ALLOREACTIVE CLONED T CELL LINES

I. Interactions Between

Cloned Amplifier and Cytolytic T Cell Lines*

BY ANDREW L. GLASEBROOK\footnote{ Supported by U. S. Public Health Service Research grant AI-4197.} AND FRANK W. FITCH

From the Committee on Immunology and the Department of Pathology, The University of Chicago, Chicago, Illinois 60637

A population of T cells has been described that can promote or enhance the proliferation and differentiation of Lyt-2,3+ cytolytic T lymphocyte (CTL)\textsuperscript{1} precursor cells in mixed leukocyte culture (MLC) (1-3). Termed amplifier T cells, they have been characterized as Lyt-1+ cells that appear to be stimulated by specific alloantigens, perhaps I region-encoded (1, 2, 4). Amplifier T cells are required for the release of nonspecific soluble factors that mediate the enhancement of cytolytic activity (5-12). However, efforts to further characterize the nature of the interacting T lymphocytes, the antigens to which they respond, and the soluble factors involved in the response have been hampered by the heterogeneous nature of the participating cell populations. Generally, attempts to derive functioning amplifier or cytolytic cell hybridomas have been unsuccessful (13), although T cell hybrids that have suppressor functions have been developed (14).

Recently, it has become possible to maintain CTL in long-term culture (15-18). With few exceptions (18), supernatant fluids (SF) from antigen- or mitogen-stimulated lymphoid cells have been used to immortalize cells from MLC in the absence of added alloantigen (15-17), with the cloning of CTL following only after successful long-term culture of the cells (16, 19). The cytolytic cell lines derived in this manner (15-17, 19) are able to proliferate independently of alloantigen in the presence of SF alone. In an attempt to develop clones of the T cells that interact in MLC, we have used a different strategy, which includes the cloning of secondary MLC cells with alloantigen and conditioned SF. With such a procedure, we have isolated a cloned amplifier T cell line, L2, which, when cultured with alloantigen, permits another cloned T cell line, L3, to proliferate and express specific cytolytic activity (20). L2 and L3 cells have now been kept growing continuously in culture for 21 mo, and during that time several other amplifier and cytolytic T cell lines have been derived, cloned, and analyzed. This paper describes the interactions occurring between cloned lines of noncytolytic

\* Supported by U. S. Public Health Service Research grant AI-4197.
\[ Supported by training grants from the U. S. Public Health Service (GM-00093-20) and the Institute of General Medical Sciences (5T32GM07197).

\[ Abbreviations used in this paper: CML, cell-mediated lympholysis; Con A, concanavalin A; CTL, cytolytic T lymphocyte(s); DMEM, Dulbecco's modified Eagle's medium; FACS II, fluorescence-activated cell sorter; FCS, fetal calf serum; \textsuperscript{[3]}H\textsuperscript{2}TdR, tritiated thymidine, LCA SF, SF from Con A-stimulated rat spleen cell cultures; 2-ME, 2-mercaptoethanol; Mls, M locus; MHC, major histocompatibility complex; MLC, mixed leukocyte culture(s); MLR, mixed lymphocyte reaction(s); Mls, M locus; MOPS, morpholinopropane-sulfonic acid; MTLA, mouse thymic lymphocyte antigen; SF, supernatant fluid(s).
and cytolytic T cells in vitro. We show that cloned noncytolytic cells are amplifier T cells responding to M locus (Mls) determinants. Such amplifier cells are functionally radioresistant and secrete soluble amplifier factor(s) that are neither antigen specific nor H-2 restricted. The cytolytic cell clones, which require both SF and irradiated splenic feeder cells for proliferation, are activated by the amplifier cell clones in a polyclonal fashion.

Materials and Methods

Animals. Adult mice of the inbred strains C57BL/6 (B6) (H-2b, Mlsb), C3H/HeJ (H-2k, MlsC), CBA/J (H-2u, Mlsd), AKR/J (H-2k, Mlsa), BALB/c (H-2k, Mlsa), DBA/2 (H-2b, Mlsb), A/J (H-2k, Mlsa), and (C57BL/6 × DBA/2)F1 (BDF1) were obtained from The Jackson Laboratory, Bar Harbor, Maine. The C57BL/10 congenic mice, B10.BR, and B10.D2 were bred in our own colony (The University of Chicago, Chicago, Ill.), whereas B10.AL and B10.OH male mice were kindly provided by Dr. Donald C. Shreffler (Washington University, St. Louis, Mo.). Adult female Lewis and BN rats were obtained from Microbiological Associates, Walkersville, Md.

MLC. Spleen cell suspensions were prepared in Dulbecco's modified Eagle's medium (DMEM) (Grand Island Biological Co., Grand Island, N. Y.) with loose-fitting glass tissue homogenizers (Belco Glass, Inc., Vineland, N. J.) and then resuspended in DMEM supplemented with 2% heat-inactivated fetal calf serum (FCS) (KC Biological, Inc., Lenexa, Kans.), 5 × 10⁻⁵ M 2-mercaptoethanol (2-ME), 10 mM morpholinopropane-sulfonic acid (MOPS), penicillin/streptomycin, and additional (21) amino acids (culture medium). Primary MLC were prepared by mixing 25 × 10⁶ viable responder spleen cells with 25 × 10⁶ irradiated (1,400 rad) (model 143 cesium irradiator, JLS Shepherd & Associates, Glendale, Calif.) DBA/2 splenic feeder cells for 14 days of culture medium in 50-ml tissue culture flasks (Falcon 3013, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). The flasks were incubated upright in a humidified atmosphere of 5% CO₂ at 37°C. Secondary MLC were prepared by collecting primary MLC cells after 14 d of culture and restimulating 3.5 × 10⁶ viable cells with 25 × 10⁶ irradiated (1,400 rad) DBA/2 spleen cells in 20 ml of culture medium in 50-ml tissue culture flasks. (Falcon 3013, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). The flasks were incubated upright in a humidified atmosphere of 5% CO₂ at 37°C. Secondary MLC were prepared by collecting primary MLC cells after 14 d of culture and restimulating 3.5 × 10⁶ viable cells with 25 × 10⁶ irradiated (1,400 rad) DBA/2 spleen cells in 20 ml of culture medium.

MLC that contained cloned T cell lines were established by transferring 1.6 × 10⁶ cloned T cells (100 µl, 7-8 d after previous transfer) to 3-ml tissue culture wells (17-mm Diam; Linbro 24-well plate, 76-033-05, Linbro Chemical Co., Hamden, Conn.) at a final density of 10⁷ cell/ml. Each well contained 6 × 10⁶ irradiated (1,400 rad) spleen cells or an equivalent volume of culture medium, and 33% SF or culture medium, in a final vol of 1.5 ml. Culture plates were then incubated in a 37°C, 5% CO₂ humidified incubator.

Marbrook Cultures. Membrane filters (0.2 µm) (Metrical GA-8, Gelman Sciences, Inc., Ann Arbor, Mich.) and Tygon plastic tubing (Norton Co., Plastics and Synthetics Div., Tallmadge, Ohio) were boiled three times in distilled water. The membrane filter was then secured on the inner tube of a single-chambered Marbrook vessel (B-9000, Bio-Research Glass, Inc., Vineland, N. J.) with Tygon tubing. Cultures were established by transferring 1.6 × 10⁶ cloned T cells, or culture medium, to the lower (outer) chamber that contained 10⁶ cell/ml. Each well contained 6 × 10⁶ irradiated (1,400 rad) spleen cells or an equivalent volume of culture medium, and 33% SF or culture medium, in a final vol of 3.5 ml. After the cells had settled, the upper (inner) chamber was replaced and received 1.6 × 10⁶ cloned T cells, 2 × 10⁶ irradiated (1,400 rad) DBA/2 spleen cells, and 33% SF from concanavalin A stimulated rat spleen cell cultures (LCA SF) or culture medium, in a final vol of 1.6 ml. Cultures were then incubated at 37°C.

Production of SF. (a) SF from secondary MLC cultures (MLC SF): Secondary MLC were prepared by restimulating cells from primary C57 anti-DBA/2 MLC with irradiated DBA/2 spleen cells after 14 d of culture as described previously. After 48 h of culture, the SF were collected and centrifuged at 3,000 g for 5 min, then filtered through a 0.2-µm Nalgene filter (Nalge Co., Nalgene Labware Div., Rochester, N. Y.), and stored at −20°C. (b) LCA SF: Lewis rat spleen cells were cultured at a density of 1.25 × 10⁶ cells/ml of culture medium (without MOPS) that contained 2.5 µg/ml concanavalin A (Con A) (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) in glass bottles. After 48 h of culture, the SF were collected and centrifuged at 3,000 g for 10 min. The SF were then absorbed twice with Sephadex G-25 (Pharmacia Fine
Chemicals, Inc.) (0.2 g/100 ml SF) to remove Con A, filtered through a 0.2-μm Nalgene filter, and stored at -20°C.

Cloning Procedure. Secondary MLC cells were harvested 48 h after initiation of culture, and then centrifuged and resuspended in either MLC or LCA SF at a concentration of 20-0.2 cell/ml. A 0.1-ml aliquot was then added to each microwell of a tissue culture plate (6.4-mm diam; Costar 3596, Costar, Data Packaging, Cambridge, Mass.) to which 10⁶ irradiated (1,400 rad) DBA/2 spleen cells in 0.1 ml of medium (DMEM, 20% FCS, 5 × 10⁻⁵ M 2-ME, and penicillin/streptomycin) had been added previously. The cloning plate was incubated at 37°C, and 0.1 ml of additional medium (DMEM, 50% SF, 11% FCS, and 5 × 10⁻⁵ M 2-ME) was added to each well after 4 d of incubation. Clusters of cells appeared in the cloning wells after 6–9 d of incubation.

Assay for Cell-mediated Lympholysis (CML). Cytolytic activity was measured in a ⁵¹Cr release assay as described by Cerottini et al. (21) with modifications (22), except that assays were 3.5 h in length. The percentage of specific cytotoxicity at various effector:target cell ratios was calculated by the formula:

\[
\text{Percent specific lysis} = \left( \frac{\text{counts per minute}_{\text{experimental}} - \text{counts per minute}_{\text{spontaneous release}}}{\text{counts per minute}_{\text{maximum release}} - \text{counts per minute}_{\text{spontaneous release}}} \right) \times 100.
\]

Maximum release was determined by freeze-thaw lysis of target cells. Lytic units per culture were calculated from individual dose-response curves by taking the reciprocal of that culture fraction that caused 50% specific lysis of target cells; therefore, one lytic unit is defined as the number of effector cells required to lyse 50% of 10,000 target cells within 3.5 h (21, 22).

Target Cells. P-815 (H-2b), RBL5 (H-2b), and AKR-A (H-2k) tumor cells maintained in culture were used as target cells. Murine Con A blast target cells were obtained by culturing spleen cells in culture medium that contained 1 μg/ml Con A for 2-4 days. Rat Con A blasts were generated in a similar manner except that MOPS was not added to the culture medium, and the Con A concentration was increased to 2.5 μg/ml.

Mixed Lymphocyte Reaction (MLR). Cultures were established in triplicate wells of a flat-bottom microwell tissue culture plate (Costar 3596, Costar, Data Packaging). Each well contained 10⁶ irradiated (1,400 rad) spleen cells, 33% SF or culture medium, and 10,000 cloned T cells, in a total vol of 0.3 ml. The plates were incubated at 37°C, and after various intervals of culture, 1 μCi of tritiated thymidine ([³H]TdR) (2 Ci/mmol, The Radiochemical Centre, Amersham England) was added to each well. After incubation for 4 h at 37°C, cells were then collected onto filter paper strips with a cell harvester (Otto Hiller, Madison, Wis.), and counted in a Searle Isocap/300 liquid scintillation counter (Searle Radiographics, Inc., Des Plaines, Ill.). Results are expressed as disintegrations per minute ± SEM, with the channels ratio method for conversion from counts per minute. The stimulation index was calculated relative to cultures with syngeneic cells.

Alternatively, cells were collected from MLC cultures and resuspended in culture medium. A 0.1-ml aliquot was then transferred to triplicate wells of a flat-bottom microwell culture plate and pulsed with 1 μCi [³H]TdR for 4 h.

T Cell Fractionation. Splenic T cells were enriched for by the method of Julius et al. (23) with glass wool-nylon wool-column fractionation.

Antiserum and Treatment. Anti-mouse thymic lymphocyte antigen (MTLA) serum was raised in goats by the method of Sauser et al. (24), and was specific for T cells by a variety of tests (data not shown). T cells were depleted from spleen cell populations by treating twice with anti-MTLA plus rabbit complement. Spleen cells were incubated with anti-MTLA serum (1:10) for 30 min at 4°C, followed by incubation with rabbit complement (1:5) for 45 min at 37°C.

Anti-Lyt-1.2 and anti-Lyt-2.2 sera, generously provided by Dr. Harvey Cantor, were prepared according to Shen et al. (25) and were specific for the appropriate Lyt alloantigen by a number of criteria, which included reduction of cytotoxic titer after absorption of the anti-Lyt sera with the appropriate B6 congenic strains, negative staining with the appropriate B6 congenic cells, and patterns of fluorescence that were identical to those obtained with monoclonal antibodies.
of anti-Lyt specificity (data not shown). For determination of Lyt phenotype, cells were incubated with anti-Lyt-1.2 (1:30) or anti-Lyt-2.2 (1:60) serum for 30 min on ice. After staining with fluoresceinated anti-Ig (1:20) for 30 min at 4°C, the cells were resuspended in medium for analysis with the fluorescence-activated cell sorter (FACS II, Becton Dickinson & Co., Mt. View, Calif.). In some instances the cells were preabsorbed with 10% human heat-aggregated serum to reduce Fc binding, which was not found to affect the staining patterns.

**Results**

**Proliferative and Cytolytic Properties of Cloned T Cell Lines.** Primary B6 anti-DBA/2 or A/J anti-DBA/2 MLC cells were collected after 14 d of culture and restimulated at low density with irradiated DBA/2 alloantigen (responder cell:stimulator cell ratio of 1:7). Secondary MLC cells were cloned 48 h after restimulation by limiting dilution in microwells with irradiated DBA/2 cells and either MLC or LCA SF. Clones of cells appeared 6–9 d after culturing, and all wells were screened for the expression of cytolytic activity. Cell clones of interest were recloned with conditions yielding growth in <5% of the cloning wells and maintained by weekly passaging with irradiated alloantigen and either MLC or LCA SF. When the cell clones had been maintained in culture for >1 mo after recloning, the proliferative and cytolytic properties of several cell clones were measured.

Cell clones were cultured with irradiated DBA/2 alloantigen and either culture medium or LCA SF, and assayed at various intervals for cell recovery, [³H]T𝑑R incorporation, and cytolytic activity (Fig. 1). Cell lines L3 and B18, of B6 origin, and C25, of A/J origin, did not proliferate appreciably when cultured with the original stimulating DBA/2 alloantigen unless either LCA SF (Fig. 1 d and e) or MLC SF (data not shown) was added. Peak [³H]T𝑑R incorporation was observed on day 5, whereas total viable cell recoveries peaked between days 5 and 7 compared with the initial addition of $1.6 \times 10^4$ clone cell/culture. Expressed in terms of lytic units per culture with P-815 (H-2b) mastocytoma as the target cell, the cytolytic activities of cell lines L3 and B18, and C25 cultured with alloantigen and LCA SF were 109- to 330-fold greater than the lytic activity of cells cultured only with alloantigen (Fig. 1 f). When expressed in terms of lytic units per $10^6$ cells recovered from culture, however, the amount of cytolytic activity generated in cultures that contained alloantigen and either culture medium or LCA SF was, on a cell-cell basis, nearly identical (20). Therefore, the cytolytic activities of L3, B18, and C25 are related primarily to the number of cells developing during culture.

On the other hand, cell lines L2 and Fa13, of B6 origin, and C14, of A/J origin, proliferated when cultured with DBA/2 alloantigen and culture medium (Fig. 1 a). The cell lines uniformly reached peak levels of [³H]T𝑑R incorporation after 3 d of culture, whereas, paradoxically, total viable cell recoveries increased up to 5 d, and declined thereafter. The inclusion of LCA SF significantly increased the proliferative response of the cell lines while not affecting the kinetics of the response (Fig. 1 b). Although L2, Fa13, and C14 cells proliferated in culture, these cells never expressed any detectable cytolytic activity (Fig. 1 c). The addition of Con A to a $^{51}$Cr release assay to promote nonspecific lysis (26) did not result in significant cytolytic activity (data not shown).

All of the cell lines isolated, both cytolytic and noncytolytic, were diploid in nature and expressed the Thy-1.2 antigen (data not shown). The expression of Lyt-1 and Lyt-2 alloantigens was measured both by indirect immunofluorescence (Fig. 2) and
Fig. 1. Proliferative and cytolytic properties of T cell clones. MLC were established by transferring 1.6 x 10^5 cloned noncytolytic (L2, Fa15, C14 [a-c]) or cytoytic (L3, B18, C25 [d-f]) T cells to 3-ml tissue culture wells 7-8 d after previous transfer. Each well contained irradiated DBA/2 spleen cells and either 33% LCA SF (-----) or culture medium (---). Cells were collected from individual wells after various periods of culture and assayed for viable cell recovery (a, d), [H]Tdr incorporation (b, e), and cytolytic activity (lytic units per culture) (c, f). The mean value obtained from duplicate cultures is shown.

absorption (data not shown). The cytoytic cell lines L3 and B18 were determined to be Lyt-1^-,2^+ (Fig. 2b-c). The noncytoytic L2 cells were Lyt-1^-,2^- (Fig. 2d), whereas the noncytoytic Fa13 and Fa15 cells were Lyt-1^+,2^- (Fig. 2e and f). The Fa13 cell
Fluorescence intensity

Fig. 2. Expression of Lyt alloantigens. Cell clones were grown in MLC with irradiated DBA/2 alloantigen and SF. After 4-6 d of culture, viable cells were collected by sedimentation of dead cells and debris through Ficoll (Pharmacia Fine Chemicals, Inc.) -Hypaque (Winthrop Laboratories, New York). Incubation with anti-Lyt-1.2 (1), anti-Lyt-2.2 (2), or normal B6 serum (3) was followed by staining with a fluoresceinated anti-Ig. The FACS II was used to quantitate the immunofluorescent staining. (a) B6 thymus cells, (b) L3 cells, (c) B18 cells, (d) L2 cells, (e) Fa13 cells, (f) Fa15 cells.

line (Fig. 2c) expressed high levels of the Lyt-1 antigen compared with thymocytes (Fig. 2a) or Fa15 cells (Fig. 2f).

The noncytolytic cell lines L2, Fa13, and C14 proliferate in response to alloantigen stimulation in the absence of any added SF. To examine the specificity of stimulation, the proliferative responses of the noncytolytic cell lines were measured in cultures prepared with various stimulating spleen cell populations.

Specificity of Proliferation of Noncytolytic Cell Lines. The peak proliferative responses of the noncytolytic cell lines L2, Fa13, Fa15, and C14 were measured in MLR prepared with irradiated spleen cells bearing different H-2 and Mls antigens (Fig. 3). L2, Fa13, and Fa15 cells, all of B6 (H-2b, Mlsb) origin, proliferated when cultured with the original stimulating DBA/2 (H-2d, Mlsa) spleen cells or with AKR/J (H-2k, Mlsb) spleen cells that are identical to DBA/2 cells at the Mls but different at H-2 (stimulation index from 6 to 82). Culture of the cell lines, however, with spleen cells syngeneic at the Mlsb but identical with the H-2 of DBA/2 (B10.D2 or BALB/c, H-2b) or AKR/J (B10.BR, H-2b) spleen cells resulted in levels of [3H]TdR incorporation equivalent to the background responses obtained with syngeneic B6 (H-2b, Mlsb)
INTERACTIONS BETWEEN CLONED T CELL LINES

Fig. 3. Specificity of MLR responses exhibited by the noncytolytic T cell clones. The noncytolytic T cell clones L2, Fa13, Fa15, and C14 (10⁴), obtained 7–8 d after previous transfer, were cultured with 10⁶ irradiated spleen cells of differing H-2 and Mls phenotype in microwell cultures for 2–4 d. Cultures were pulsed with 1 μCi [³H]Tdr for 4 h and then collected onto filter-paper strips for counting. Peak values are shown for [³H]Tdr incorporation and are expressed as mean disintegrations per minute ± SEM for triplicate cultures. Results are shown from a representative experiment repeated three times.

spleen cells. When the cell lines were cultured with C3H/HeJ (H-2k) or A/J (H-2a) spleen cells expressing weakly stimulating Mls c determinants, MLR responses were slightly higher. MLR responses, however, obtained with CBA/J (H-2k, Mls b) spleen cells were equivalent to or higher than those obtained with DBA/2 and AKR/J spleen cells.

The MLR responses of an A/J-derived cell line, C14 (H-2a, Mls c), were similar to those of the other cell lines, except that C14 cells also proliferated when cultured with Mls b spleen cells (BALB/c, B10.BR, and B10.D2) with the exception of B6 spleen cells (Fig. 3). C14 cells did not proliferate when cultured with spleen cells expressing syngeneic Mls c determinants (C3H/HeJ, H-2k) (A/J, H-2a). Cloned L2, Fa13, Fa15, and C14 cells, therefore, appear to react towards Mls determinants independently of H-2 as there is no cell proliferation when cultured with syngeneic Mls spleen cells.

The Mls reactivity of the noncytolytic cell lines was further investigated with representative L2 cells. Because Mls determinants are expressed primarily on B cells
FIG. 4. Mls reactivity of the noncytolytic L2 cell clone. L2 cells (10⁴), obtained 7-8 d after previous transfer, were cultured in microwells that contained 10⁶ irradiated B6 spleen cells, DBA/2 spleen cells, BDF₁ spleen cells, DBA/2 T cells, or T cell-depleted DBA/2 spleen cells, and 33% LCA SF or culture medium. After 3 d of culture, each culture was pulsed with 1 μCi [³H]TdR for 4 h, then the cells collected onto filter-paper strips and counted. Results shown are from a representative experiment repeated twice and are expressed as mean disintegrations per minute ± SEM for triplicate cultures.

(27), L2 cells were cultured with nylon-wool-enriched T cells, T cell-depleted spleen cells, or whole DBA/2 spleen cells. Peak MLR responses are shown in Fig. 4. Compared with cultures of L2 cells prepared with B6 spleen cells, significant proliferative responses were observed when L2 cells were cultured with whole or T cell-depleted DBA/2 spleen cells (stimulation index of 33 and 34, respectively), but not when cultured with DBA/2 T cells (stimulation index of 1.3). The addition of syngeneic adherent cells to these cultures did not reconstitute the proliferative response of L2 cells (data not shown). The culture of L2 cells with DBA/2 T cells in the presence of LCA SF, however, increased [³H]TdR incorporation 88-fold, which indicates the ability of the irradiated DBA/2 T cell-enriched stimulating cells to function as feeder cells. The proliferation of L2 cells was not a result of backstimulation by the irradiated stimulating cells because L2 cells proliferated equally well whether cultured with BDF₁ spleen cells, T cell-depleted DBA/2 spleen cells, or with whole DBA/2 spleen cells. The noncytolytic cell lines, therefore, appear to proliferate specifically in response to Mls stimulation.

Specificity of Cytolytic Cell Lines. The cytolytic specificity of the cloned cell lines was determined by lysis of spleen cell Con A blasts and tumor target cells bearing different alloantigens. Several patterns of reactivity were observed (Table I). Cloned L3 and A6 cells lysed target cells bearing H-2D<sup>d</sup>, whereas cloned B18 and C25 cells lysed target cells bearing H-2K<sup>d</sup>. Cell line C25 also lysed H-2<sup>k</sup> tumor cells (AKR-A), but not H-2<sup>k</sup> Con A blasts, which suggests that the lysis of AKR-A tumor cells by C25 cells is not a result of H-2<sup>k</sup> reactivity. Cell lines L3, B18, and C25 lysed 50% of 10,000 P-B15 target cells bearing the original stimulating alloantigens (H-2<sup>d</sup>) at effector:target cell ratios of 0.3, 0.5, and 1.7, respectively.

In contrast, several cloned cell lines express cytolytic activity not specifically...
| Target cells | H-2 | B6 anti-DBA/2 origin | A/J anti-DBA/2 origin |
|--------------|-----|----------------------|----------------------|
|              | K   | I   | S   | D   | L3 | B18 | A2 | A4 | A6 | B6 | B8 | G14 | G20 | C25 | C16 | E11 |
| Mouse        |     |     |     |     |    |     |    |    |    |    |    |     |     |     |     |
| P815 mastocytoma | d   | —   | —   | d   | +++* | ++++ | —  | +++ | ++++ | ++++ | ++++ | ++± | ++++ | ++± | —   | —   |
| B10.D2 Con A blast | d   | d   | d   | d   | ++++ | ++++ | —  | —   | —   | —   | —   | —   | —   | —   | —   |
| B10.A Con A blast | k   | k   | d   | d   | ++++ | —   | —   | ++± | ++++ | —   | ++± | ++± | ++± | ++± | —   | —   |
| AKR-A lymphoma | k   | —   | k   | k   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   |
| B10.BR Con A blast | k   | k   | k   | k   | —   | —   | —   | ++± | —   | —   | —   | —   | —   | —   | —   |
| B10.AL Con A blast | k   | k   | k   | d   | ++++ | —   | —   | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| B10.OH Con A blast | d   | d   | d   | k   | —   | ++± | —   | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| RBL3 lymphoma | b   | —   | —   | b   | —   | +   | —   | —   | —   | —   | —   | —   | —   | —   | —   |
| Rat          |     |     |     |     |    |     |    |    |    |    |    |     |     |     |     |
| Lewis Con A blast Ag-B1 | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | —   | +   |
| BN Con A blast Ag-B3 | —   | —   | —   | ND | ++± | —   | —   | ND | ++± | —   | —   | —   | —   | —   | —   |

* Percent specific lysis during a 3.5-h $^{51}$Cr release assay at an effector:target cell ratio of 10:1. ++++ ≥ 75%; +++ ≥ 50%; ++ ± 25%; + ≥ 10%; — < 10%.

‡ ND, not done.
ANDREW L. GLASEBROOK AND FRANK W. FITCH

TABLE II

Expression of Cytolytic Activity Is Amplified by Antigen-Specific Stimulation of Amplifier T Cells

| Stimulator spleen cells | H-2 | Mls | Responding cells per culture | Amplification |
|-------------------------|-----|-----|-------------------------------|---------------|
|                         |     |     | L2 | L3 | L2 + L3 |                  |
| B6                      | b   | b   | 0.0* | 1 | 3 | 3‡            |
| C3H/HeJ                 | k   | c   | 0.0 | 1 | 4 | 4             |
| AKR/J                   | k   | a   | 0.0 | 2 | 104 | 52           |
| BALB/c                  | d   | b   | 0.0 | 7 | 12 | 2             |
| DBA/2                   | d   | a   | 0.0 | 6 | 124 | 21            |

L2 cells and/or L3 cells (1.6 × 10⁶) were cultured with 6 × 10⁶ irradiated (1,400 rad) spleen cells in 1.6 ml total culture medium. After 5 d of incubation, cytolytic activity per culture was measured in a 3.5-h ⁵¹Cr release assay with P-815 tumor target cells. Results are shown from a representative experiment repeated four times.

* Lytic units per culture.
‡ Ratio of lytic units per culture determined for cultures of L2 + L3 cells compared with lytic units for cultures of L3 cells alone.

directed towards H-2K² or H-2D⁴ alloantigens. Cell lines A4, B8, G14, and E11 lysed both H-2k and H-2d target cells. Cell line G20 lysed syngeneic RBL5 tumor target cells as well as all other target cells, whereas cell lines A2, B6, and C16 lysed only tumor target cells and not the equivalent Con A blasts. Two of the cell lines, G14 and G20, lysed both Lewis and BN rat spleen cell Con A blasts in addition to several murine target cells.

Amplifier T Cell Augmentation of the Cytolytic Response. Cloned noncytolytic and cytolytic cell lines were cultured together with irradiated spleen cells in the absence of SF to study the possible amplifier activity of the noncytolytic cell clones. Table II shows the results obtained in a representative experiment in which cloned noncytolytic L2 cells and cytolytic L3 cells, both of B6 origin, were cultured with different stimulating spleen cells. Cytolytic activity per culture was measured with P-815 (H-2d) target tumor cells because the expression of cytolytic activity by cloned L3 cells is directed towards H-2D⁴ alloantigens.

Cloned L3 cells cultured with only irradiated spleen cells expressed low levels of cytolytic activity, which was greatest in cultures that contained H-2d spleen cells. However, cultures of L2 and L3 cells together with irradiated spleen cells bearing Mls° antigen (AKR/J, DBA/2), which stimulate proliferation of L2 cells, developed high levels of cytolytic activity (amplification from 20.7 to 57.7 times). Both proliferation of L2 cells and amplification of cytolytic activity was dependent upon stimulation by Mls antigens rather than by antigens of the major histocompatibility complex (MHC). Culture of L2 and L3 cells with irradiated spleen cells (B6, BALB/c, C3H/HeJ) that did not stimulate proliferation of L2 cells resulted in low levels of cytolytic activity per culture (amplification from 1.7 to 3.8 times). Comparable results were obtained when L3 cells, before culture with L2 cells, were maintained with syngeneic spleen cells to avoid the transmission of allogeneic stimulating cell antigens.

Regardless of the H-2 haplotype of the stimulating spleen cells, the cytolytic specificity of L3 cells was always directed against H-2D⁴ alloantigens (data not shown). Proliferation of cloned L2 cells stimulated by the appropriate Mls alloantigen can, therefore, amplify the expression of cytolytic activity by cultures that contain cloned cytolytic L3 cells. Judged by their ability to promote L3 cell proliferation, several other cloned
noncytolytic T cell lines (Fa13, Fa15, C14) that function as amplifier cells have been identified; all noncytolytic amplifier T cell clones isolated thus far proliferate in response to Mls stimulation.

**Irradiation Sensitivity of Cell Lines.** Cloned T cell lines L2 and L3 were cultured separately or together with irradiated DBA/2 alloantigen in the presence or absence of LCA SF. In some cases, L2 cells or L3 cells were exposed to 1,500 rad of gamma radiation before the initiation of culture. Peak MLR and cytolytic responses obtained in a representative experiment are shown in Table III. In the absence of LCA SF, L2 cells cultured with alloantigen proliferated but did not express cytolytic activity, whereas the inclusion of LCA SF increased the MLR response 1.6-fold. Depending upon whether LCA SF was included or not, irradiation of L2 cells reduced the MLR responses 96 and 97%, respectively. L3 cells cultured with alloantigen alone did not proliferate and expressed only low levels of cytolytic activity, whereas the addition of LCA SF to cultures that contained L3 cells and alloantigen increased the MLR and cytolytic responses 208- and 192-fold, respectively. Irradiated L3 cells, however, did not proliferate or express cytolytic activity when cultured with alloantigen and LCA SF.

When L3 cells were cultured with L2 cells and alloantigen, there was a 29-fold increase in cytolytic activity per culture compared with L3 cells cultured with alloantigen alone. In such cultures prepared with irradiated L2 cells, the cytolytic response of L3 cells was increased 14-fold. No demonstrable cytolytic activity developed in such cultures when L3 cells, rather than L2 cells, were irradiated. The proliferative response measured in cultures that contained L2 cells, L3 cells, and alloantigen was equivalent to the sum of the proliferative responses observed in

### Table III

| Responding cells | LCA SF | $[^3]H$Tdr incorporation* | Lytic units per culture‡ |
|------------------|--------|--------------------------|-------------------------|
| L2               |        | 23,186 ± 1,724           | 0.0                     |
| L2 (1,500 rad)   | ±      | 59,523 ± 5,918           | 0.0                     |
| L3               |        | 654 ± 66                 | ND                      |
| L3 (1,500 rad)   | +      | 2,343 ± 88               | ND                      |
| L2 + L3          |        | 668 ± 48                 | 8 ± 0.4                 |
| L2 + L3 (1,500 rad) |    | 139,600 ± 4,749          | 1,486 ± 146             |
| L2 + L3 (1,500 rad) |    | 453 ± 47                 | 0.01                    |
| L2 + L3 (1,500 rad) |    | 413 ± 38                 | 0.01                    |

* L2 and/or L3 cells (10^4; ± 1,500 rad) were cultured with 10^6 irradiated (1,400 rad) DBA/2 spleen cells in 0.3 ml total medium. After 3 d of incubation, cultures were pulsed with 1 μCi of $[^3]H$Tdr for 4 h. Results are expressed as mean disintegrations per minute ± SEM for triplicate cultures.

‡ L2 cells and/or L3 cells (1.6 × 10^4; ± 1,500 rad) were cultured with 6 × 10^6 irradiated (1,400 rad) DBA/2 spleen cells in a total of 1.6 ml culture medium. After incubation for 5 d at 37°C, cytolytic activity per culture was measured in a 3.5-h $^{10}$Cr release assay with P-815 tumor target cells. Results are expressed as mean lytic units per culture ± SEM for triplicate cultures.
ANDREW L. GLASEBROOK AND FRANK W. FITCH

TABLE IV
Amplifier Cell Help Is Mediated by Soluble Factors

| Culture vessel* | Upper chamber | Lower chamber | Lytic units per culture |
|-----------------|---------------|---------------|-------------------------|
| 1               | DBA           | L3/DBA        | 0                       |
| 2               | L2/DBA        | L2 + L3/DBA   | 185                     |
| 3               | L2/DBA        | L3/DBA        | 135                     |
| 4               | L3/DBA        | L2/DBA        | 0                       |
| 5               | L3/LCA SF     | DBA           | 152                     |

* Cultures were established in Marbrook culture vessel chambers separated by a 0.2 μm membrane filter. L2 and/or L3 cells were cultured with irradiated DBA/2 spleen cells for 5 d, after which time the cells were collected and cytolytic activity per chamber was measured in a 3.5-h 51Cr release assay with P-815 tumor target cells. Where indicated, LCA SF was included at a final concentration of 33%. Results are shown from a representative experiment repeated three times.

cultures prepared with irradiated L2 cells, normal L3 cells, and alloantigen and cultures prepared with normal L2 cells, irradiated L3 cells, and alloantigen. The cloned noncytolytic L2 cells, therefore, function as amplifier T cells that, when cocultured with cloned cytolytic L3 cells and alloantigen, stimulate L3 cell proliferation with a concomitant increase in the expression of cytolytic activity per culture. The proliferation of L2 cells is sensitive to irradiation, but the L2 cell amplifier function is radioresistant. Both proliferation and expression of cytolytic activity by L3 cells are radiosensitive. To determine whether L2 cell amplification of the L3 cell cytolytic response was mediated via soluble factors or cell-cell interactions, we measured cytolytic activity when L2 cells and L3 cells were physically separated in Marbrook culture vessels.

Mode of Amplification. Various combinations of L2 cells, L3 cells, and alloantigen were cultured in Marbrook culture chambers separated by a 0.2-μm membrane filter. After 5 d of culture, the contents of each chamber were assayed for cytolytic activity. Table IV shows the lytic units per chamber observed for various combinations of L2 and L3 cells with alloantigen. The culture of L3 cells with L2 cells and alloantigen in the lower chamber (culture 2) resulted in a 61-fold increase in cytolytic activity compared with L3 cells cultured with alloantigen alone (culture 1). The culture of L2 cells with alloantigen in the upper chamber, however, resulted in a 44-fold increase in the expression of cytolytic activity by L3 cells cultured with alloantigen in the lower chamber (culture 3). Reversing the location of cells, the culture of L3 cells in the upper chamber and L2 cells in the lower chamber, both with alloantigen, also resulted in a significant increase in the expression of cytolytic activity by L3 cells (culture 4). The integrity of the membranes in these experiments was shown by the fact that cytolytic activity could not be demonstrated in the lower chamber of culture 4, which indicates that L3 cells did not leak downwards, nor L2 cells upwards. Whereas L3 cells require splenic feeder cells for proliferation and the expression of cytolytic activity...
INTERACTIONS BETWEEN CLONED T CELL LINES

Table V

Comparison of SF

| Responding cell | SF | T cell-depleted stimulator cells | Lytic units per culture |
|-----------------|----|---------------------------------|-------------------------|
| L3              | Culture medium     | +                              | 2                       |
|                 | MLC SF             | +                              | 227                     |
|                 | LCA SF             | +                              | 453                     |
|                 | L2 SF*             | +                              | 48                      |
| L3              | Culture medium     | +                              | 2                       |
|                 | L2 SF‡             | +                              | 16                      |
|                 | L2 SF‡             | −                              | 1                       |

L3 cells (1.6 × 10⁶) were cultured with 6 × 10⁶ irradiated (1,400 rad) T cell-depleted DBA/2 spleen cells and 33% 48-h MLC SF, LCA SF, L2 SF, or an equivalent volume of culture medium, in a total vol of 1.6 ml. Cytolytic activity per culture was measured after 5 d of incubation in a 3.5-h ⁵¹Cr release assay with P-815 tumor target cells. Results are shown from two representative experiments repeated two times each.

* L2 SF collected from cultures of L2 cells prepared with irradiated DBA/2 spleen cells.
‡ L2 SF collected from cultures of L2 cells prepared with irradiated T cell-depleted BDF₁ spleen cells.

(20), culture 5 shows that direct interaction between feeder cell and L3 cell is not required. Rather, this effect is mediated by soluble factors. Therefore, cultures of L2 cells stimulated by Mls antigens secrete soluble factor(s) that amplify the proliferation and expression of cytolytic activity by L3 cells.

This was further shown by the ability of SF collected from cultures of L2 cells prepared with irradiated DBA/2 spleen cells to amplify the L3 cell cytolytic response (Table V). L2 SF was collected after 48 h of culture and compared with the ability of 48-h MLC SF and LCA SF, and culture medium, to enhance the expression of L3 cell cytolytic activity when cultured with T cell-depleted alloantigen. The data from a representative experiment is shown in Table V. The addition of L2 SF to cultures of L3 cells with alloantigen resulted in a 23-fold increase in cytolytic activity per culture compared with the addition of culture medium. The addition of MLC SF or LCA SF resulted in 112- and 225-fold increases in cytolytic activity, respectively. The differences in amplification of cytolytic activity produced by the different SF may relate to the number of cells required for SF production. The MLC SF and LCA SF were produced by 1.8 × 10⁶ or 12.5 × 10⁶ responding cells, respectively, whereas an equivalent amount of L2 SF was produced by only 10⁵ L2 cells. L2 cell cultures, therefore, secrete soluble amplifying factor(s) upon specific Mls stimulation.

SF was also collected from cultures of L2 cells (Lyt-1⁻) prepared with irradiated T cell-depleted BDF₁ spleen cells (Table V). In a different series of experiments, such 48-h L2 SF in the presence of irradiated T cell-depleted DBA/2, spleen cells were effective in promoting L3 cell proliferation (sevenfold increase), which indicates that the L2 cell amplifying factor did not act through or require an Lyt-1⁺ T cell present in the stimulating cell population. Therefore, an Lyt-1⁺ T cell is not necessarily required for augmentation of cytolytic activity, although the possibility that L2 cells may have lost the ability to express Lyt-1 antigens with continuous in vitro culture
Table VI

Interactions Between Amplifier and Cytolytic Cell Lines Are Nonspecific

| Amplifier cell | Cytolytic cell | Lytic units per culture |
|----------------|----------------|-------------------------|
| L2 (B6, Lyt-1⁻) | —              | 0.0                     |
| Fa13 (B6, Lyt-1⁺) | —              | 0.0                     |
| Fa15 (B6, Lyt-1⁺) | —              | 0.0                     |
| C14 (A/J) | —              | 0.0                     |
| — | L3 (B6, Lyt-2⁺) | 7 ± 0.6                 |
| L2 | L3              | 393 ± 68                |
| Fa13 | L3             | 251                     |
| Fa15 | L3              | 166 ± 27                |
| C14 | L3              | 238 ± 24                |
| — | B18 (B6, Lyt-2⁺) | 1.6 ± 0.3               |
| L2 | B18             | 23 ± 2                  |
| Fa13 | B18            | 48                      |
| Fa15 | B18             | 36 ± 1                  |
| C14 | B18             | 66 ± 5                  |
| — | C25 (A/J)      | 0.1                     |
| L2 | C25             | 4.1 ± 0.7               |
| Fa15 | C25            | 5.2 ± 0.9               |
| C14 | C25             | 4.6 ± 0.5               |

Cloned amplifier and/or cytolytic cells (1.6 × 10⁴) were cultured with 6 × 10⁶ irradiated (1,400 rad) DBA/2 spleen cells in 1.6 ml total culture medium. After 5 d of incubation, cytolytic activity per culture was measured in a 3.5-h ⁵¹Cr release assay with P-815 (H-2⁵) tumor target cells. Results are expressed as mean lytic units per culture ± SEM for triplicate cultures.

The expression of cytolytic activity in cultures that contained cytolytic cell lines were increased >14-fold by the inclusion of noncytolytic amplifier cell lines. The cytolytic activity expressed by each cell line remained specific for H-2D<sup>d</sup> or H-2K<sup>d</sup> alloantigens, whether B6- or A/J-derived amplifier cell lines were included (data not shown). Amplifier cell help, therefore, is not antigen specific because individual amplifier cell lines could augment equally well the expression of cytolytic activity by...
H-2D<sup>a</sup> (L3)- or H-2K<sup>d</sup> (B18, C25)-reactive cytolytic cell lines. Also, amplifier cell help was not H-2 restricted because B6-derived amplifier cell lines (L2, Fa13, Fa15) could augment the expression of cytolytic activity by an A/J-derived cytolytic cell line (C25), and vice versa. The expression of Lyt-1 alloantigens on the amplifier cell surface was not necessary for cooperation with cytolytic cell lines in the expression of cytolytic activity. L2 cells (Lyt-1<sup>-</sup>) were as effective as Fa15 cells (Lyt-1<sup>+</sup>) in amplifying the cytolytic response by cultures that contained cytolytic cell lines. The amplification of cytolytic activity by the cloned amplifier cell lines is, therefore, neither antigen specific nor H-2 restricted nor Lyt-1 dependent.

**Discussion**

The present study confirms previous reports that amplifier T cells can augment the expression of cytolytic activity in MLC (1–3), and extends those observations with cloned T cell lines rather than heterogeneous cell populations. Our results demonstrate that amplifier cell lines that are stimulated by Mls determinants secrete soluble factor(s), which, in turn, induce the proliferation of cytolytic cell lines reactive toward the original sensitizing H-2 alloantigens, thereby amplifying the expression of cytolytic activity per culture. Functionally, the soluble amplifying factors are neither antigen specific nor H-2 restricted and are able to stimulate the proliferation of Lyt-2<sup>+</sup> cytolytic cell lines in the absence of specific alloantigen. These results suggest a pathway in which products derived from amplifier cells responsive to Mls determinants produce polyclonal activation of primed CTL.

The antigens that stimulate proliferation of the cloned amplifier T cell lines in the absence of SF appear to be Mls determinants, which are known to induce strong MLR responses (28). It is somewhat surprising that none of the four amplifier cell lines described are reactive towards H-2 alloantigens because cell populations that contain amplifier T cells are capable of augmenting the cytolytic response when stimulated by H-2 alloantigens in MLC (1, 2, 4). Both of the strain combinations used in our studies, A/J anti-DBA/2 and C57 anti-DBA/2, involved H-2K<sup>+</sup> I or entire H-2 differences, respectively, as well as incompatibilities at the Mls. The Mls, which is not linked to H-2 or closely linked to any other histocompatibility locus, has four codominant determinants; Mls<sup>a</sup>d are strongly stimulatory in vitro, Mls<sup>e</sup> is much weaker, and Mls<sup>b</sup> may be nonstimulatory (28). The strongly stimulating Mls determinant, Mls<sup>a</sup>, is expressed on DBA/2 spleen cells and was involved in sensitization of the responding cells in vitro. A possible explanation is that our culture conditions for MLC sensitization of cells may not favor the development of H-2-reactive amplifier cell clones. Attempts to augment the expression of cytolytic activity toward H-2K antigens by including I-region stimulation have not succeeded with culture conditions similar to ours (29). An important difference here may be our use of FCS instead of the homologous mouse serum used by others in studying H-2 proliferative responses (30). Also, the kinetics of the proliferative responses toward Mls and H-2 alloantigens may be different, so that at the time of cloning there were greater numbers of Mls-responsive cells.

A significant degree of cross-reactivity among Mls alloantigens was observed when the amplifier cell lines were cultured with spleen cells different from the Mls allotype of the original stimulating cell. The most consistent cross-reactivity in terms of cell proliferation occurred with Mls<sup>d</sup>. Little or no cross-reactivity occurred with Mls<sup>b</sup> or
Mls<sup>a</sup> antigens with the exception of cell line C14, which exhibited strong MLR responses when cultured with Mls<sup>b</sup> spleen cells from strains other than B6. Similar findings have been reported by Peck et al. (31), who observed strong secondary MLR responses when purified T blast cells from primary MLC were cultured with spleen cells not sharing the Mls allele carried by the original stimulating spleen cells. On the basis of the observed MLR responses, we conclude that Mls determinants, like H-2 alloantigens, are cross-reactive as a result of shared antigenic specificities that the amplifier cell clones recognize. The inability of B6 spleen cells (Mls<sup>b</sup>) to stimulate proliferation of cell line C14 may be a result of the lack of expression on the B6 cells of the particular Mls antigenic specificity recognized by C14 cells.

The specificity of the cytolytic cell lines was quite varied. Several cell lines were specific for the H-2D- or H-2K-region alloantigens of the original stimulating cell. Other cell lines lysed target cells bearing different H-2 alloantigens. In some cases, this could be demonstrated only by using tumor target cells, whereas Con A blast target cells bearing comparable H-2 antigens were not lysed. Because many of the tumor target cells commonly used express viral antigens, the expression of new or altered antigenic structures on tumor cell surfaces may explain some of these results because allosensitized CTL can be cross-reactive towards allo-determinants and modified-self determinants (32). It is of interest that the antigenic determinants recognized by two cytolytic cell lines were expressed on both rat and mouse spleen cells. McKearn et al. (33) have reported similar cross-reactivity in these species with monoclonal antibodies directed against rat Ag-B antigens. Final determination of the antigenic specificities recognized by the cell lines will be accomplished by selective blocking of target cell lysis with antisera directed against known public and private specificities.

It has been proposed that two signals are necessary for the induction of primary CTL responses in vitro. The first signal is provided by interaction of receptors on Lyt-2<sup>+</sup> CTL precursors with sensitizing alloantigens, which are coded for primarily, but not exclusively, by the K and D regions of the MHC. A second signal is provided by a nonspecific stimulus that is delivered by amplifier cells (Lyt-1<sup>+</sup>) activated, perhaps, by I-region determinants. The demonstration that nonspecific SF derived from MLC are able to amplify primary CTL responses is consistent with a two-signal model that involves soluble factors (5-12). In contrast, the second signal alone appears to be sufficient for the activation of secondary CTL responses (9-11). SF collected from mitogen- or antigen-stimulated spleen cell cultures are able nonspecifically to trigger cell proliferation and cytolytic activity of cells from secondary MLC in the absence of the original sensitizing alloantigen (9-11). By using this approach, it has been possible to maintain continuously proliferating cytolytic T cell lines in vitro (15-17).

Our results are consistent with the view that specific alloantigen (signal 1) is not essential for proliferation of CTL. Cloned cytolytic L3 cells, specific for H-2D<sup>4</sup> alloantigens, proliferated and concomitantly expressed specific cytolytic activity when cultured with cloned amplifier L2 cells and irradiated third-party CBA/J (H-2<sup>k</sup>) spleen cells. In this combination, the CBA/J spleen cells served as a source of Mls alloantigen that stimulated proliferation of L2 cells and the release of SF (signal 2). Levels of cytolytic activity were the same if L3 cells, before culture, were maintained with syngeneic spleen cells to avoid transmission of antigenic material that could be derived from allogeneic stimulating cells. These findings are in agreement with those
of Ryser et al. (9) and Wagner and Rollinghoff (11) who found that trypsin-treated alloimmune CTL responded to mitogen- or MLC-derived factors (signal 2) in the absence of alloantigen (signal 1) as readily as untreated cells. Immune T cells can, therefore, be stimulated to proliferate with the expression of cytolytic activity in the absence of signal 1 (alloantigen).

The amplifying factor(s) collected from cultures of cloned amplifier cells and irradiated stimulating alloantigen appear to be functionally similar to the murine lymphokines reported by Ryser et al. (9) and Wagner and Rollinghoff (11) that promote cytolytic responses by secondary MLC cells in a polyclonal manner. These lymphokines, as well as the amplifying factor delivered by our cell lines, are neither antigen specific nor strain specific, and do not require specific alloantigen for the stimulation of immune T cells. However, our cloned cytolytic T cell lines are unlike those reported by other investigators in that they do not proliferate when cultured alone with amplifying SF (15–17, 19); irradiated splenic feeder cells are nonspecifically required in addition (20). Although culture with the original stimulating alloantigen and SF results in the greatest stimulation (20), spleen cells of all H-2 haplotypes tested supported the growth of the cytolytic cell lines when cultured with LCA SF (data not shown). Recent experiments would indicate that adherent accessory cells present in splenic feeder cell populations supply nonspecific labile factors that function together with amplifier cell-derived factors to stimulate proliferation of the cytolytic cell lines (C. T. Lutz. Manuscript in preparation.).

Why our T cell lines should require SF and feeder cells for growth, whereas the other reported T cell lines require only SF, is unclear. In contrast to our method for deriving cloned cell lines, others (16, 19) have maintained bulk MLC cells with LCA SF alone and then cloned CTL after continued passaging in vitro. Maintenance of bulk MLC cells with LCA SF alone may provide selective pressure that favors those cell types that respond to LCA SF stimulation in the absence of feeder cells.

Whereas the amplifying factor(s) delivered by the cell lines are neither antigen specific nor strain specific, antigen-specific stimulation of the amplifier cell lines is required for factor production. Our results show that the stimulus for production of amplifying factors can not be confined solely to I region-encoded antigens. I-region antigens of the MHC have been convincingly shown to augment cytolytic responses in vitro via stimulation of Lyt-1+ amplifier T cells (1, 2, 7, 11). Our amplifier cell lines, which were derived from MLC involving H-2 and Mls differences but are reactive towards Mls antigens, clearly demonstrate that Mls antigens can function in the augmentation of CTL responses. The data also establish that amplifier cell surface expression of Lyt-1 alloantigens is not required for augmentation of cytolytic activity. Whereas several systems in which amplification of cytolytic activity has been described involved MHC differences (1, 6–8, 11), many involved stimulation by MHC as well as Mls antigens (3, 9, 11, 12). Also, antigen stimulation is not essential for amplifying factor production because mitogenic stimulation of Lyt-1+ spleen cells can result in lymphokine production (11, 12, 34). The nature of the stimulating antigens responsible for amplifier effects can be determined conclusively only by using amplifier T cell clones. The isolation of amplifier cell clones, all of which are reactive toward Mls antigens, was a fortuitous event related to the particular experimental conditions employed, and we do not presume that only Mls antigens stimulate the amplification of cytolytic activity. Therefore, we are working to establish amplifier cell clones that are MHC reactive.
Cell clones isolated from immune responses will be very useful in characterizing those responses in terms of the participating cell types, the antigens to which they respond, and the soluble factors involved. The derivation of cell clones will also facilitate the manufacture of monoclonal antibodies directed against cell surface proteins and serve as useful probes for analysis of the receptor molecules involved in specific immune interactions.

Summary

Several T cell clones have been derived by limiting dilution of secondary mixed leukocyte culture cells stimulated by H-2- and M locus (Mls)-disparate spleen cells. When examined for the expression of cytolytic activity and the ability to proliferate, these cell clones can be classified into two major categories. One type of cell is noncytolytic; when cultured with irradiated spleen cells, such clones proliferate in response to Mls determinants. Some, but not all, of these clones express Lyt-1 alloantigens. The other type of cell is cytolytic; these clones do not proliferate when cultured with irradiated allogeneic spleen cells unless supernatant fluid (SF) is added. These cytolytic clones express Lyt-2 alloantigens. Some cytolytic clones are specific for H-2K<sup>d</sup> and others for H-2D<sup>d</sup> alloantigens. Still other cytolytic cell clones exhibit cross-reactive lysis of different H-2-bearing tumor and Con A blast target cells. Noncytolytic T cell clones, when stimulated by Mls antigens, were examined for their ability to promote proliferation of cytolytic T cell clones. All of the noncytolytic cell clones tested were able to promote proliferation of cytolytic cell clones with the concomitant expression of cytolytic activity directed toward the original stimulating alloantigen (H-2<sup>d</sup>). Amplification of cytolytic activity was dependent upon stimulation of the noncytolytic amplifier T cell clones by Mls antigens. Specific alloantigen (signal 1), however, was not required for proliferation of the cytolytic cell clones; the amplifying signal (signal 2), delivered by the amplifier cell clones, was sufficient alone to promote proliferation of the cytolytic cell clones. Whereas proliferation of the amplifier cells was radiosensitive, the generation of the soluble amplifying signal was radioresistant. Amplification of cytolytic activity was observed when either amplifier cells were physically separated from responding cytolytic cells in Marbrook cultures or when cytolytic cells were cultured with SF collected from amplifier cell cultures. The amplifying factors were neither antigen specific nor strain specific and could be produced by Lyt-1<sup>−</sup> cells. The availability of cloned T cell lines that retain specific biologic function offers unique opportunities to characterize cell surface proteins and cell-cell interactions.

The authors wish to thank Dr. Donald C. Shreffler for providing congenic mice and Dr. Harvey Cantor for providing both congenic mice and Lyt antisera. We are indebted to Dr. Gary Nabel and Dr. Michael Loken for determining the Lyt phenotypes of the cell lines, to John Ely for karyotype analysis, and to Charles Lutz for nylon-wool enrichment of T cells. The excellent technical assistance of Yukio Hamada, Laverne Decker, Daisy Freeman, and Francis Mills is gratefully acknowledged.

Received for publication 1 November 1979 and in revised form 4 January 1980.

References

1. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly<sup>+</sup> cells in the generation of killer activity. J. Exp. Med. 141:1390.
INTERACTIONS BETWEEN CLONED T CELL LINES

2. Bach, F. H., M. L. Bach, and P. M. Sondel. 1976. Differential function of major histocompatibility complex antigens in T-lymphocyte activation. Nature (Lond.). 259:273.

3. Pilarski, L. M. 1977. A requirement for antigen-specific helper T cells in the generation of cytotoxic T cells from thymocyte precursors. J. Exp. Med. 145:709.

4. Nagy, Z., B. E. Elliott, and M. Nabholz. 1976. Specific binding of K- and I-region products of the H-2 complex to activated thymus-derived (T) cells belonging to different Ly subclasses. J. Exp. Med. 145:1545.

5. Altman, A., and I. R. Cohen. 1975. Cell-free media of mixed lymphocyte cultures augmenting sensitization in vitro of mouse T lymphocytes against allogeneic fibroblasts. Eur. J. Immunol. 5:437.

6. Plate, J. M. D. 1976. Soluble factors substitute for T-T-cell collaboration in generation of T-killer lymphocytes. Nature (Lond.). 260:329.

7. Bach, F. H., C. Grillot-Courvalin, O. J. Kupperman, H. W. Sollinger, C. Hayes, P. M. Sondel, and M. L. Bach. 1977. Antigenic requirements for triggering of cytotoxic T lymphocytes. Immunol. Rev. 35:76.

8. Finke, J. H., C. G. Oroz, and J. R. Battisto. 1977. Splenic T-killer cells can be generated by allogeneic thymic cells in conjunction with assisting factor. Nature (Lond.). 267:335.

9. Ryser, J.-C., J.-C. Cerottini, and K. T. Brunner. 1978. Generation of cytolytic T lymphocytes in vitro. IX. Induction of secondary CTL responses in primary long-term MLC by supernatants from secondary MLC. J. Immunol. 120:370.

10. Uotila, M., H. N. Rode, and J. Gordon. 1978. Blastogenic factor: its role in the mixed leukocyte reaction. Eur. J. Immunol. 8:133.

11. Wagner, H., and M. Rollinghoff. 1978. T-T-cell interactions during in vitro cytotoxic allograft responses. I. Soluble products from activated Lyt+ T cells trigger autonomously antigen-primed Ly23+ T cells to cell proliferation and cytolytic activity. J. Exp. Med. 148:1523.

12. Okada, M., G. R. Klimpel, R. C. Kuppers, and C. S. Henney. 1979. The differentiation of cytotoxic T cells in vitro. I. Amplifying factor(s) in the primary response is Lyt 1+ cell dependent. J. Immunol. 122:2527.

13. Goldsby, R. A., B. A. Osborne, E. Simpson, and L. A. Herzenberg. 1977. Hybrid cell lines with T-cell characteristics. Nature (Lond.). 267:707.

14. Taussig, M. J., J. R. F. Corvalan, R. M. Binns, and A. Holliman. 1979. Production of an H-2-related suppressor factor by a hybrid T-cell line. Nature (Lond.). 277:305.

15. Gillis, S., and K. A. Smith. 1977. Long-term culture of tumor specific cytotoxic T cells. Nature (Lond.). 268:154.

16. Nabholz, M., H. D. Engers, D. Collave, and M. North. 1978. Cloned T cell lines with specific cytolytic activity. Curr. Top. Microbiol. Immunol. 81:176.

17. Rosenberg, S. A., S. Schwarz, and P. J. Spiess. 1979. In vitro growth of murine T cells. II. Growth of in vitro sensitized cells cytotoxic for alloantigens. J. Immunol. 121:1951.

18. Dennert, G. 1979. Cytolytic and proliferative activity of a permanent T killer cell line. Nature (Lond.). 277:476.

19. Baker, P. E., S. Gillis, and K. A. Smith. 1979. Monoclonal cytolytic T-cell lines. J. Exp. Med. 149:273.

20. Glasebrook, A. L., and F. W. Fitch. 1979. T cell lines which cooperate in the generation of specific cytolytic activity. Nature (Lond.). 278:171.

21. Cerottini, J.-C., H. D. Engers, H. R. MacDonald, and K. T. Brunner. 1974. Generation of 117 cytotoxic T lymphocytes in vitro. I. Response of normal and immune spleen cells in mixed cytotoxic T lymphocytes in vitro. I. Response of normal and immune spleen cells in mixed leukocyte cultures. J. Exp. Med. 140:703.

22. Weiss, A., and F. W. Fitch. 1977. Macrophages suppress CTL generation in rat mixed leukocyte cultures. J. Immunol. 119:510.
23. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* 3:645.

24. Sauser, D., C. Anckers, and C. Bron. 1974. Isolation of mouse thymus-derived lymphocyte specific surface antigens. *J. Immunol.* 113:617.

25. Shen, F.-W., E. A. Boyse, and H. Cantor. 1975. Preparation and use of Ly antisera. *Immunogenetics.* 2:591.

26. Tartof, D., and F. W. Fitch. 1977. Immunologically specific cytolytic activity induced in long-term mixed leukocyte culture cells by concanavalin A. *J. Immunol.* 118:35.

27. von Boehmer, H., and J. Sprent. 1974. Expression of M locus determinants by B cells but not T cells. *Nature (Lond.)* 249:363.

28. H. Festenstein. 1973. Immunogenetic and biological aspects of *in vitro* lymphocyte allotransformation (MLR) in the mouse. *Transplant. Rev.* 15:62.

29. Engers, H. D., and H. R. MacDonald. 1975. Generation of cytolytic T lymphocytes in vitro. *Contemp. Top. Immunol.* 5:145.

30. Peck, A. B., and F. H. Bach. 1975. Mouse cell-mediated lympholysis assay in serum-free and mouse serum supplemented media: culture conditions and genetic factors. *Scand. J. Immunol.* 4:53.

31. Peck, A. B., H. Wigzell, C. Janeway, Jr., and L. C. Andersson. 1977. Environmental and genetic control of T cell activation *in vivo*. A study using isolated alloantigen-activated T cell clones. *Immunol. Rev.* 35:146.

32. Lemmonier, F., S. J. Burakoff, R. N. Germain, and B. Benacerraf. 1977. Cytolytic thymus-derived lymphocytes specific for allogeneic stimulator cells cross-react with chemically modified syngeneic cells. *Proc. Natl. Acad. Sci. U. S. A.* 74:1229.

33. McKearn, T. J., F. W. Fitch, D. E. Smilek, M. Sarmiento, and F. P. Stuart. 1979. Properties of rat anti-MHC antibodies produced by cloned rat-mouse hybridomas. *Immunol. Rev.* 47:91.

34. Watson, J., S. Gillis, J. Marbrook, D. Mochizuki, and K. A. Smith. 1979. Biochemical and biological characterization of lymphocyte regulatory molecules. I. Purification of a class of murine lymphokines. *J. Exp. Med.* 150:849.