CLONAL HETEROGENEITY IN THE FUNCTIONAL REQUIREMENT FOR Lyt-2/3 MOLECULES ON CYTOLYTIC T LYMPHOCYTES: ANALYSIS BY ANTIBODY BLOCKING AND SELECTIVE TRYPSINIZATION

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Despite extensive investigation over the past decade, the mechanism of specific target cell recognition and lysis by cytolytic T lymphocytes (CTL)[1] remains obscure (for recent reviews, see ref. 1 and 2). One promising approach to this problem has been the derivation of antisera (or monoclonal antibodies) that have the ability to block cytolytic activity in the absence of complement. Although early studies using heterologous antisera met with mixed results (reviewed in ref. 3), more recent experiments with monoclonal antibodies have clearly defined at least two antigenic structures on CTL, which may be implicated in antigen recognition and/or cytolysis. Thus, monoclonal antibodies directed against the Lyt-2/3 molecular complex (4–10) or the LFA-1 surface antigen (11, 12) have been shown to block CTL activity in a variety of experimental systems.

A major difficulty in the interpretation of antibody blocking experiments is to ascertain whether or not the structure(s) recognized by the antibodies is actually implicated in the functional activity being measured. In this context, the recent development of T cell cloning technology (reviewed in ref. 13) has provided homogeneous populations of CTL that can be subjected to somatic cell genetic and biochemical analysis. Using antibody selection methods on a mutagenized CTL clone, Dialnyas et al. (14) were able to isolate a Lyt-$2^-/3^-$ variant which failed to express cytolytic activity, thus suggesting a crucial role for this molecular complex in the cytolytic mechanism. In apparent contrast to these findings, Giorgi et al. (15) obtained a spontaneous Lyt-2^- variant of another CTL clone that retained cytolytic function. Conflicting results regarding the possible role of Lyt-2/3 molecules in cytolysis were also obtained in proteolytic digestion experiments. Whereas Fan and Bonavida (9) concluded that there was a good correlation between loss of Lyt-2 antigenic determinants and loss of CTL activity in trypsinized populations of alloimmune cells, Ledbetter et al. (10) found that CTL activity was considerably more resistant to trypsin than Lyt-2/3 antigens.

In view of these apparently contradictory findings, we reinvestigated the role of the

[1] *Abbreviations used in this paper:* CTL, cytolytic T lymphocyte; DME, Dulbecco's modified Eagle's medium; MLC, mixed leukocyte culture; MLTC, mixed leukocyte/tumor cell culture; MSV, murine sarcoma virus-murine leukemia virus.

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Lyt-2/3 molecular complex in T cell-mediated cytolysis at the clonal level. In agreement with an earlier report (8), we show that there is considerable clonal heterogeneity in the ability of CTL to be blocked by monoclonal anti-Lyt-2 or anti-Lyt-3 antibodies. Furthermore, by using quantitative trypsinization methods on selected CTL clones, we demonstrate that this heterogeneity of inhibition most probably reflects a true heterogeneity in the requirement for Lyt-2/3 molecules in antigen recognition by these cells. Based on these findings, a novel function of the Lyt-2/3 molecules in the stabilization of CTL antigen receptors will be proposed.

Materials and Methods

Derivation and Maintenance of CTL Clones. Clone L3 was derived by limiting dilution from a long-term in vitro primed C57BL/6 anti-DBA/2 secondary mixed leukocyte culture (MLC) population as described elsewhere (16). Clone C10 was derived by limiting dilution from an in vivo primed C57BL/6 anti-DBA/2 secondary MLC population (17).

C57BL/6 CTL clones against murine sarcoma virus-murine leukemia virus (MSV)-associated antigens were derived by micromanipulating single cells from in vivo primed secondary mixed leukocyte/tumor cell (MLTC) cultures. These clones were selected for cross-reactive cytolytic activity by simultaneous testing against MSV-infected syngeneic (MBL-2) and uninfected allogeneic (P-815) tumor target cells.

All clones were maintained by weekly restimulation of $1–5 \times 10^4$ cloned cells with 5×10^6 irradiated (2,000 rad) antigenic (allogeneic or syngeneic virus-infected) spleen cells in 2 ml of Dulbecco's modified Eagle's medium (DME) supplemented with 2% fetal bovine serum, additional amino acids (17), 5×10^{-5} M 2-mercaptoethanol, and 2–3% (vol/vol) supernatant from phorbol myristic acetate-stimulated EL-4 leukemia cells as a source of interleukin 2 (18). Cell recovery after 5–7 d ranged from 0.2 to 1×10^6 viable cloned cells.

Monoclonal Antibodies. Monoclonal rat IgG antibodies against nonpolymorphic determinants of Lyt-2 (53-6.7) and Lyt-3 (53-5.1) antigens were kindly provided by Dr. J. Ledbetter, Stanford University, CA. Monoclonal rat IgM antibodies against Thy-1.2 (AT83) were kindly provided by Dr F. Fitch, University of Chicago, Chicago, IL. Monoclonal mouse IgG antibodies against H-2K^b (B8-24) were kindly provided by Dr G. Köhler, Basel Institute for Immunology, Basel, Switzerland. A monoclonal rat antibody that inhibits T cell-mediated cytolysis (H35-89.9) was kindly provided by Dr. M. Pierres and Dr. P. Golstein, Centre d'Immunologie INSERM-CNRS, Marseille, France. The properties of all of these reagents have been described in detail elsewhere (7, 12, 19, 20).

Trypsin Treatment of CTL Clones. Cloned cells were washed twice in serum-free DME and resuspended at a concentration of 1×10^6 cells/ml in the presence of various concentrations of trypsin (type XI, DPCC treated, Sigma Chemical Co., St. Louis, MO). After 30 min at 37°C, 15% (vol/vol) bovine serum was added to block trypsin activity, and the cells were washed two additional times at 4°C. Treated or control cells were then analyzed for surface marker expression and cytolytic activity as described below.

Flow Microfluorometry. Expression of surface Lyt-2, Lyt-3, Thy-1.2, H-2K^b, and H35.89.9 antigens on CTL clones was quantitated by flow microfluorometry as described in detail elsewhere (21). Briefly, aliquots of 3×10^5 cells were incubated sequentially at 4°C with optimal concentrations (1–5 μg) of monoclonal antibodies followed by fluoresceinated rabbit anti-rat or rabbit anti-mouse Ig (20–50 μg). Samples were passed on a FACS II flow cytometer (Becton, Dickinson & Co., Sunnyvale, CA) gated to exclude nonviable cells. Between 5,000 and 20,000 cells were accumulated for each histogram.

Inhibition of Cytolytic Activity. Cytolytic activity of cloned CTL was assessed by mixing various numbers of cells with 2×10^3 ^{51}Cr-labeled target cells (either P-815, of DBA/2 origin, or MSV-infected MBL-2, of C57BL/6 origin) in 200 μl final volume in round-bottomed microtiter wells. After 3 h at 37°C, plates were centrifuged and supernatants were removed and counted in a well-type scintillation counter. Percent specific ^{51}Cr release was calculated as described elsewhere (17). In the trypsin treatment experiments, a minor modification of this protocol was used (22). Briefly, mixtures of CTL and labeled target cells were centrifuged and incubated for 30 min at

37°C. EDTA was then added to a final concentration of 10 mM and the plates left for an additional 90 min, at which time ^{51}Cr release was assessed. In this way, the effective duration of the assay was restricted to 30 min (22). No re-expression of Lyt-2 or Lyt-3 antigens on trypsinized clones was observed within this 30-min period (data not shown).

For the antibody inhibition studies, a fixed number (usually 4×10^3) of cloned CTL were preincubated with various concentrations of monoclonal antibodies in a volume of 100 μl for 20 min at 20°C. Then 2×10^3 ^{51}Cr-labeled target cells (100 μl) were added for an additional 3 h, and ^{51}Cr release was assessed. To normalize results in certain experiments, results are expressed as percent inhibition according to the formula: percent inhibition of cytolysis = 1 − ([^{51}Cr release with inhibitor]/[^{51}Cr release without inhibitor]) × 100.

Results

Differential Effect of Anti-Lyt-2/3 Antibodies on CTL Clones C10 and L3. We previously demonstrated a marked heterogeneity in the inhibitory effect of monoclonal anti-Lyt-2 antibodies on cytolysis by CTL populations and short-term clones (8). To investigate this phenomenon in greater detail, representative clones of the inhibited (L3) and uninhibited (C10) phenotype were established as long-term cell lines and tested for their susceptibility to inhibition by monoclonal antibodies directed against Lyt-2 or Lyt-3. As shown in Fig. 1, clone L3 was inhibited in a dose-dependent fashion by either anti-Lyt-2 or anti-Lyt-3 antibodies, with 50% inhibition at doses of 10 ng per well. On the other hand, clone C10 was not significantly inhibited by doses of either antibody as high as 5 μg per well. This dramatic dissociation between the two clones was not a property of the particular antibodies used because IgM or IgG monoclonal antibodies directed against either polymorphic or nonpolymorphic determinants on the Lyt-2 molecule behaved in a similar fashion (data not shown).

Expression of Lyt-2/3 Antigens by clones L3 and C10. One trivial explanation for the observed failure of clone C10 to be inhibited by anti-Lyt-2/3 antibodies would be lack of the corresponding antigenic structures. This possibility was excluded by quantitative flow microfluorometry. As shown in Fig. 2, clones L3 and C10 appeared

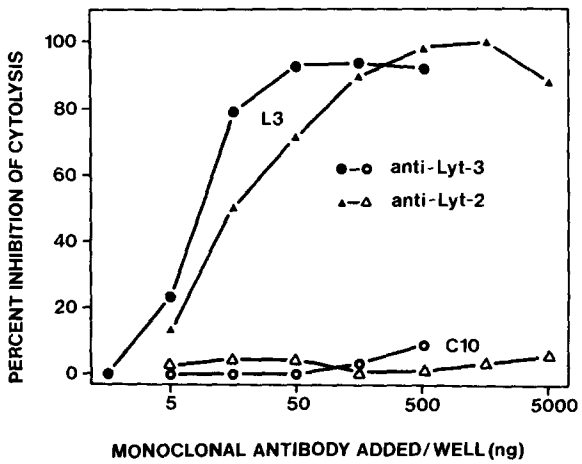

FIG. 1. Differential inhibitory effect of monoclonal anti-Lyt-2/3 antibodies on CTL clones L3 and C10. Cloned CTL were incubated with the indicated amount of anti-Lyt-2 or anti-Lyt-3 monoclonal antibody and subsequently tested for cytotoxicity against ^{51}Cr-labeled P-815 (DBA/2) target cells at a 2:1 ratio. For purposes of comparison, data are expressed as percent inhibition relative to control lysis in the absence of inhibitor (59% and 69% for L3 and C10, respectively).

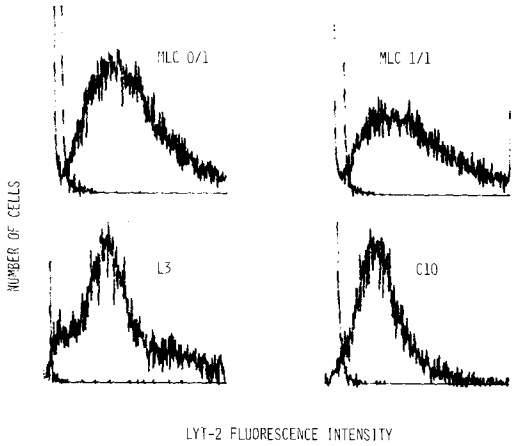

FIG. 2. Quantitative expression of Lyt-2 antigens on CTL clones. Aliquots of 3×10^5 cells from CTL clones L3 and C10 were incubated with monoclonal rat anti-Lyt-2 antibodies (2 μg in 100 μl), followed by fluoresceinated rabbit anti-rat immunoglobulin. Samples were run on an FACS II flow cytometer gated to accumulate 20,000 viable cells. Each fluorescence histogram (expressed in arbitrary linear units) is compared with a control histogram obtained with the fluorescent conjugate alone. Primary (0/1) and in vivo primed secondary (1/1) C57BL/6 anti-DBA/2 MLC populations were included as an internal control.

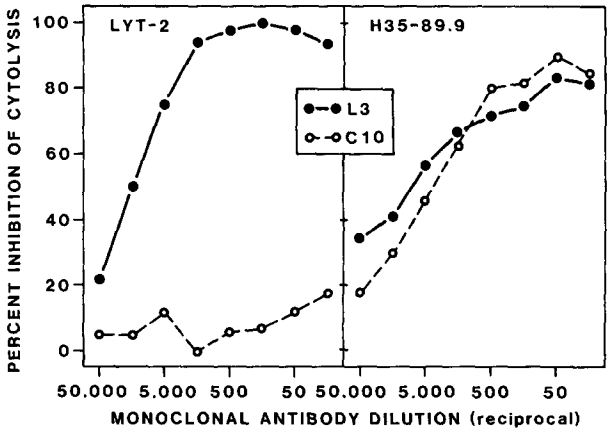

FIG. 3. Failure of a "LFA-1-like" monoclonal antibody to dissociate between CTL clones L3 and C10. Cloned CTL were preincubated with various dilutions of monoclonal antibodies directed against either Lyt-2 or H35-89.9 (a molecular complex similar to LFA-1; see ref. 12) and subsequently tested for cytotoxicity against P-815 target cells at a 2:1 ratio. For purposes of comparison, data are expressed as percent inhibition relative to control lysis in the absence of antibody (34% and 67% for L3 and C10, respectively).

to express comparable amounts of Lyt-2 molecules. Similar results were obtained for Lyt-3 (data not shown).

Failure of Another Cytolysis-inhibiting Antibody to Dissociate between L3 and C10. To determine whether the dissociation of inhibition of cytolytic activity of clones C10 and L3 was unique for antibodies directed against the Lyt-2/3 molecular complex, we also investigated the effect of an independent monoclonal antibody (H35-89.9) which has been reported to inhibit CTL activity (12). H35-89.9 immunoprecipitates two membrane polypeptides of apparent 180,000 and 94,000 mol wt and is thus

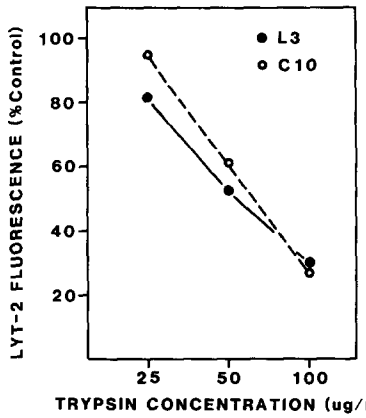

FIG. 4. Trypsin sensitivity of Lyt-2 antigenic determinants on clones L3 and C10. Aliquots of 2 × 10^6 cells from CTL clones L3 and C10 were treated with the indicated concentrations of trypsin and analyzed for the expression of Lyt-2 antigens by flow microfluorometry (see Fig. 2). Fluorescence intensity is expressed as a percentage of the untreated controls.

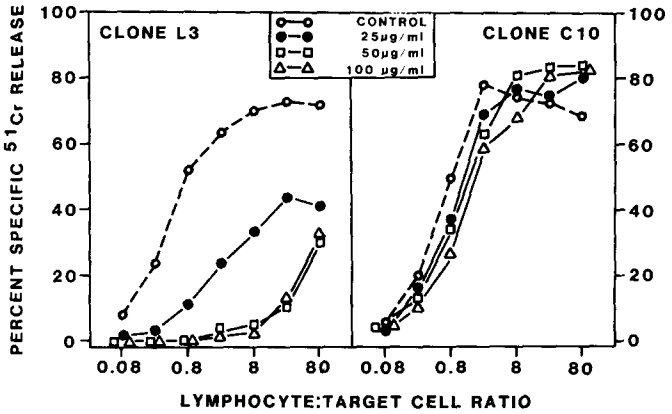

FIG. 5. Differential effect of trypsin treatment on the cytolytic activity of clones L3 and C10. Aliquots of 2 × 10^6 cells from CTL clones L3 and C10 were treated with various concentrations of trypsin and assayed for cytolytic activity against P-815 target cells at various effector/target cell ratios using a short-term (30 min) modification of the ^{51}Cr release assay (22).

unrelated to anti-Lyt-2/3, although similar to the anti-LFA-1 antibody described by Davignon et al. (11). When tested against CTL clones L3 and C10, H35-89.9 inhibited cytolytic activity to a comparable extent (Fig. 3).

Effect of Trypsin Treatment on Lyt-2/3 Expression and Cytolytic Activity of Clones L3 and C10. The extreme trypsin sensitivity of the Lyt-2/3 antigenic complex is well documented (10, 23). It was therefore of interest to examine in parallel the effect of trypsin treatment on Lyt-2/3 expression and cytolytic activity of clones L3 and C10. In agreement with previous studies of murine thymocytes by Ledbetter et al. (10), moderate doses of trypsin (25–100 µg/ml for 30 min) were sufficient to remove Lyt-2 antigens from clones L3 and C10 (Fig. 4). When these trypsin-treated cells were tested in parallel for their cytolytic activity using a short-term (30 min) ^{51}Cr release assay (22), a striking dissociation was observed. Whereas clone L3 lost 90% and 99% of its cytolytic activity after treatment with 25 µg/ml and 50 µg/ml of trypsin, respectively,

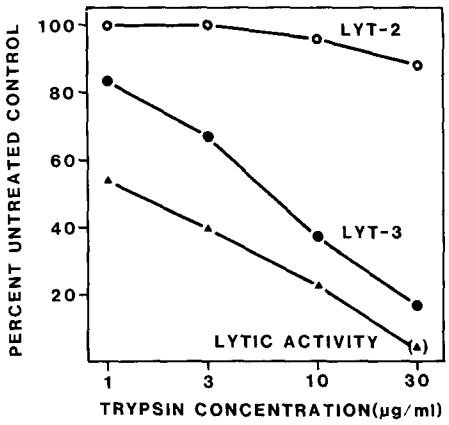

FIG. 6. Correlation between Lyt-2/3 expression and cytolytic activity in trypsin-treated L3 cells. Aliquots of CTL clone L3 (2×10^6 cells) were exposed to the indicated concentrations of trypsin and divided into three aliquots. Two aliquots were stained with monoclonal anti-Lyt-2 or monoclonal anti-Lyt-3 antibodies and analyzed by flow microfluorometry (cf., Fig. 2). The third aliquot was assayed for cytolytic activity against P-815 target cells at various effector/target cell ratios (cf. Fig. 5). Cytolytic activity was converted to lytic units (17) and expressed as a percentage of the mock-treated control. Relative Lyt-2 or Lyt-3 fluorescence intensity was likewise expressed as a percentage of the control.

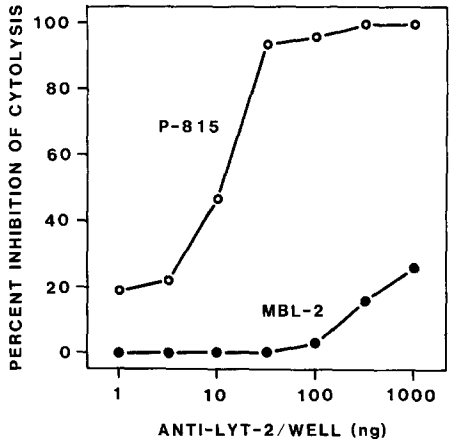

FIG. 7. Differential blocking effect of monoclonal anti-Lyt-2 antibodies on specific and cross-reactive lysis mediated by a C57BL/6 anti-MSV CTL clone. A micromanipulated CTL clone derived from a day-7 MLTC population was expanded and assayed for cytolytic activity against its specific target (MBL-2) or against a third-party allogeneic target (P-815) in the presence or absence of various concentrations of monoclonal anti-Lyt-2 antibodies. For purposes of comparison, data are presented as percent inhibition relative to control lysis in the absence of antibody (31% and 81% against MBL-2 and P-815, respectively, at an effector/target ratio of 5:1). Similar results were obtained with four other clones derived in the same experiment (not shown).

clone C10 was resistant to treatment with up to 100 μg/ml (Fig. 5). With lower doses of trypsin (1–30 μg/ml), clone L3 lost cytolytic activity in a dose-dependent fashion that paralleled loss of the Lyt-3 antigenic determinant (Fig. 6). In this dose range, no significant effect of trypsin treatment on the expression of other cell surface antigens, such as Thy-1, H-2K^b, Lyt-2, or H35-89.9 by clone L3, could be detected (data not shown).

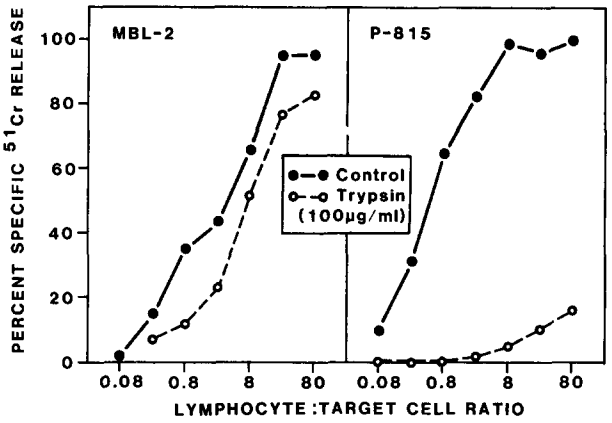

FIG. 8. Differential effect of trypsin treatment on specific and cross-reactive lysis mediated by a C57BL/6 anti-MSV CTL clone. The same clone as in Fig. 7 was treated with trypsin (100 μg/ml) and assayed for cytotoxicity against MBL-2 or P-815 target cells at various effector:target cell ratios using a short-term ^{51}Cr-release assay. Untreated cloned CTL were included as a positive control. Similar results were obtained with a second clone (not shown).

Effect of Anti-Lyt-2 Antibodies and Trypsin Treatment on Clones with Cross-Reactive Lytic Activity. Results obtained with clones C10 and L3 indicated a clear dissociation in their apparent requirement for Lyt-2/3 molecules in the killing process. Furthermore, as shown elsewhere (8), this dissociation correlates in general with in vivo priming. To determine whether such a dissociation could be observed in lytic activities mediated by a single clone, we took advantage of the fact that a significant proportion of in vivo primed C57BL/6 anti-MSV clones lyse uninfected allogeneic (P-815) target cells (24, 25). When five such clones (isolated by micromanipulation) were tested for their ability to lyse specific (MBL-2) or cross-reactive (P-815) target cells in the presence or absence of various concentrations of anti-Lyt-2 antibodies, a marked dissociation was observed. As shown for a representative clone in Fig. 7, lysis of MBL-2 target cells was not significantly inhibited at any antibody concentration tested (up to 1 μg/well), whereas lysis of P-815 target cells was completely inhibited, even at low antibody doses (10 ng/well). Furthermore, when two of these clones were treated with 100 μg/ml trypsin (a dose that removed 90% of the Lyt-2 antigenic determinants), only lysis of P-815 target cells was affected (Fig. 8). Thus, two independent specificities of a single CTL clone could be dissociated either by antibody blocking or by trypsin treatment.

Discussion

The experiments described in this communication provide direct evidence for clonal heterogeneity in the requirement for Lyt-2/3 molecules in T cell-mediated cytolysis. Whereas certain C57BL/6 anti-DBA/2 CTL clones (such as L3) were inhibited by low doses (10 ng) of monoclonal anti-Lyt-2 or anti-Lyt-3 antibodies, other clones (such as C10) were not inhibited by concentrations as high as 5 μg. Treatment of these clones with doses of trypsin sufficient to cleave Lyt-2/3 antigenic determinants from the cell surface confirmed this apparent dissociation in the sense that "inhibited" clones lost cytolytic activity, whereas "uninhibited" clones did not. Furthermore,

cross-reactive C57BL/6 anti-MSV CTL clones were derived that exhibited an inhibited, trypsin-sensitive phenotype when tested against allogeneic third-party (P-815) target cells and an uninhibited, trypsin-resistant phenotype when tested against syngeneic (MBL-2) target cell. Taken together, these data lead to the paradoxical conclusion that Lyt-2/3 molecules are essential for some CTL/target cell interactions but not for others.

The implications of these findings for the functional role of Lyt-2/3 molecules on CTL warrant further discussion. In the first place, the ability of trypsin-treated CTL clones (which lack detectable Lyt-2/3 antigenic determinants) to effectively lyse target cells argues strongly against the possibility that these molecules function either as antigen receptors or as an essential component of the lytic machinery on CTL. Although the presence of partially degraded Lyt-2/3 molecules on the surface of trypsin-treated cells cannot be ruled out, the observed correlation between trypsin resistance and resistance to inhibition with monoclonal anti-Lyt-2/3 antibodies provides compelling evidence that such CTL do indeed recognize and destroy target cells in a manner which is functionally independent of the Lyt-2/3 complex.

The results obtained with the trypsin-sensitive CTL clones (such as L3) should be interpreted with caution. Whereas loss of cytolytic activity of these cells correlated quantitatively with loss of Lyt-3 antigenic determinants, it is difficult to formally exclude the possibility that this correlation may reflect the trypsin sensitivity of another protein that is unrelated to Lyt-2/3. Despite these limitations, however, the trypsin sensitivity data are consistent both with antibody blocking experiments (Fig. 1) and with the finding that Lyt-$2^-/3^-$ variants of clone L3 fail to exhibit specific cytolytic activity (14). Taken together, these results lead to the conclusion that CTL vary clonally in their functional dependence upon a trypsin-sensitive structure that cannot be dissociated from Lyt-2/3.

Previous attempts to correlate Lyt-2/3 antigenic expression with cytolytic function at either the population (9, 10) or clonal (14, 15) level have led to conflicting results. In view of our past (8) and present findings, it is tempting to speculate that these observed experimental discrepancies could have resulted simply from heterogeneity in Lyt-2/3 requirements for killing in the particular CTL populations or clones under study. Indeed, the noncytolytic Lyt-$2/3^-$ variant CTL clone of Dialnyas et al. (14) was derived from the same parental CTL clone (L3) that was shown to be trypsin sensitive and anti-Lyt-2 inhibitable in the present study. Similarly, the alloimmune spleen cell population used as a source of CTL in the study of Ledbetter et al. (10) would be expected, on the basis of our results, to contain a majority of CTL that are not inhibited by anti-Lyt-2 antibodies. It is thus not surprising that these authors found cytolytic activity to be more resistant to trypsin treatment than Lyt-2/3 expression. Unfortunately, the parental CTLL-2 line corresponding to the Lyt-2^- variant CTL clone that retained cytolytic activity in the study of Giorgi et al. (15) was not characterized for inhibition of function by anti-Lyt-2 antibodies; however, based on our results with C10 and other similar CTL clones, we would expect their original line to be uninhibited. As for the experiments of Fan and Bonavida (9), the high concentrations of trypsin used by these authors (1–5 mg/ml) make any comparisons with the present study very difficult.

No apparent qualitative or quantitative differences in the expression of Lyt-2/3 antigens on inhibited or uninhibited CTL clones could be detected in the present

study. Thus, clones L3 and C10 expressed comparable amounts of Lyt-2 and Lyt-3 antigens as assessed by flow microfluorometry. Furthermore, when exposed to various doses of trypsin, Lyt-2 and Lyt-3 antigenic determinants were lost in a parallel fashion on clones L3 and C10. Preliminary analysis of immunoprecipitated surface ^{125}I-labeled Lyt-2/3 antigens on these clones by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions indicates a similar pattern with a major broad band of approximately 40,000 mol wt (26). More detailed comparisons of peptides immunoprecipitated from these and other CTL clones (using two-dimensional gel electrophoresis) will be required to assess possible structural microheterogeneity in the expression of Lyt-2/3 antigens.

Of particular interest in the present study was the failure of another cytolysis-inhibiting monoclonal antibody (H35–89.9) to differentially inhibit CTL clones L3 and C10 that were heterogeneous with respect to anti-Lyt-2 inhibition. As described elsewhere (12), H35–89.9 reacts with two surface polypeptides of 94,000 and 180,000 apparent mol wt on the surface of B and T cells, and this antibody inhibits mitogen-induced T (but not B) cell proliferation as well as T cell-mediated cytolysis. Thus, in many respects the determinant recognized by H35–89.9 is similar to the LFA-1 antigen described by Davignon et al. (11). The fact that clones L3 and C10, in addition to a larger series of 50 micromanipulated C57BL/6 anti-DBA/2 CTL clones (A. L. Glasebrook and H. R. MacDonald, unpublished data), were uniformly inhibited by H35–89.9 in a manner that was independent of their susceptibility to inhibition by anti-Lyt-2 antibodies argues strongly in favor of a unique mechanism governing anti-Lyt-2 inhibition (vide infra).

An important question arising from our data is whether the apparent requirement for Lyt-2/3 molecules on certain CTL clones (such as L3) is related to antigen recognition or to some aspect of the cytolytic process itself. On the one hand, direct evidence against the latter possibility comes from the observation that other CTL clones (such as C10) in which Lyt-2/3 molecules have been either blocked (by antibodies) or removed (by trypsin) are still able to mediate cytolysis. On the other hand, additional independent evidence in favor of an involvement of Lyt-2/3 molecules in antigen recognition is provided by our recent demonstration that other functions of selected CTL clones, including antigen-dependent proliferation and the secretion of lymphokines such as interferon-γ and macrophage-activating factor, can be inhibited by monoclonal anti-Lyt-2/3 antibodies in a manner that strictly parallels inhibition of cytolysis (26). The fact that several (presumably independent) functional activities of cloned CTL exhibit a similar dependence upon Lyt-2/3 molecules strongly suggests that a common initial pathway (most likely antigen recognition) is involved.

How then can the differing inhibition phenotypes of CTL clones be reconciled with a consistent model for the role of the Lyt-2/3 molecular complex in T cell-mediated antigen recognition? Given that Lyt-2/3 molecules do not constitute the CTL antigen receptor, one attractive hypothesis would be that these molecules somehow function to stabilize the interaction between the putative receptor and the appropriate antigenic determinant(s) on the target cells. Irrespective of how this stabilization process might occur at the molecular level, one important corollary to this postulate would be that, as the number and/or affinity of CTL antigen receptors increase, the requirement for Lyt-2/3 molecules to stabilize the binding would decrease. Thus, in an operational sense, the requirement of any particular CTL clone to express Lyt-2/3 antigens would

be inversely proportional to either the number and/or affinity of its antigen receptors.

It is instructive to consider the trypsin results obtained with cross-reactive CTL clones in the light of this hypothesis. Because trypsin treatment eliminated the allospecific lytic activity of these clones without affecting their anti-MSV activity, it could be argued that these cloned CTL have two antigen receptors, one trypsin-sensitive (anti-allo) and the other trypsin-resistant (anti-MSV). Although this hypothesis cannot be formally disproved, it is nevertheless difficult to reconcile with the fact that both trypsin-sensitive and trypsin-resistant lytic activities were observed for other anti-allo (Fig. 5) and anti-MSV (H. R. MacDonald, unpublished data) CTL clones. Thus, we prefer to interpret our results in the context of a single receptor (altered self) model (27) in which the cross-reactive CTL receptor has relatively high affinity for one antigen (in this case MSV) and low affinity for the other (allo). As discussed in detail above, the dissociation of lytic specificities of these clones after trypsin treatment would result as a consequence of the fact that trypsin-sensitive Lyt-2/3 molecules are required to stabilize low affinity receptor binding, rather than as a consequence of any direct effect of trypsin on the receptor itself.

Finally, although our hypothesis cannot be experimentally tested in the absence of any molecular definition of CTL antigen receptors, it is nevertheless interesting to compare some aspects of anti-Lyt-2/3 inhibition at the clonal level with what might be predicted by such a "receptor affinity" model. First, the observed inhibition of lytic activity of CTL clones by monoclonal anti-Lyt-2 antibodies is very heterogeneous. In contradistinction to the extreme situations represented by clones such as L3 and C10 (this report), data obtained with a larger number of CTL clones (reference 8 and unpublished results) suggest that the degree of inhibition of lytic activity of individual clones is distributed in a continuous (rather than "all or none") fashion. Such a continuous distribution would be consistent with the concept of a wide range of receptor affinities. Second, the degree of inhibition of CTL by anti-Lyt-2 antibodies, measured either at the population or clonal level, is dramatically reduced when the CTL have been derived from precursors selected by in vivo priming with the appropriate antigens. Thus, CTL populations or clones obtained either from alloimmune peritoneal exudate cells (8) or from restimulated populations of alloimmune spleen (this report) are much more difficult to inhibit with anti-Lyt-2 antibodies than CTL obtained in primary MLC. These differences in susceptibility to inhibition are not restricted to alloreactive CTL because most CTL clones recognizing H-2-restricted syngeneic antigens, such as MSV (this report), are likewise resistant to inhibition by monoclonal anti-Lyt-2 antibodies when derived from in vivo primed cell populations. If CTL responses, like antibody responses (28), select in vivo for precursors of high affinity, it is reasonable to assume that such differences in affinity might be reflected in the clonal progeny of such cells maintained in vitro. In such a case, quantitative inhibition experiments of CTL clones with monoclonal anti-Lyt-2/3 antibodies, such as those described in this report, may prove to be a useful (and unique) tool for making operational estimates of the relative affinity of CTL antigen receptors.

Summary

While it is well established that murine cytolytic T lymphocytes (CTL) express the Lyt-2/3 molecular complex on their surface, conflicting results have been reported concerning the role of this complex in CTL activity. In the present study this question

was reinvestigated at the clonal level. Although different (H-2^b anti-H-2^d) CTL clones expressed comparable amounts of Lyt-2/3 molecules, as assessed by quantitative flow microfluorometry, the activity of some clones was inhibited by low doses (10 ng) of monoclonal anti-Lyt-2 or anti-Lyt-3 antibodies (in the absence of complement), whereas other clones were not inhibited by either antibody at doses as high as 5 μg. Treatment of these clones with doses of trypsin sufficient to cleave Lyt-2/3 antigenic determinants from the cell surface resulted in a similar dissociation: clones that were inhibited by antibodies lost cytolytic activity, whereas "uninhibited" clones were unaffected by trypsin treatment. Moreover, the dissociation observed among different alloreactive clones could be demonstrated within self-H-2-restricted (H-2^b anti-MSV) clones exhibiting cross-reactivity with normal H-2^d products. The lytic activity of these clones against the relevant syngeneic target cells was unaffected by anti-Lyt-2 antibodies or trypsin, whereas their cross-reactivity on H-2^d target cells was abolished by either treatment.

These results provide direct evidence for clonal heterogeneity in the requirement for Lyt-2/3 molecules in CTL-mediated lysis. It is proposed that the function of Lyt-2/3 molecules is to stabilize the interaction between CTL receptors and the corresponding antigens on the target cells and that the requirement for such a stabilization is correlated with low number and/or affinity of CTL receptors.

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