Cell surface polypeptides of murine T-cell clones expressing cytolytic or amplifier activity

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ABSTRACT Murine cytolytic T-cell and amplifier T-cell clones derived from secondary unidirectional mixed leukocyte cultures were labeled with $^{125}$I by the lactoperoxidase method, and their polypeptide profiles were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. All cytolytic T-cell clones derived from the same mouse strain yielded similar cell surface polypeptide profiles. However, profiles obtained with three amplifier T-cell clones were strikingly different from each other as well as from those of cytolytic T-cell clones. Comparison of these profiles with those obtained from mixed leukocyte culture cells, whole spleen cells, glass wool/nylon-purified splenic T cells, and thymocytes indicates that cytolytic T-cell clones have a characteristic cell surface polypeptide profile that distinguishes this type of lymphocyte from most other lymphocytes.

Subpopulations of lymphocytes have been distinguished on the basis of antigenic determinants associated with cell surface molecules. Expression of Thy-1 and surface immunoglobulin have been used to distinguish between T cells and B cells (1). These cells have been classified further into subpopulations on the basis of expression of Ly cell surface antigens (2). Physical and chemical methods also have been used to study cell surface molecules that are characteristic of functionally distinct lymphocytes. Trowbridge et al. (3) analyzed cell surface polypeptides on NaDodSO$_4$/polyacrylamide gels and demonstrated that two bands, T200 and T25, were characteristic of T cells and that T25 was analogous to the polypeptide bearing the Thy-1 antigens. Cell surface radiolabeling with either $^{125}$I or boro$^{3}$$^{3}$Hhydride has also been used to distinguish B cells from thymocytes (4) and T cells (5) and to distinguish antigen-activated splenic T cells from either unstimulated splenic T cells or thymocytes (6). Furthermore, it has been found that a polypeptide, T145, appears to be expressed exclusively on cytolytic T cells (7).

Analysis of cell surface molecules of lymphocyte subpopulations by using either immunological or biochemical approaches has been complicated by the heterogeneous nature of the cell populations available for study. Although enrichment of cells having particular characteristics could be achieved to some extent with various procedures, the resulting preparations of cells always were heterogeneous and consisted of mixtures of several cell types. Recently, it has been possible to obtain T-cell clones that express specific functions (8–10). Two such T-cell clones have been derived in this laboratory: one expresses specific cytolytic T-cell (Tc) activity against target cells bearing the H-2$^{d}$ antigen and the other expresses amplifier or helper T-cell (Th) activity (11).

We report here the analysis of cell-surface polypeptides of these and other T-cell clones by using lactoperoxidase-catalyzed iodination and NaDodSO$_4$/polyacrylamide gel electrophoresis. We find that the polypeptides expressed on the surfaces of Tc clones derived from mixed leukocyte cultures (MLCs) give electrophoresis profiles characteristic for Tc clones derived from a given mouse strain. Moreover, the polypeptide band profile of Tc clones is distinctly different from profiles obtained from total MLC cells, thymocytes, and splenic T cells. Unlike the Tc clones, Th clones exhibit variations in cell surface polypeptide profiles that appear to be independent of mouse strain origin.

MATERIALS AND METHODS

Mice. C57BL/6 (H-2$^{b}$), DBA/2 (H-2$^{d}$), and A/J (H-2$^{e}$) mice were obtained from The Jackson Laboratory.

Isolation and Expansion of Murine T-Cell Clones. T-cell clones were derived from unidirectional secondary C57BL/6 anti-MLC (2) or A/J anti-MLC MLC as described (11). Clones were assayed for cytolytic activity by testing for ability to lyse P-815 (H-2$^{4}$) mastocyte target cells, initially in a $^{51}$Cr-release microcytotoxicity assay (12) and then in a 3-hr $^{51}$Cr-release cytotoxicity assay (13). Clones showing no cytolytic activity were tested for ability to amplify cytolytic activity levels of Tc clones as described (11).

Cell Surface Radioiodination and Extraction of Proteins for Electrophoresis. Cloned T cells were grown in the presence of irradiated spleen cells and supernatant fluid obtained from 48-hr cultures of Lewis rat spleen cells stimulated with concanavalin A (LCA SF) as described (11) and were harvested on day 5 of culture. Secondary (C57BL/6 anti-MLC/2) MLC were prepared as described for the derivation of clones L3 and L2 (11) with the exception that the cells were harvested on the fifth day after restimulation. Splenic T cells were obtained by passage of whole C57BL/6 spleen cells over glass wool/nylon wool (14). T-cell clones, MLC cells, C57BL/6 splenic T cells, C57BL/6 thymocytes, and C57BL/6 whole spleen cells were centrifuged over Ficoll/Hypaque gradients prior to iodination in order to remove dead cells, erythrocytes, and cellular debris (15).

The cells were washed and resuspended in Dulbecco’s phosphate-buffered saline at 10–20 × 10$^{6}$ cells per ml and were labeled with Na$^{125}$I by using the lactoperoxidase procedure (16). Less than 1.0% of the cells in each preparation were permeable to trypan blue stain. After iodination, the cells were washed with phosphate-buffered saline and were solubilized by resuspension in 1 ml of 0.5% Nonidet P-40/0.15 M NaCl/0.1 M Tris-HCl pH 7.4: the suspensions were placed on ice for 20 min and then the insoluble material was removed by centrifugation at 85,000 g-hr at 5°C. Aliquots from each supernatant fraction were diluted 1:3 with 8% (wt/vol) NaDodSO$_4$/20% (vol/vol) 2-mercaptoethanol/20% (wt/vol) sucrose

Abbreviations: MLC, mixed leukocyte culture; Th, helper T cell; Tc, cytolytic T cell; LCA SF, Lewis concanavalin A supernatant factor; DATB, N’/N’-diallyltartardiamide.

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crose/0.004% bromophenol blue/0.2 M Tris-HCl, pH 7.0. Samples were placed in a boiling water bath for 2 min prior to electrophoresis.

**NaDodSO4/Polyacrylamide Gel Electrophoresis and Autoradiography.** Discontinuous polyacrylamide gel electrophoresis was performed on slabs essentially as described by Laemmli (17) except that N',N'-diallyltartardiamide (DATD) was used as crosslinking agent because it has been reported that DATD yields better resolution of membrane glycoproteins than is usually obtained on gels crosslinked with methylenebisacrylamide (18). Long, thin gels (24 cm x 14 cm x 0.75 mm) were used in these analyses because we have found that lymphocyte polypeptides, particularly high molecular-weight components, are better separated on these gels than on the usual short (10 cm) gels. Some other modifications were introduced to facilitate polymerization. Briefly, the acrylamide/DATD solution was degassed for 3 min under vacuum, the concentrations of DATD and ammonium persulfate were increased to 0.23 and 0.3%, respectively, in the main gel, and the concentrations of acrylamide and DATD were increased to 6 and 0.15%, respectively, in the stacking gel. The combs used to form the sample wells were removed immediately after the stacking gel had polymerized (0.5 hr) because it was found that further delay resulted in strong adherence of the gel to the comb and damage to the wells upon removal.

**Reagents.** Lactoperoxidase and glucose oxidase were obtained from Sigma. Carrier-free Na$^{25}$I (350–600 mCi/ml; 1 Ci = 3.7 x 10$^{10}$ bequerels) was a product of Amersham. The reagents used in electrophoresis, including N,N',N',N'-tetramethylethylenediamine, NaDodSO$_4$, acrylamide, DATD, glycine, 2-mercaptoethanol, and Coomassie brilliant blue, were obtained from Bio-Rad.

**RESULTS**

**Identification of Tc and Th Clones.** Several cytolytic and noncytolytic clones were assayed for ability to lyse P-815 mastocyte target cells, by the 3-hr $^{51}$Cr-release cytotoxicity assay (13), at various effector:target ratios. Table 1 shows the specific lysis obtained by each clone at several ratios; also shown is the ratio at which 50% of the targets were lysed. As observed in the initial screening, clones A4, A6, B15, B18, L3, B28, C25, E8, and E11 were found to be cytolytic for P-815 mastocyte targets. Although most of these clones were highly cytolytic, producing 50% lysis at ratios ranging from 0.36 to 13, one, B28, produced barely detectable levels (8% lysis at a ratio of 30). Cytolytic activity could not be detected in clones L2, Fa13, and C23. None of the clones listed in Table 1 was found to be cytolytic against either C57BL/6 or A/J concanavalin A blasts (data not shown). These clones have been designated as T cells based on expression of Thy-1.2 as detected by anti-Thy-1.2 (AKR anti-C1H) antisera (unpublished observations).

It has been reported that the noncytolytic clone L2 can exert an amplifier (helper) effect on the proliferative and cytolytic activities of clone L3 (11). When tested in a similar manner, C23 and Fa13 effected a 5- and 11-fold enhancement, respectively, on L3 (data not shown). In addition, C23 and Fa13 had growth requirements similar to those of L2 because, unlike L3 or any other Tc clone we have isolated, C23 and Fa13 could proliferate in the absence of LCA SF but only when cultured in the presence of irradiated DBA/2 (allogeneic) spleen cells (data not shown). Thus, noncytolytic clones C23 and Fa13 are biologically similar to the amplifier clone L2 in both growth requirements and ability to amplify the cytolytic effects of L3. Details of growth requirements, amplifier and cytolytic activities, and characterization of the Ly phenotypes of some of the T cell clones listed in Table 1 will be described elsewhere.

**Cell Surface Polypeptides of Tc and Th Clones.** T-cell clones expressing either cytolytic activity against P-815 mastocyte targets or amplifier effects on the cytolytic activity of L3 cells were surface-labeled with Na$^{25}$I and lactoperoxidase. Proteins obtained from 80,000 g-hr supernatants of Nonidet P-40 extracts of the cells were analyzed on NaDodSO$_4$/polyacrylamide gels. Fig. 1 shows an autoradiograph of radiolabeled polypeptides obtained from various clones of either C57BL/6 or A/J origin. The overall profiles from C57BL/6 Tc clones A4, A6, B15, B18, B28, and L3 were similar to each other, although there were slight variations in both the electrophoretic mobilities and relative intensities of some of the bands. The overall profiles of Tc clones of A/J origin (C25, E8, and E11) were similar. However, these A/J Tc clone profiles were distinctly different from those of the C57BL/6 Tc clones. These differences were most evident in the high molecular weight region, 150,000–205,000, but some differences also were detectable in smaller components. Although Tc clones of the same mouse origin exhibit similar profiles, they were not identical. For example, a triplet banding pattern was evident in the high molecular weight region of the A4 and A6 profiles but less detectable in the other C57BL/6 Tc clone profiles. The reason for these slight variations is not clear although it is likely that the clones may vary with respect to densities of individual cell surface polypeptides, particularly as a function of time in culture. L3, which has been maintained in culture for more than 1½ years without detectable loss in cytolytic function, has on occasion produced a triplet pattern in the high molecular weight region after surface iodination (see Fig. 2).

The relative intensity of these bands has varied over months in culture. Similar fluctuations also have been observed in some of the other Tc clone profiles. It should be pointed out that the fluctuations observed are manifested in the relative intensities of the bands in this region rather than in their disappearance or reappearance or in changes in their electrophoretic mobilities. No novel bands have appeared during the time that these cells have been maintained in culture.

### Table 1. Cytolytic activity* of C57BL/6 and A/J T cell clones†

<table>
<thead>
<tr>
<th>T cell clone</th>
<th>Mouse strain</th>
<th>% lysis at various effector:target ratios†</th>
<th>ETo$^{51}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>C57BL/6</td>
<td>0.3 1.0 3.0 5.0 10.0 30.0</td>
<td>2.1</td>
</tr>
<tr>
<td>A6</td>
<td>C57BL/6</td>
<td>43 68 90 93 93 30.0</td>
<td>0.4</td>
</tr>
<tr>
<td>B15</td>
<td>C57BL/6</td>
<td>8 19 47 ND 13.0</td>
<td></td>
</tr>
<tr>
<td>B18</td>
<td>C57BL/6</td>
<td>32 68 92 95 0.55</td>
<td></td>
</tr>
<tr>
<td>L3</td>
<td>C57BL/6</td>
<td>43 80 93 ND 0.36</td>
<td></td>
</tr>
<tr>
<td>B28</td>
<td>C57BL/6</td>
<td>ND 2 6 8</td>
<td></td>
</tr>
<tr>
<td>Fa13</td>
<td>C57BL/6</td>
<td>— — — —</td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td>C57BL/6</td>
<td>— — — —</td>
<td></td>
</tr>
<tr>
<td>C25</td>
<td>A/J</td>
<td>32 44 63 1.7</td>
<td></td>
</tr>
<tr>
<td>E8</td>
<td>A/J</td>
<td>40 70 88 ND 0.45</td>
<td></td>
</tr>
<tr>
<td>E11</td>
<td>A/J</td>
<td>23 53 86 ND 0.88</td>
<td></td>
</tr>
<tr>
<td>C23</td>
<td>A/J</td>
<td>— — — —</td>
<td></td>
</tr>
</tbody>
</table>

* Determined by 3-hr $^{51}$Cr-release assay.
† Clones were derived from unidirectional secondary MLC, of which the indicated mouse strain was the source of responding cells.
‡ Percentage P-815 mastocyte targets lysed at indicated ratios. ND, not done; —, activity not detectable.
§ Effector:target ratio at which 50% lysis of P-815 mastocyte target cells was obtained. Spontaneous lysis of P-815 mastocytes averaged 7%.
C57BL/6 Th clones L2 and Fa13 and A/J Th clone C23 showed a broad variability in the cell surface iodination profiles, apparently regardless of strain origin (compare L2 and Fa13 in Fig. 1). However, because only a few Th clones have been isolated thus far, it is not known whether the variability is related to mouse strain origin or to different types of Th clones.

Results shown in Fig. 1 have been observed in separate experiments done 1, 5, 3, 6, and, in the case of L3, 10, 12, and 15 months after clone isolation. Iodinations done 3 or 5 days after the cells were plated yielded similar profiles. It is unlikely that differences in polypeptide profiles seen between the Th clones are due solely to differences in levels of proteases or other factors endogenous to the cells, unless these differences are present in the Th clones and not in the Tc clones.

These data indicate that Tc clones produce similar cell surface iodinated polypeptide profiles on NaDodSO4/polyacrylamide gels. In addition, the polypeptide profile of the Tc clone appears to be strain-dependent. On the other hand, Th clones show variability in cell surface polypeptide profiles, even among clones derived from the same mouse strain.

Comparison of Cell Surface Polypeptides of T-Cell Clones with Those of Secondary MLC, Thymocytes, Spleen Cells, and Fractionated Splenic T Cells. Preparations of C57BL/6 T-cell clones L3, L2, and Fa13, C57BL/6 thymocytes, C57BL/6 spleen cells, C57BL/6 splenic T cells, and unidirectional, secondary C57BL/6 anti-DBA/2 MLC cells were labeled with $^{125}$I and lactoperoxidase; 80,000 g-hr supernatants of Nonidet P-40 extracts of each were obtained and analyzed on NaDodSO4/8.5% polyacrylamide gels. The iodinated cell surface polypeptide profiles of T-cell clones L3, L2, and Fa13 were distinct from those of thymocytes, spleen cells, and splenic T cells, particularly in the high molecular weight region (159,000-212,000) (Fig. 2). Although the T-cell clones were derived from secondary MLC, it should be noted that not all the bands represented in the MLC profile are accounted for in the T-cell clone profiles, and vice versa. Because all C57BL/6 Tc clones have a cell surface iodinated polypeptide profile similar to that of L3, bands present in the MLC but not L3 profile (indicated by *) may correspond to noncytolytic cells (including other Th cells) present in MLC. On the other hand, certain bands were present in both Tc (L3) and Th (Fa13) clone profiles which were not well represented in the MLC profile (indicated +). It is probable that these bands are made evident by the enrichment obtained through cloning.

In order to resolve smaller molecular weight components,
we also obtained polypeptide profiles from each cell preparation on 10 and 15% gels (data not shown). No iodinated cell surface polypeptides smaller than 14,400 daltons, which corresponds to the position of β2-microglobulin in this electrophoretic system, were detected.

Because MLCs contain mostly T cells, it is not surprising that the MLC profile contains many bands that also are present in the other T-cell or thymocyte profiles. It should be noted that some of these "common" bands vary in intensity relative to each other between the various profiles. In addition, we have found that purified splenic T cells are deficient in a band of 212,000 daltons and also in a band of 33,000 daltons as compared to whole spleen cells, whereas a band of 14,400 daltons in whole spleen cell profiles is enriched by T-cell purification (data not shown).

**DISCUSSION**

The successful cloning of murine T lymphocytes has made available homogeneous populations of cells for study of the cell surface molecules of functionally distinct cells. We have found that cell surface iodination profiles of all Tc clones obtained from one mouse strain are similar on NaDodSO4/polyacrylamide gels. The surface-iodinated polypeptide profiles of Tc clones isolated from another mouse strain also are similar to each other but markedly different from those obtained from the first strain. These differences are most evident in the molecular weight region 159,000–205,000. Although apparently characteristic, we cannot be certain that the cell surface polypeptide profile of the Tc clone is unique for this type of T cell because we have studied relatively few clones of other types of T cells. The cell surface polypeptide profiles of the Th cell clones L2, Fa13, and C23 appear to show a broad variability, regardless of strain origin. Comparison of Tc and Th clone cell surface polypeptide profiles with those obtained from MLC, thymocytes, and splenic T cells indicates that the cloned T cells have profiles that are distinct from those of the more heterogeneous preparations.

It should be noted that the Tc and Th clones examined in this study require the presence of both LCA SF and irradiated spleen cells for growth, and thus it is possible that some of the bands present in the profiles of the Tc and Th clones as well as of the MLC preparations may correspond to externally acquired polypeptides. No difference in band profiles has been observed in clones grown in the presence of either allogeneic (DBA/2) or syngeneic (C57BL/6 or A/J) irradiated spleen cells. Other investigators have derived Tc clones that do not require the presence of irradiated spleen cells for growth (8–10), although these cells do not appear to have the high levels of cytolytic activity expressed by the Tc clones in this study. It will be of interest to compare cell surface iodination profiles of such clones to those obtained in the present work.

Because electrophoretic mobilities of certain polypeptides (particularly glycoproteins) are different on DATD-crosslinked gels than on gels crosslinked with methylenebisacrylamide (18) and because the use of 24-cm gels provides a finer resolution of polypeptides than is obtainable with 10-cm gels, it is difficult to correlate individual bands observed on these gels with those obtained by other investigators using different electrophoretic methods. However, certain conclusions seem warranted.

The whole-spleen-cell profile contains a unique band at position 212 (Fig. 2) which is removed when T cells are passed over nylon wool/glass wool and which is not detectable in any of the other profiles. This band probably corresponds to the B-cell component of molecular weight 245,000 on gels crosslinked with bisacrylamide previously shown by Dunlap et al. (6) to be removed by nylon wool/glass wool treatment. Other investigators have identified an iodinatable T-cell component, T200, that appears as a broad diffuse band in 10-cm gels crosslinked with bisacrylamide (3, 5). This band is resolved into multiple components, ranging from 159,000 to 205,000 daltons under the electrophoretic conditions described in this report.

The most marked differences in electrophoretic mobilities of cell surface polypeptides from the various T-cell clones as well as thymocytes, splenic T cell, whole spleen cells, and MLC cells are found in this high molecular weight region. Some of the differences in electrophoretic mobilities of bands in this region between different cell types may only reflect modification of prosthetic groups (i.e., glycosylation, phosphorylation, etc.). However, preliminary results obtained with monoclonal (hybridoma) antibodies directed against components in this region indicate that these bands can be separated into antigenically distinct polypeptides, some of which are detectable only on T-cell clones or secondary MLC cells but not on thymocytes (unpublished data). It should be noted that bands that correspond to Ly antigens or the Tc antigen T145 (7) are not obvious in either the MLC or Tc clone profiles presented in this report. Identification of the polypeptide bands bearing these antigens will probably require immune precipitation with specific antibody (19, 20) or radiolabeling with boro4H3H)hydride (7). Finally, T-cell component Thy 1, which has been identified as polypeptide band T25 on 10-cm gels crosslinked with bisacrylamide (3), has been found, with the use of anti-Thy 1.2 monoclonal antibodies, to be expressed on the surfaces of all the cloned T cells in this study. Immune precipitates obtained with these antibodies indicate that T25 corresponds to a series of closely migrating bands spanning the molecular weight region 29,000–36,000 on 24-cm gels crosslinked with DATD (unpublished data).

Recent experiments (unpublished data) have indicated that the cytolytic activities of the Tc clones described in this report are directed against major histocompatibility antigens; however, the antigenic specificity of each clone is distinct and directed against either H-2K or H-2D antigens. Differences in antigenic specificity of cytolytic activity of T-cell clones are not reflected in differences in the electrophoretic profiles of cell surface polypeptides on NaDodSO4/polyacrylamide gels as described in this report. It may be possible to detect differences between antigen receptors of cytolytic cells by the technique of two-dimensional electrophoresis (21) or by using hybridoma antibodies that recognize clones of a particular cytolytic specificity; such antibodies may be directed against the antigen receptor and may be useful for identification and characterization of such receptors.

Although the cell surface polypeptide iodination profiles of the Tc clones on NaDodSO4/polyacrylamide gels appear to be characteristic for these cells, the basis for the strain differences in polypeptide profiles is not clear. The most distinguishing polypeptides of the cytolytic cells are not located in positions expected for polypeptides corresponding to class I (K or D equivalent) or class II (Ia-like) antigens. It is possible that these strain differences are due to cell surface molecules that are not H-2 related.

Recent studies with Th clones L2 and Fa13 indicate that these cells respond to stimulation by Mls antigens rather than major histocompatibility complex antigens. Because the gene products of the Mls locus are not well defined, it is not possible at present to determine whether L2 and Fa13 respond to the same or different Mls determinants. The marked differences in the cell surface polypeptide profiles of L2, Fa13, and C23 support the notion that these Th clones express biological differences that are not revealed by the assays used in these experiments.
The data reported indicate that cloned T lymphocytes expressing cytolytic activities present a cell surface polypeptide profile on NaDdSO₄/polyacrylamide gels that is characteristic of these cells and that is strain-dependent. On the other hand, analyses of three Th clones indicate that these cells vary significantly from each other in their cell surface polypeptides, regardless of strain origin. The interpretation from these studies is that cell surface polypeptide profiles definitive of functionally distinct lymphocytes can be obtained through the use of cloned cells. The most obvious electrophoretic differences between cloned Tc and Th as well as between these and the more heterogeneous preparations of thymocytes, splenic T cells, and total MLC cells occur in the molecular weight region 159,000-205,000 and require the use of 24-cm-long gels for detection. Although this high molecular weight region does not contain known class I or class II antigens, it is clear from immune precipitation studies using monoclonal antibodies directed against components in this region that some of these polypeptides bear antigens that distinguish Tc clones from Th clones (unpublished data).

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