Development of large granular lymphocytes with anomalous, nonspecific cytotoxicity in clones derived from Ly-2+ T cells

KENDALL SHORTMAN, ANNE WILSON, ROLAND SCOLLAY, AND WEI-FENG CHEN

The Walter and Eliza Hall Institute, Post Office Royal Melbourne Hospital, Victoria 3050, Australia

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ABSTRACT T cells cultured at limit dilution for 8 days in a concanavalin A-stimulated, filler-cell and growth factor-supported system produced cytolytic clones with high efficiency. These clones were not specific, lysing a wide range of targets, syngeneic and allogeneic, of tumor and normal cell origin. Lysis was a cell-mediated phenomenon but was not blocked by anti-Ly-2. One H-2-2-negative target was lysed, but one was resistant. Xenogeneic (human) tumor cells were not lysed. The cells in the clones were large, vacuolated, granular lymphocytes. They originated from single Ly-2+ responder cells and not from irradiated filler cells. Therefore, activated lymphocyte killers and other natural killer-like cells may be differentiated elements of the Ly-2+ T-cell lineage.

Cytolytic T lymphocytes (CTL) arise by proliferation and differentiation from nonlytic precursors within the Ly-2+ subset of murine T lymphocytes (1). CTL are considered to exhibit a high level of immunological specificity. Another group of lymphoid-like cells active against a broad range of target cells has also been described. These include natural killer cells (NK cells), preexistent in the animal, and certain induced or activated lymphocyte killers (ALK) (2-5). Cells of this type have the morphological form of large granular lymphocytes (LGL) (2, 6-8). Although relationships between these various cytolytic cell types have been proposed (4), direct evidence has been lacking. Here we demonstrate that mature murine Ly-2+ T cells give rise to clones of LGL with the capacity to kill a wide range of targets.

The system employed was designed to measure the frequency of nonlytic CTL precursors by the limit-dilution approach (9, 10). T cells, cultured at levels of about one cell per well, and stimulated with concanavalin A (Con A) in the presence of irradiated spleen filler cells and growth factors, grow rapidly for 9 days with a near-100% cloning efficiency (10). With phytohemagglutinin (PHA) included to mediate lysis of tumor target cells, those clones that are cytolytic may be detected, regardless of their specificity, in a radioautographic In-release assay (9, 11).

Clones derived from Ly-2+ T cells, but not those from Ly-2- T cells, were cytolytic in this universal assay. The low cell input and strict linear fit to the zero-order Poisson relationship demonstrated the clonal nature of the response. The effector cells were shown to be Thy-1- derivatives of the responders rather than of the irradiated fillers (9). It is the cells in such clones that we now show to have an unexpected LGL morphology and to kill most target cells, even in the absence of lectin mediation.
together with, when required, PHA-M (1:100) or α-methylmannoside (0.1 M). After centrifugation, the cells were incubated for 4 hr at 37°C. A sample of each supernatant was exposed overnight at -70°C against x-ray film with enhancing screens. A black spot represented a positive culture, with 200 allospecific CTL being sufficient to register above the 5–9% spontaneous release. In some cases the supernatants were counted in a gamma counter to determine the percent specific release.

Antibody Blocking of Cytolysis. Anti-Ly-2 supernatant from rat anti-Ly-2 hybridoma 53-6.7 (IgG2a) was added directly to the cytotoxic assay (1:3 final dilution) and left in the assay mixture for 4 hr.

Labeling with Monoclonal Antibodies. For anti-Ly-2, cells were stained with biotin-conjugated rat monoclonal protein (clone 53-6.7), followed by fluorescein-conjugated avidin (Vector Laboratories, Burlingame, CA). For anti-Thy-1, cells were stained with rhodamine-conjugated rat anti-Thy-1.2 protein (clone 30-H12). In double-stained preparations this was followed by a fluorescein-conjugated mouse anti-Thy-1.1 (clone HO-22-1). All conjugated antibodies were prepared from supernatants of cultures grown in this institute.

Analysis and Sorting of Fluorescent-Labeled Cells. A FACS II instrument (Becton Dickinson) was used, dead cells being excluded by low-angle light scatter. Fluorescence-sorting thresholds were based on preliminary analysis of stained lymph node cells, by using unstained preparations and stained cells of inappropriate allotype as controls. Reanalysis of sorted fractions routinely showed <2% cross-contamination.

RESULTS

Lectin-Enhanced Versus Direct Cytolysis of P815. When CBA mouse spleen cells were cultured in our Con A-stimulated system at a level of 10 cells per well (around one Ly-2+ T cell per well) and assayed at day 8 or 9 on P815 tumor targets in the presence of PHA, a high proportion of cultures showed cytolytic activity, averaging 32% lysis above background (Fig. 1). When the PHA was omitted, we anticipated a reduction to 1/20th to 1/100th in the number of positive cultures, to the level expected of the CBA anti-H-2d allospecific nonlytic CTL precursor frequency. Instead, a high proportion of positive cultures was still obtained (Fig. 1), and lytic activity per culture remained relatively high. No lysis (with or without PHA) was seen in cultures of filler cells alone. Similar results were obtained with (i) lymph node cells or cortisone-resistant thymocytes as responders; (ii) sorted, >98% pure Ly-2+ T cells cultured at levels down to 0.3 cells per well; and (iii) lymphoid cells from C57BL/6 mice. The effect was not unique to our cytotoxic assay system. The same direct cytolysis of P815 was obtained by using gamma counting to measure isotope release and by using 51Cr instead of 111In–oxine to label the cells. Substituting mouse serum for fetal calf serum in the assay medium gave the same result.

Relationship Between Clones Giving Direct and Lectin-Mediated Cytolysis. The same clones were responsible for both the direct and the lectin-mediated lysis of P815. This was demonstrated by splitting cultures at day 8 and assaying with or without PHA. As shown in Table 1, 95% of cultures showing direct cytolysis also scored positive in the presence of lectin. Similar correspondence was obtained with lower responder-cell inputs. Thus, the strict single Ly-2+ precursor relationship previously established for clones assayed in the presence of PHA (9) must also apply to clones giving direct cytolysis.

Cytolysis Is Not Due to Con A Carry-over. Con A, like PHA, can cause specific CTL to kill an inappropriate target. However, the direct lysis was not mediated by residual Con A from the initial mitogenic stimulus. α-Methylmannoside (0.1 M) abrogated completely Con A-mediated lysis of P815 by MLR-derived CTL of inappropriate specificity, even with Con A concentrations 10 times those initially in our cultures. Yet, as Fig. 1 shows, lysis was unaffected by this sugar. Washing cells, with or without α-methylmannoside, did not prevent direct cytotoxicity.

Cytolysis Is Not Due to a Nonspecific Lymphotoxin. The cultured cells did not release detectable amounts of a soluble

Table 1. Clones showing direct lysis correspond to clones showing lectin-mediated lysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cultures, no.</th>
<th>Proportion of cultures</th>
</tr>
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<tbody>
<tr>
<td>Total cultures assayed</td>
<td>400</td>
<td>—</td>
</tr>
<tr>
<td>Positive on P815 with PHA</td>
<td>129</td>
<td>0.32</td>
</tr>
<tr>
<td>Positive on P815 alone</td>
<td>103</td>
<td>0.26</td>
</tr>
<tr>
<td>Positive on both, observed</td>
<td>98</td>
<td>0.25</td>
</tr>
<tr>
<td>Expected by coincidence</td>
<td>33</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Cultures of CBA spleen cells (eight cells per well) were split at day 8, one-half being assayed in the presence of PHA and the other half, in the absence of PHA.
nonspecific factor causing target cell lysis. Supernatants of the original 5-day cultures failed to lyse P815 (4 hr at 37°C). Supernatants obtained by incubating washed cells from each well with cytotoxic assay medium for 2 or 4 hr likewise failed to lyse P815.

Metal Dependence. The direct cytolysis was completely eliminated by adding EDTA (5 mM), showing that divalent metals were required as they are for lysis by normal CTL.

Unlabeled Target Inhibition. Addition of a 20-fold excess of unlabeled P815, along with the 111In-labeled P815, caused a reduction to \( \sim 1/10th \) in the lysis by individual cultures, a decrease similar to that obtained with conventional allo-specific CTL.

Lack of Blockage with Anti-Ly-2. Addition of monoclonal anti-Ly-2 to the assay caused no detectable decrease in direct cytolysis by cells in these clones, although it caused a marked decrease in allo-specific lysis by MLR-derived CTL (Table 2).

Lytic Efficiency. The cells harvested from the clones gave direct lysis of P815 even at low effector-to-target ratios (Fig. 2). However, they were only 1/10th as efficient as allo-specific, MLR-generated CTL preparations.

Range of Target Cells Lysed. The high proportion of clones capable of direct cytolysis was not just a feature of P815 target cells (Table 3). A range of tumor lines, including the murine NK target YAC, and the syngeneic tumors C1.18 and R1, were all lysed with high efficiency, although the degree of lysis was sometimes less than with P815. Likewise, there was a high frequency of clones lysing normal macrophages (syngeneic and allogeneic) and Con A blasts (syngeneic and allogeneic), generally with less efficiency than the lysis of P815. Two HLA+ human lines (including a preferred human NK target) and two HLA- lines were also tested. Killing was negligible in the absence of PHA (Table 3), but all four were lysed with about the same frequency as allo-specific targets in the presence of PHA.

Influence of Target Cell H-2 on Lysis. One surface H-2-negative variant target, R1 (TL-') (13), was lysed, although with lower frequency and extent than R1, the H-2-positive line of origin. The H-2-negative Krebs ascites line showed negligible lysis. However, this line was not killed, even in the presence of PHA, and may be a resistant target.

Morphology of Cells in Cytolytic Clones. The appearance of cells in the lytic clones was examined, initially by assaying samples of cultures for direct cytolysis at day 8 and then making smears of individual positive cultures at day 9. An alternative was to pool positive cultures, remove dead filler cells, and then prepare smears. Finally, the simplest approach was to grow sorted Ly-2+ T cells at all cells per well, pool all cultures (most of which were positive), and remove damaged cells before smearing. All approaches gave the same result (Fig. 3). The vast majority of cells were large- to medium-sized, vacuolated, and granulated, although the granules were generally larger than those found in LGL in vivo. Many such cells were in division. Control cultures of filler cells alone yielded only a few viable cells of very diverse morphological type.

Origin of Cells in the Cytolytic Clones. The majority of LGL in these clones appeared to come from the single responder T cell rather than from the filler cells, as assessed by using responders and fillers of differing Thy-1 allotype. AKR responder cells grown on CBA fillers were as effective as the CBA on CBA combination in producing directly cytotytic clones. The cells in such clones were found to be 79% Thy-1.1-positive and 7% Thy-1.2-positive (average of four experiments) and thus were pre-
dominantly of donor allotype. Because the Thy-1.1 staining reagents gave weaker fluorescence than the Thy-1.2 reagents, the Thy-1.1 values are a minimal estimate. No double-positive cells were seen. The same result was obtained whether the starting population was unsorted or consisted of Ly-2\(^-\) AKR lymphoid cells. The direct cytolysis of P815 was the same regardless of whether 1\% or 10\% Thy-1.2-positive cells were found and thus appeared independent of filler-cell contamination.

**DISCUSSION**

The striking aspect of these findings is the high frequency with which individual mature Ly-2\(^-\) T cells gave rise to clones of LGL with an ability to directly lyse most target cells in a nonspecific manner. This may be a consequence of a particularly effective "cocktail" of growth and differentiation factors produced by the Con A-stimulated filler cells in these cultures. These nonspecific clones appeared wherever we expected to find specific CTL. The exact relationship between CTL and these nonspecific LGL remains to be established, but clearly they both arise from the Ly-2\(^-\) pool. The Ly-2\(^-\) population might include separate CTL and LGL precursors or, alternatively, a single common precursor. In any case, this clonal analysis demonstrates that these different cytolytic lymphoid cells are closely related.

Are all forms of LGL, whether ALK or NK, members of the Ly-2\(^-\) T-cell lineage? The present results provide an argument for this view but do not prove it. The clones lyse YAC, a typical NK target, but also lyse P815, which is not (2, 3). Our cytolytic clones are Con A- and growth factor-stimulated and thus not selected by antigen. It is possible that selective stimulation and inhibition in vitro might decrease this broad cytolytic capacity to just the peculiar pattern considered diagnostic of NK cells. Further study of the morphology, surface markers, and specificity potential of cells in these clones may clarify this point.

These findings complicate interpretations of limit-dilution CTL-clone specificity analyses. A culture scored positive for cytolysis of a given target might be composed of specific CTL but might also include polyspecific LGL, depending on conditions. Several experiments suggesting generation of CTL specificity diversity in clones derived from pre-T or stem cells (14, 15) must now be reassessed, because the culture conditions may well have generated nonspecific LGL.

What is the basis of the nonspecific lysis by these clones? We have not yet completely excluded the possibility that the actual killing is done by a minor, filler cell-derived element merely activated by the LGL, but this seems unlikely in view of the extent of lysis. Target cell major histocompatibility antigen does not appear to be essential because the major histocompatibility complex-negative line R1 (TL\(^-\)) was also killed. Generation of a range of specificity variants of a normal T-cell receptor could explain the results, but again the extent of lysis argues against this. A new developmental step, from Ly-2\(^-\) T cell to a NK-like LGL with a new "universal" receptor is one explanation although universal in this context does not include human cells. Another is that LGL represent an extremely differentiated and potent form of CTL, with such a high cytolytic potential that even a low-affinity, transient interaction with the target cell leads to a lethal hit.

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