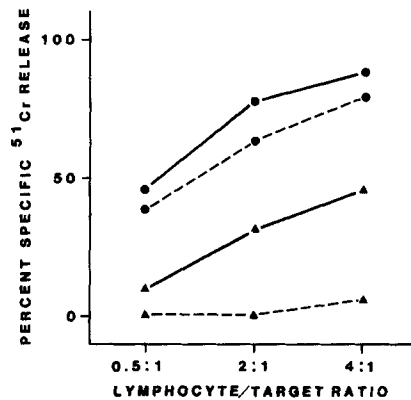


FIGURE 10. Specific cytolytic activity of clones A11 (●) and A2 (▲) before (—) and after (---) modulation of T3 surface antigen. The two clones were cultured in the presence or in the absence of saturating amounts of OKT3 mAb for 24 h at 37°C. After extensive washing, graded numbers of cells were mixed with 5×10^5 ⁵¹Cr-labeled PHA-activated lymphoblasts bearing the sensitizing alloantigens, and cytolytic activity was determined after 4 h.

that the lack of inhibitory activity of B9.4 or OKT4 mAb on some CTL clones could simply be explained by the lack of the corresponding structure on the effector cells. However, heterogeneity of inhibition was observed also among CTL clones bearing the corresponding molecules. Thus, although the majority of primary MLC-derived CTL clones expressing T8 molecules were either completely or strongly inhibited by B9.4 mAb, ~15% were inhibited minimally or unaffected. Interestingly, the susceptibility (or the lack thereof) of these clones to inhibition by B9.4 mAb did not correlate with the amount of T8 antigen expressed on the cell surface. Similarly, among T4⁺ CTL clones, some were completely inhibited by OKT4 mAb, whereas others were unaffected even by an excess of antibody.

In the clonal analysis performed by Meuer et al. (3) all the T8⁺ CTL clones examined were found to be susceptible to inhibition by anti-T8 mAb. In contrast, Malissen et al. (8) reported that only a minority of T8⁺ CTL clones were susceptible to inhibition by B9.4 mAb. These apparently conflicting findings can be reconciled on the basis of the data reported here, as well as the results obtained in the mouse concerning the role of the Lyt-2/3 antigen complex in CTL activity. As shown by MacDonald et al. (12), there is considerable heterogeneity in the susceptibility of murine Lyt-2/3⁺ CTL to inhibition by mAb directed against Lyt-2 or Lyt-3 antigenic determinants. Moreover, the proportion of CTL clones that are unaffected by anti-Lyt-2/3 antibodies appears to be dependent on the source of CTL. In the study by MacDonald et al. (12), <20% of CTL clones derived from MLC populations established with unprimed responder lymphocytes were not inhibited by anti-Lyt-2 mAb as compared to 60–75% of CTL clones obtained from *in vivo* immunized populations. In this context, it is noteworthy that the human CTL clones studied by Malissen et al. (8), were derived from an allosensitized individual. As shown here, only a small fraction of human T8⁺ CTL clones derived from primary MLC populations was unaffected by B9.4 mAb. It is therefore likely that the results of Meuer et al. (3),

who used T8⁺ CTL clones derived from primary MLC populations, can be explained by the limited number of CTL clones examined. Taken together, all these results point to a striking functional similarity between the mouse Lyt-2/3 and the human T8 molecules, since in both instances, the resistance of CTL clones bearing these antigens to inhibition by the corresponding antibodies appears to correlate quantitatively (but not absolutely) with *in vivo* priming.

While Meuer et al. reported that T4⁺ CTL clones were inhibited by anti-T4 mAb, opposite results were obtained by Spitz et al. (7). From the work presented here, it is evident that, as in the case of T8 molecules, the requirement for T4 molecules in the cytolytic activity of T4⁺ CTL exhibits clonal heterogeneity. It has been suggested that T8 and T4 molecules can act as associative recognition structures by virtue of their binding to the constant region of MHC class I or class II molecules, respectively (26). Alternatively, as proposed by MacDonald et al. (27) to explain the clonal heterogeneity in the requirement for Lyt-2/3 molecules in the lytic activity of murine CTL, it can be speculated that the role of T8 and T4 molecules is to stabilize the binding of CTL receptors to the corresponding antigens. In either case, one would expect that CTL clones with high affinity receptors do not require additional surface molecules to mediate their function and therefore are not inhibited by anti-T8 or -T4 antibodies. Comparative studies using CTL clones derived from both unprimed and primed lymphoid cell populations might help to clarify this issue. Perhaps more importantly, the present study indicates that heterogeneity may also exist in the requirement of T3 molecules for CTL activity. Several lines of evidence suggest that T3 molecules are closely associated with T cell antigen receptors (24, 25, 28, 29). Firstly, as already mentioned, the determinants recognized by mAb directed against clonotypic structures of CTL clones were found to be absent from the cell surface after modulation of T3 molecules by anti-T3 mAb. In addition, modulation of T3 molecules resulted in a marked reduction of the activity of such clones. Secondly, it has been shown that soluble anti-T3 mAb can inhibit the proliferative response of T cell clones to insolubilized anti-clonotypic mAb. Finally, it appears that detectable amounts of molecules bearing clonotypic determinants can be co-precipitated by anti-T3 mAb. It should be emphasized, however, that the number of CTL clones used in all these studies has been very limited. Indeed, our work indicates that about two-thirds of the CTL clones derived from primary MLC populations required T3 molecules for target cell lysis. In contrast, the remaining clones, irrespective of their T4/T8 phenotype, did not appear to be dependent upon T3 molecules as indicated by the lack of inhibitory activity of anti-T3 mAb. In support of this contention is the demonstration that the cytolytic activity of a CTL clone resistant to inhibition by anti-T3 mAb was unaffected by antibody-induced modulation of T3 molecules. This finding strongly suggest that T3 molecules and the clonotypic structures, which are thought to act as antigen receptors, are not always physically and functionally linked on the CTL surface. Further work using antibodies against clonotypic structures of CTL clones that are independent of T3 molecules is needed to ascertain directly this point. Moreover, it remains to be determined whether the proportion of T3-independent CTL is increased after *in vivo* priming.

Summary

In an attempt to define the requirement of T8, T4, and T3 surface molecules in functional interactions occurring between human cytolytic T lymphocytes (CTL) and specific target cells, we have analyzed a large number of CTL clones derived from primary mixed lymphocyte culture (MLC) T cell populations for their susceptibility to inhibition by monoclonal antibodies (mAb) directed against these surface antigens. In most experiments, MLC T cells were stained with B9.4 (anti-T8) or OKT4 (anti-T4) mAb, separated into positive and negative cells using a fluorescence-activated cell sorter (FACS) and cloned under limiting conditions. While the lytic activity of the majority of T8⁺ CTL clones was inhibited by B9.4 mAb, ~15% of these clones were unaffected even in the presence of excess antibody. Flow cytometric analysis of T8 antigen in individual clones did not show any correlation between the amount of T8 antigen expressed, the magnitude of cytolytic activity and the susceptibility (or lack thereof) to inhibition by B9.4 mAb. Of the 16 T4⁺ CTL clones analyzed, 7 were resistant to inhibition by OKT4 mAb even at doses 10-fold higher than that sufficient for complete inhibition of susceptible clones. Again, no correlation was found between the amount of T4 antigen expressed and the susceptibility to inhibition by the corresponding antibody.

The same sets of T8⁺ and T4⁺ CTL clones were also analyzed for their susceptibility to inhibition by OKT3 mAb. Although all of the clones expressed the T3 surface antigen, only 15/23 T8⁺ clones and 9/14 T4⁺ clones were inhibited by anti-T3 mAb. To further document this clonal heterogeneity, we selected two T3⁺ T4⁻ T8⁺ CTL clones that had no concomitant NK-like activity. One clone was resistant to inhibition by OKT3 mAb, whereas the other was highly susceptible. Incubation with OKT3 mAb resulted in modulation of the T3 molecules in both clones. Following modulation, however, the cytolytic activity of the resistant clones was unaffected, whereas the lytic activity of the susceptible clone was abrogated. These results thus indicate extensive clonal heterogeneity in the requirement for T3, T4, and T8 molecules in CTL function. Moreover, it appears that T3 molecules are not always physically and functionally linked to CTL receptor structures.

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References

1. Moretta, L., M. C. Mingari, A. Moretta, and A. S. Fauci. 1982. Human lymphocyte surface markers. *Semin. Hematol.* 19:273.
2. Martz, E., W. Haegy, and S. H. Gromkowski. 1982. The mechanism of CTL-mediated killing: monoclonal antibody analysis of the roles of killer and target cell membrane proteins. *Immunol. Rev.* 72:73.
3. Meuer, S. C., R. E. Hussey, J. C. Hodgdon, T. Hercend, S. F. Schlossman, and E. L. Reinherz. 1982. Surface structures involved in target recognition by human cytotoxic T lymphocytes. *Science (Wash. DC)*. 218-417.
4. Chang, T. W., P. C. Kung, S. P. Gingras, and G. Goldstein. 1981. Does OKT3 monoclonal antibody react with an antigen recognition structure on human T cells? *Proc. Natl. Acad. Sci. USA.* 78:1805.
5. Evans, R. L., D. W. Wall, C. D. Platsoucas, and F. Siegal. 1981. Thymus dependent

- membrane antigens in man: inhibition of cell mediated lympholysis by monoclonal antibodies to TH₂ antigen. *Proc. Natl. Acad. Sci. USA.* 78:544.
6. Landegren, U., U. Ramstedt, I. Axberg, M. Ullberg, M. Jondal, and H. Wigzell. 1982. Selective inhibition of human T cell cytotoxicity at levels of target recognition or initiation of lysis by monoclonal OKT3 and Leu-2a antibodies. *J. Exp. Med.* 155:1579.
 7. Spits, H., J. Borst, C. Terhorst, and J. E. De Vries. 1982. The role of T cell differentiation markers in antigen specific and lectin dependent cellular cytotoxicity mediated by T8⁺ and T4⁺ human cytotoxic T cell clones directed at class I and class II MHC antigens. *J. Immunol.* 128:1563.
 8. Malissen, B., N. Rebai, A. Liabeuf, and C. Mawas. 1982. Human cytotoxic T cell structures associated with expression of cytolysis. I. Analysis at the clonal level of the cytolysis inhibiting effect of 7 monoclonal antibodies. *Eur. J. Immunol.* 12:737.
 9. Moretta, A., G. Pantaleo, M. C. Mingari, G. Melioli, L. Moretta, and J. C. Cerottini. 1984. Assignment of human natural killer (NK)-like cells to the T-cell lineage. Single allospecific T cell clones lyse specific or NK-sensitive target cells via distinct recognition structures. *Eur. J. Immunol.* In press.
 10. Reinherz, E. L., S. Meuer, K. A. Fitzgerald, R. E. Hussey, H. Levine, and S. F. Schlossman. 1982. Antigen recognition by human T lymphocytes is linked to surface expression of the T3 molecular complex. *Cell.* 30:735.
 11. Sarmiento, M., A. L. Glasbrook, and F. W. Fitch. 1980. Monoclonal antibodies block cytolysis by T cells. *J. Immunol.* 125:2665.
 12. MacDonald, H. R., N. Thiernesse, and J. C. Cerottini. 1981. Inhibition of T cell-mediated cytolysis by monoclonal antibodies directed against Lyt-2: heterogeneity of inhibition at the clonal level. *J. Immunol.* 126:1671.
 13. Malissen, B., M. Jonker, N. Rebai, H. Balner, and C. Mawas. 1982. In vitro and in vivo effects of cytolysis-inhibiting monoclonal antibodies raised against cloned lines of human cytotoxic T lymphocyte. *In Current Concepts in Human Immunology and Cancer Immunomodulation.* 91.
 14. Moretta, A., M. C. Mingari, B. F. Haynes, R. P. Sekaly, L. Moretta, and A. S. Fauci. 1981. Phenotypic characterization of human cytolytic T lymphocytes generated in mixed lymphocyte culture. *J. Exp. Med.* 153:213.
 15. Moretta, A., R. S. Accolla, and J. C. Cerottini. 1982. IL-2 mediated T cell proliferation in human is blocked by a monoclonal antibody directed against monomorphic determinants of HLA-DR antigens. *J. Exp. Med.* 155:599.
 16. Moretta, A., G. Corte, M. C. Mingari, and L. Moretta. 1982. Human T cell subpopulations defined by a monoclonal antibody. II. Evidence of cell cooperation in the response to alloantigens and generation of cytolytic cells. *J. Immunol.* 128:20.
 17. Moretta, A., G. Pantaleo, L. Moretta, M. C. Mingari, and J. C. Cerottini. 1983. Quantitative assessment of the pool size and subset distribution of cytolytic T lymphocytes within human resting or alloactivated peripheral blood T cell populations. *J. Exp. Med.* 158:571.
 18. Moretta, A., M. C. Mingari, J. C. Cerottini, A. S. Fauci, and L. Moretta. 1983. Relationship between expression of Fc γ receptors or Ia antigens and cytolytic activities of alloactivated human T cells. *Clin. Immunol. Immunopathol.* 26:232.
 19. Moretta, L., M. C. Mingari, R. P. Sekaly, A. Moretta, B. Chapuis, and J. C. Cerottini. 1981. Surface markers of cloned human T cells with various cytolytic activities. *J. Exp. Med.* 154:569.
 20. Moretta, A. M., Colombatti, and B. Chapuis. 1981. Human spleen as a source of T cell growth factor. *Clin. Exp. Immunol.* 44:262.
 21. Moretta, A., G. Pantaleo, L. Moretta, J. C. Cerottini, and M. C. Mingari. 1983.

- Direct demonstration of the clonogenic potential of every human peripheral blood T cell. Clonal analysis of HLA-DR expression and cytolytic activity. *J. Exp. Med.* 157:743.
22. Haynes, F. B. 1981. Human T lymphocyte antigens as defined by monoclonal antibodies. *Immunol. Rev.* 57:127.
 23. Meuer, S. C., O. Acuto, R. E. Hussey, J. C. Hodgdon, K. A. Fitzgerald, S. F. Schlossman, and E. L. Reinherz. 1983. Evidence for the T3-associated 90 KD heterodimer as the T-cell antigen receptor. *Nature (Lond.)*. 303:808.
 24. Meuer, S. C., K. A. Fitzgerald, R. A. Hussey, J. C. Hodgdon, S. F. Schlossman, and E. L. Reinherz. 1983. Clonotypic structures involved in antigen-specific human T cell function: relationship to the T3 molecular complex. *J. Exp. Med.* 157:705.
 25. Bigler, R. D., D. E. Fisher, C. Y. Wang, E. A. Rinnoy kan, and H. G. Kunkel. 1983. Idiotypic-like molecules on cells of a human T cell leukemia. *J. Exp. Med.* 158:1000.
 26. Meuer, S. C., S. F. Schlossman, and E. L. Reinherz. 1982. Clonal analysis of human cytotoxic T lymphocytes: T4⁺ and T8⁺ effector T cells recognize products of different major histocompatibility complex regions. *Proc. Natl. Acad. Sci. USA.* 79:4395.
 27. MacDonald, H. R., A. L. Glasebrook, C. Bron, A. Kelso, and J. C. Cerottini. 1982. Clonal heterogeneity in the functional requirement for Lyt-2/3 molecules on cytolytic T lymphocytes (CTL): possible implications for the affinity of CTL antigen receptors. *Immunol. Rev.* 68:89.
 28. Meuer, S. C., J. C. Hodgdon, R. E. Hussey, J. P. Protentis, S. F. Schlossman, and E. L. Reinherz. 1983. Antigen-like effects of monoclonal antibodies directed at receptors on human T cell clones. *J. Exp. Med.* 158:988.
 29. Reinherz, E. L., S. C. Meuer, K. A. Fitzgerald, R. E. Hussey, J. C. Hodgdon, O. Acuto, and S. F. Schlossman. 1983. Comparison of T3-associated 49- and 43-kilodalton cell surface molecules on individual human T cell clones: evidence for peptide variability in T-cell receptor structures. *Proc. Natl. Acad. Sci. USA.* 80:4104.