AN ANTIGEN RECEPTOR-DRIVEN, INTERLEUKIN 2-INDEPENDENT PATHWAY FOR PROLIFERATION OF MURINE CYTOLYTIC T LYMPHOCYTE CLONES

BY RICHARD L. MOLDWIN, DAVID W. LANCKI, KEVAN C. HEROLD, AND FRANK W. FITCH

From the Committee on Immunology, and the Department of Pathology, University of Chicago, Pritzker School of Medicine, Chicago, Illinois 60637

According to a current model for T cell activation, stimulation of the T cell receptor for antigen (TCR)\(^1\) induces an increase in the density of cell surface IL-2-R (1, 2), as well as the secretion of lymphokines, including IL-2. Cellular proliferation occurs as the result of binding of the secreted IL-2 to the newly induced receptors (3). This model seems to apply directly to Th, but not to most CTL. Conventional CTL do not secrete IL-2; rather, they require exogenous IL-2 in addition to alloantigen for continued growth in culture (4). In mixed leukocyte cultures (MLC), Th secrete IL-2, which provides the signal for proliferation of both Th and IL-2-dependent CTL (5).

However, not all CTL are dependent upon IL-2 produced by Th. Several investigators have described cloned CTL that can proliferate in response to appropriate antigenic challenge in the absence of exogenous IL-2 (6–8). Some of these Th-independent CTL secrete IL-2, and for these CTL, the IL-2-driven model for T cell proliferation also appears to be applicable. However, other helper cell-independent CTL do not secrete detectable amounts of IL-2 (8). The nature of the proliferative signal for these cells has not been determined.

Recently in our laboratory, we have been able to activate some of our cloned lines of CTL in the absence of exogenous IL-2 by using either specific antigen in the form of T cell–depleted allogeneic spleen cells or P-815 mastocytoma cells, or by using immobilized mAbs directed against the TCR. The stimulated, cloned CTL synthesize DNA and enter into cell cycle. They secrete macrophage activating factor (MAF) after stimulation, but by several criteria they do not synthesize IL-2. The proliferative response of these CTL after stimulation of the TCR is not inhibited by anti–IL-2-R antibodies, which do block IL-2-induced

This research was supported by grants AI-04197, AI-18061, and CA-19266 from the National Institutes of Health, U. S. Public Health Service. R. Moldwin was supported by Medical Scientist Training Program Grant 5T32 GM-07281 from the U. S. Public Health Service. K. Herold was supported by Training Grant T32 AI-07090, U.S. Public Health Service. Address correspondence to Dr. Frank W. Fitch, Department of Pathology, Box 414, University of Chicago Hospitals and Clinics, 5841 S. Maryland Ave., Chicago, IL 60637.

\(^1\)Abbreviations used in this paper: DPBS, Dulbecco’s phosphate buffered saline solution; dpm, disintegrations per minute; MAF, macrophage activating factor; MLC, mixed leukocyte cultures; PI, propidium iodide; SF, supernatant fluid; TCR, T cell antigen receptor.
proliferation of these cells. Our observations indicate that for conventional CTL there exists a TCR-driven pathway for proliferation that is independent of IL-2.

Materials and Methods

Animals. C57BL/6, CBA, and DBA/2 mice, 6–8 wk of age were purchased from The Jackson Laboratory, Bar Harbor, ME.

Cell Lines. CTL clones L3 (4), dB45, and BC9 (9) were used; these cloned cells are Thy-1⁺, LFA-1⁺, Lyt-2⁺, and L3T4⁻. CTL clones L3, dB45, and BC9 are H-2Ld-reactive. Th clone L2 is Mls⁺-reactive and is Thy-1⁺, LFA-1⁺, Lyt-2⁻, and L3T4⁺ (4).

Cloned T lymphocytes were maintained routinely by weekly passage on allogeneic spleen cells (irradiated with 2,000 rad from a 137Cesium source) as a source of stimulating alloantigen, together with conditioned medium containing 5–10 U/ml of exogenous murine IL-2, either supernatants from secondary mixed lymphocyte cultures (10), or supernatants from PMA-stimulated EL-4 cells (EL-4 SF) (11). The cell lines were recloned at regular intervals by limiting dilution. Culture wells were harvested on the sixth, seventh, or eighth day of the culture cycle, and the cloned cells were isolated by Ficoll-Hypaque gradient centrifugation (12). These cells were washed twice and suspended in culture medium. ([DME, H-21; Gibco Laboratories, Grand Island, NY] containing 5% FCS [KC Biological, Inc., Lenexa, KS], 10 mM 3-[N-morpholino]propanesulfonic acid, 5 × 10⁻⁵ M 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, and additional amino acids [4, 13]).

The IL-2-dependent cell line CTLL-20 (14) (generously provided by K. Smith, Dartmouth Medical School, Hanover, NH) was used in the IL-2 assays. Several tumor lines were used in these studies. EL-4 is a T cell lymphoma of C57BL/6 origin which produces IL-2 when stimulated with PMA (10 ng/ml) (Sigma Chemical Co., St. Louis, MO) (11). AKR-A is a T cell lymphoma of AKR (H-2k) origin. P-815 mastocytoma cells are of DBA/2 origin and express K⁺ and L⁺ on the cell surface. P-815 cells do not express IL-2-R or secrete IL-2 (unpublished observations).

Monoclonal Antibodies. The origin and characterization of the mAbs used in these studies are listed in Table I. mAb 384.5 is a clonotypic antibody that reacts with the antigen receptor of CTL clone L3 (15). Hybridomas 3C7 and 7D4 were generously provided by T. Malek at the National Institutes of Health, Bethesda, MD (16–18), and hybridoma PC61.5 was generously provided by M. Nabholz of the Swiss Institute of Experimental Cancer Research (19); the mAbs secreted by these hybridomas react with different epitopes on the IL-2-R. Hybridoma KJ16-133 was generously provided by P. Marrack at the National Jewish Hospital, Denver, CO (20). Hybridoma F23.1 was generously provided by M. Bevan of the Scripps Clinic and Research Foundation, La Jolla, CA (21). mAb KJ16-133 and mAb F23.1 appear to react with the same allotypic determinant on the TCR. This determinant is expressed on CTL clone dB45 and on Th
clone L2. The two antibodies, mAb K16-133 and mAb F23.1, had the same functional effects on our cell lines and were used interchangeably. The other hybridomas were derived in this laboratory (22, 23). Hybridoma culture supernatants and ascites were used for the studies in this paper. mAb PC61.5 and mAb 7D4 were partially purified by ammonium sulfate precipitation, as previously described (24).

**Lymphokine Preparations.** Culture medium from PMA-stimulated EL-4 cells was the usual source of murine IL-2. Lymphokine-containing supernatants from various cloned CTL and Th were obtained by culturing $10^7$ cloned T cells with either immobilized mAb 584.5 or mAb F23.1 in 4 ml of culture medium for 3, 6, or 18 h. In other experiments, $5 \times 10^5$ cloned CTL and Th were cultured with either $3.3 \times 10^5$ mitomycin C-treated P-815 cells, $10^6$ DBA/2 splenocytes (irradiated 2000 rad from a $^{137}$Cesium source), or immobilized mAb 384.5 or mAb F23.1 for various periods of time in flat-bottomed microtiter wells (#3590; Costar, Cambridge, MA) in a total volume of 0.2 ml. The cells were then removed by centrifugation at 1500 g for 5 min and the supernatants were collected for assay of lymphokine titers.

**T Cell Depletion.** T cells were selectively removed from spleen cell populations by treating with monoclonal anti-Thy-1.2 antibody (AT83A) and rabbit complement (25). Briefly, $10^8$ spleen cells were incubated for 30 min at 4°C with 100 μl AT83A culture supernatant in a final volume of 4 ml. The cells were washed once and resuspended in a 1:3 dilution of agar-adsorbed rabbit serum that had been pretested for complement activity. After a 45 min incubation at 37°C, cells were washed twice before addition to culture. Cell recoveries ranged from 50–70%.

**Mitomycin C Treatment of Tumor Cells.** Tumor cells were inactivated by treating them with mitomycin C (Sigma Chemical Co.). Between $10^7–10^8$ P-815 or AKR-A tumor cells were centrifuged and resuspended in 1 ml Dulbecco’s phosphate buffered saline solution (DPBS) containing 200 μg/ml mitomycin C. The cells were then incubated in the dark at 37°C for 1 h, with occasional mixing. After the incubation, they were washed three times in culture medium containing 5% FCS before addition to cultures.

**Immobilization of mAbs in Culture Wells.** To immobilize various mAbs to the surface of microtiter tray wells, mAb-containing solutions (50–100 μl) were added to microtiter wells (#3596, Costar) and incubated at 4°C for 18–24 h, or at 37°C for 2–3 h. The solutions were then aspirated, and the wells were washed two times with DPBS before the addition of cloned T cells. Binding of the antibodies to the culture wells was monitored with an ELISA assay; all anti-TCR mAb used in these studies bound to microtiter wells (data not shown).

**Thymidine Incorporation Assay.** Thymidine incorporation by cloned T cells was measured by culturing various numbers of cloned T cells with various stimuli in flat-bottomed microtiter wells (#3590 or #3596, Costar). 12 or 18 h before harvest, 1 μCi [3H]thymidine (Amersham Corp., Arlington Heights, IL) in 0.025 ml culture medium was added to each well. Cultures were harvested onto glass fiber filter strips using an automated PhD cell harvester (Cambridge Technology, Inc., Cambridge, MA) and samples were counted in a liquid scintillation spectrometer (Nuclear Chicago, Chicago, IL). Counts were corrected from counts per minute to disintegrations per minute (dpm) by the channels ratio method. Thymidine incorporation is presented as the mean ± SEM of triplicate cultures.

**DNA Content.** The relative DNA content of CTL L3 cells was measured by analyzing the binding of the fluorescent dye, propidium iodide (PI), to DNA in isolated CTL L3 cell nuclei (26). CTL L3 cells ($10^7$) were suspended in 0.25 ml DPBS, and 2 ml of staining solution (5 mg PI, 100 mg sodium citrate, 0.1 ml Triton X-100 in 100 ml deionized water) were added. The cells were mixed with a vortex mixer for 15 s and incubated on ice for 15–30 min. The resulting nuclei from the lysed CTL L3 cells were then centrifuged (1500 g for 5 min), and resuspended in 1 ml of cold Isoton II/PI (5 mg PI in 100 ml Isoton II; Coulter Electronics Inc., Hialeah, FL). Histograms were recorded using a FACS analyzer (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

**Assay for IL-2.** IL-2 activity was determined by a modification of the standard procedures (27) by measuring relative amount of uptake of 3-(4,5-alpha, methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chemical Co.) by the IL-2-dependent
cell line CTLL-20 in a 24-h assay (28). We have determined that results obtained using this assay are comparable to results obtained by measuring thymidine incorporation in CTLL-20 cells. This assay consistently detected as little as 0.1 U/ml of IL-2. Units of IL-2 were calculated by comparison to a standard (No. ISDP-841) provided by the National Cancer Institute Biological Response Modifiers Program.

Assay for MAF. MAF activity was measured by determining the titer of culture supernatant that induced 50% of maximal lysis of $^{51}$Cr-labeled P-815 cells by C57BL/6-derived bone marrow macrophages activated with lipopolysaccharide (Escherichia coli, 0127:B8; Difco Laboratories Inc., Detroit, MI) (29).

Identification of Lymphokine Messenger RNA (mRNA) in Cloned T Cells. CTL L3, CTL dB45, and Th L2 cells at a concentration of $10^7$ cells in 4 ml of culture medium were stimulated with either immobilized mAb 384.5 or mAb F23.1 in 60 x 10-mm petri dishes (Falcon #1007, Becton Dickinson & Co., Oxnard, CA). At the end of the culture period, the cells were detached from the petri dishes by adding 0.08 M EDTA in DME to a final concentration of 0.04 M. The cells were then harvested and washed in DPBS. 2.9 ml of 4.0 M guanidine thiocyanate was added to $10^7$ cells, and the RNA was isolated using the cesium chloride method, with a second precipitation of the RNA in 7.5 M guanidine hydrochloride. The samples were washed, dissolved in water, and reprecipitated with 0.05 M potassium acetate in ethanol. After rewashing, the amount of RNA present was quantified by measuring absorbance at 260 nm. 1.0 M glyoxal was added to equal amounts of RNA (~40 µg) with the modifications of procedures as described by Thomas (30). The RNA samples were then either dotted directly onto Genescreen (New England Nuclear, Boston, MA) or subjected to electrophoresis in a 1% agarose gel and transferred to nitrocellulose (Schleicher and Schuell, Inc., Keene, NH). The Northern and dot blots prepared in this manner were first hybridized to a $^{32}$P-labeled cDNA probe for IL-2 (10$^8$ cpm/µg) and autoradiograms were made. The IL-2 probe was then removed and the blots were rehybridized to the cDNA probe for IFN-γ (kindly provided by Dr. Ken-ichi Arai, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA) (31). The cDNA probes were hybridized and removed from blots using procedures modified from the manufacturer’s instructions.

Results

CTL Clones Can Proliferate in Response to Alloantigen in the Absence of Exogenous IL-2 or Accessory Cells. Previous studies in this laboratory indicated that the cytolytic T cell clone L3, as well as several other CTL clones, was dependent on IL-2 for proliferation (4, 32). Although proliferation was enhanced by including H-2Ld alloantigen, as well as exogenous IL-2, alloantigen alone did not appear to stimulate CTL L3 cells significantly under the conditions employed in these earlier studies. However, we have found that we can induce significant thymidine incorporation in several different CTL clones, including CTL L3 cells, in the absence of exogenous IL-2, using slightly different assay conditions. The results shown in Fig. 1 indicate that CTL L3 cells stimulated with alloantigen in the form of either T cell–depleted allogeneic DBA/2 splenocytes or P-815 mastocytoma cells which bear H-2Ld, or with the clonotypic mAb 384.5, which binds to the T cell receptor on CTL L3 cells, incorporate significant amounts of tritiated thymidine in a 48-h assay.

When CTL L3 cells were stimulated with the indicated numbers of either T cell–depleted DBA/2 splenocytes or P-815 mastocytoma cells, or with immobilized mAb 384.5, thymidine incorporation increased in proportion to the responding cell number (Fig. 1, A–C). When the concentration of CTL L3 cells was increased, greater numbers of DBA/2 stimulator cells were required to induce plateau levels of stimulation (Fig. 1A). At equivalent stimulating cell numbers, P-815
FIGURE 1. Dose-dependent thymidine incorporation by clone CTL L3 cells stimulated by allogeneic spleen cells, P-815 tumor cells, or immobilized mAb 384.5. CTL L3 cells (10⁵ [---]; 3.3 × 10⁴ [- -]; 1.1 × 10⁴ [---]; or 3.7 × 10³ [----]) were cultured with the indicated number of either irradiated T cell-depleted DBA/2 splenocytes (A), mitomycin C-treated P-815 mastocytoma cells (B), or various dilutions of mAb 384.5 supernatant immobilized in microtiter wells 2 h before the addition of CTL L3 cells (C). The cultures were pulsed with [³H]thymidine for the last 12 h of the 48 h incubation period. Thymidine incorporation by CTL L3 cells in control cultures prepared with C57BL/6 splenocytes was less than 1,500 dpm; by CTL L3 cells and irradiated DBA/2 splenocytes and mitomycin-treated P-815 cells alone, it was less than 2,000 dpm.
cells were substantially more stimulatory than DBA/2 splenocytes (Fig. 1B). The optimal number of P-815 stimulator cells in this experiment was $3.3 \times 10^5$ per microwell, regardless of the number of CTL L3 responder cells added to the cultures. A sharp peak in the dose response curve for P-815 cells is evident. The reduced response with a greater number of P-815 cells may be due to a nonspecific inhibitory effect of the P-815 cell membranes (33, 34), or may reflect specific inhibition by a high antigen dose. In the same experiment, T cell-depleted C57BL/6 splenocytes, which are syngeneic to CTL L3 cells, did not induce thymidine incorporation in CTL L3 cells (data not shown). In response to the immobilized clonotypic mAb 384.5 (Fig. 1C), maximal levels of thymidine incorporation were observed with CTL L3 cells when mAb 384.5 supernatants at dilutions of up to 1:1000 were used to immobilize the antibody.

Antibodies reactive with the TCR of two T cell clones, CTL L3 and CTL dB45, stimulated only those cells that expressed the appropriate determinant (mAb 384.5 for CTL L3 cells, and mAb KJ16-133 for CTL dB45 cells), as shown in Table II. In repeated experiments, mAbs directed against either Thy-1.2 (mAb AT83A) or Lyt-2 (mAb 3.155) did not stimulate thymidine incorporation by either of these cloned T cell populations (Table II). In other experiments, immobilized mAb directed against the IL-2 receptor and the LFA-1 complex failed to stimulate cloned CTL L3 and CTL dB45 cells (data not shown).

The kinetics of thymidine incorporation by CTL L3 cells following stimulation is shown in Fig. 2. CTL L3 cells were cultured with T cell-depleted syngeneic C57BL/6 or allogeneic DBA/2 spleen cells, P-815 mastocytoma cells, or AKR-A T lymphoma cells (H-2k) (Fig. 2A), or with either immobilized mAb 384.5 or immobilized mAb KJ16-133 (Fig. 2B). The response of CTL L3 cells to either P-815 cells or DBA/2 splenocytes was maximal at 48 h. The response induced by DBA/2 splenocytes consistently remained elevated through day 4, but the response to P-815 cells had returned to background levels by this time. In repeated experiments, the peak of thymidine incorporation always occurred at 48 h. The pattern of thymidine incorporation by CTL L3 cells produced by stimulation with mAb 384.5 differed somewhat from that induced by antigen,
remaining detectable up to day 4 (Fig. 2). Furthermore, as had been seen in the dose-response studies, the optimal dose of mAb 384.5 consistently induced more thymidine incorporation than that generated by optimal numbers of either P-815 cells or DBA/2 splenocytes.

**Thymidine Incorporation by CTL L3 Cells Stimulated by mAb 384.5 Is Associated with Entry into Cell Cycle.** To determine whether the thymidine incorporation observed in CTL L3 cells stimulated by immobilized mAb 384.5 represented entry into cell cycle, we analyzed the DNA content of CTL L3 cells using the fluorescent dye PI, which binds stoichiometrically to DNA (26). Fig. 3A is a histogram presenting the DNA content of CTL L3 cells cultured for 40 h in culture medium. Only 2.5% of these cells are found in S, G2, and M phases of the cell cycle; essentially all of the unstimulated CTL L3 cells are contained within a single peak corresponding to 2n DNA content of cells in the G1 phase of the cell cycle. Fig. 3B is a histogram presenting the DNA content of CTL L3 cells stimulated for 40 h with 10 U/ml IL-2 (EL-4-SF). A significant number (28%) of these cells are contained within the peaks corresponding to the S, G2, and M phases of the cell cycle. Fig. 3C is a histogram presenting the DNA content of CTL L3 cells stimulated for 40 h with immobilized mAb 384.5. 37% of these CTL L3 cells are contained within the peaks corresponding to the S, G2, and M phases of the cell cycle. Thus, the thymidine incorporation induced by mAb 384.5 reflects entry into the cell cycle.

In separate experiments, we determined cell numbers after stimulation of CTL L3 cells. A two- to sixfold increase in CTL L3 cell numbers was found 4 d after stimulation with immobilized mAb 384.5, and a two- to threefold increase in cultures prepared with P-815-stimulating cells (data not shown).

**Secretion of Lymphokines by Cloned T Cells After Stimulation of the Antigen Receptor.** Immobilized mAb 384.5 and mAb F23.1 were used to stimulate cloned T cells at a cell density (2.5 x 10⁶ cells/ml) that produced high lymphokine titers. The production of lymphokine mRNA was also studied under the same experimental conditions. IL-2 and MAF titers for the supernatants harvested 18 h after
stimulation with immobilized mAb 384.5 or mAb F23.1 are presented in Table III. High levels of MAF activity were detected in 18-h supernatants from the two cloned CTL, L3 and dB45, and from the cloned Th, L2. IL-2 activity was high in the L2 supernatant, but undetectable in the supernatants from CTL L3 or CTL dB45 cells.

Supernatants harvested at 3 and 6 h after stimulation were also assayed for IL-2 activity. At no time was IL-2 activity detected in the supernatants from the cloned CTL. IL-2 was readily detectable in supernatants from Th L2 cells as early as 3 h after stimulation with immobilized mAb F23.1. The IL-2 titers in the L2 supernatants were highest at 18 h after stimulation (data not shown).

In other experiments, both CTL L3 cells and CTL dB45 cells stimulated with DBA/2 splenocytes, P-815 mastocytes, or immobilized mAb 384.5 or mAb F23.1
1574 INTERLEUKIN 2-INDEPENDENT PROLIFERATION OF T CELLS

**Table III**

Lymphokine Production by T Cell Clones

| T cell clone | MAF*  | IL-2†  |
|--------------|-------|--------|
| CTL L3       | 17,900| 0      |
| CTL dB45     | 14,500| 0      |
| Th L2        | 25,500| 205    |

CTL L3 cells (10 × 10^6) were incubated with the immobilized mAb 384.5. CTL dB45 and Th L2 cells (10^7) were incubated with immobilized mAb F23.1. The final volume of culture medium was 4 ml. After 18 h culture supernatants were collected and assayed for MAF and IL-2 activity as described in Materials and Methods.

* MAF activity is expressed as the titer of culture supernatant that resulted in 50% maximal lysis of 51Cr-labeled target cells as described in Materials and Methods.

† IL-2 activity is expressed as U/ml as described in Materials and Methods.

consistently secreted MAF within 12 h, but neither CTL clone produced detectable levels of IL-2 (data not shown).

**Proliferation of CTL Clones Induced by Stimulation of the TCR is Not Inhibited by Anti-IL-2-R mAb.** Although proliferation of cloned CTL cells induced by stimulation of the antigen receptor was not accompanied by secretion of detectable levels of IL-2, it was possible that IL-2 was produced but that the amounts were not high enough to be detected in our assay. Therefore, we determined the effect of anti-IL-2-R antibodies on proliferation in cloned CTL cells induced by IL-2 or by stimulation of the TCR.

A mixture of two anti-IL-2-R antibodies, mAb PC61.5 plus mAb 7D4, was used in amounts sufficient to inhibit maximal levels of thymidine incorporation induced by IL-2. Proliferation of Th L2 cells, CTL dB45 cells, and CTL L3 cells induced by 20 U/ml of IL-2 was profoundly inhibited by these antibodies, as shown in Fig. 4; B, D, and F, respectively. The anti-IL-2-R mixture also blocked the proliferation of Th L2 when this clone was stimulated by either immobilized mAb F23.1 or by alloantigen in the form of CBA spleen cells, as shown in Fig. 4A. In contrast, anti-IL-2-R had no significant inhibitory effect on the proliferation of the CTL L3 cells stimulated with either immobilized mAb 384.5 or alloantigen in the form of DBA/2 spleen cells, as shown in Fig. 4E. Also, anti-IL-2-R had no significant inhibitory effect on the proliferation of CTL dB45 cells in response to immobilized mAb F23.1, or to alloantigen in the form of DBA/2 spleen cells, as shown in Fig. 4C.

In other experiments with three CTL clones, CTL L3, dB45, and BC9, mAb PC61.5 alone inhibited the proliferation induced by IL-2. In contrast, we could not detect blocking by mAb PC61.5 of the thymidine incorporation induced by P-815 mastocytes (Table IV). None of these three CTL clones secretes detectable IL-2 in response to Con A or alloantigen (data not shown).

Comparable results were obtained when mAb 3C7 or 7D4 were used alone with CTL L3 and CTL dB45 cells stimulated with P815 mastocytes (data not shown). In addition, a polyclonal rabbit anti-human IL-2 antiserum (Collaborative Research, Inc., Lexington, MA) was able to block the proliferation of CTL L3 cells induced by 5 U/ml of IL-2, but it had no inhibitory effect on the proliferation of CTL L3 cells in response to mAb 384.5 (data not shown).
Figure 4. Effect of anti-IL-2-R mAb on Th L2, CTL dB45, and CTL L3 cell proliferation induced by IL-2, allogeneic spleen cells, or immobilized mAb 384.5 or mAb F23.1. Th L2 cells (5 × 10⁴) were cultured with either immobilized mAb F23.1, irradiated CBA spleen cells (10⁶), (A), or with 20 U/ml IL-2 contained in EL-4-SF (B). CTL dB45 cells (5 × 10⁴) were cultured with either immobilized mAb F23.1, irradiated DBA/2 spleen cells (10⁶), (C), or with 20 U/ml IL-2 contained in EL-4-SF, (D). CTL L3 cells (5 × 10⁴) were cultured with either immobilized mAb 384.5, irradiated DBA/2 spleen cells (10⁶), (E), or with 20 U/ml IL-2 contained in EL-4-SF, (F). Hatched bars indicate the addition to the culture wells of a mixture containing mAb PC61.5 (final concentration 1:800) and mAb 7D4 (final concentration 1:800). Both antibodies were purified from hybridoma supernatants by ammonium sulphate precipitation. The cultures were pulsed with [³H]thymidine for the last 12 h of the 48 h incubation period.
**Response of CTL Clones to P-815 Cells Is Not Blocked by Monoclonal Anti-IL-2-R Antibody**

| Stimulus                  | L3       | dB45     | BC9       |
|---------------------------|----------|----------|-----------|
| None                      | 654 ± 114| 1,279 ± 311| 1,196 ± 61|
| P-815                     | 18,578 ± 2,480| 14,908 ± 1,914| 3,546 ± 38|
| P-815 + PC61.5            | 26,413 ± 2,284| 12,911 ± 455| 2,680 ± 58|
| IL-2                      | 77,913 ± 6,433| 86,548 ± 8,409| 37,887 ± 999|
| IL-2 + PC61.5             | 1,257 ± 318| 15,610 ± 2,850| 1,772 ± 269|

Cells of the indicated CTL clone (5 x 10⁴) were incubated with either medium, 3 x 10⁵ mitomycin C-treated P-815 mastocytoma cells, or with 3 U/ml of IL-2 (EL-4 SF). mAb PC61.5 culture supernatant at a final dilution of 1:6 was added to the indicated wells. The cultures were pulsed with [³H]thymidine for the last 12 h of the 48 h incubation period. [³H]thymidine incorporation by mitomycin C-treated P-815 mastocytoma cells alone was 155 ± 20 dpm.

**Discussion**

CTL clones that incorporate thymidine in response to stimulation with alloantigen in the absence of exogenous IL-2 have been described previously (6-8, 35, 36). Widmer and Bach (6) designated such CTL as "helper-cell independent" and suggested that such cells might produce IL-2 (or other lymphokines) which caused cell proliferation. In fact, IL-2 production has been documented for some but not all Th-independent CTL clones (8, 36); for other CTL clones, IL-2 production was not measured (7). However, studies using mixed populations of
FIGURE 5. Production of lymphokine mRNA by cloned CTL and Th stimulated with immobilized mAb 384.5 or mAb F23.1. Th L2 cells and CTL dB45 cells were stimulated with immobilized mAb F23.1 and CTL L3 cells were stimulated with immobilized mAb 384.5 for the indicated periods of time, after which the supernatants and cells were harvested, as described in Materials and Methods. Dot blots were performed using cDNA probes for IL-2 (A) and IFN-γ (B) as described in the Materials and Methods section.
stimulating cells are inherently difficult to interpret. It seemed probable that secretion of small amounts of IL-2 either by Th-independent CTL themselves or by Th remaining in the stimulating cell population accounted for the thymidine incorporation induced by alloantigen. The latter problem was avoided in the present studies by stimulating cloned CTL and Th with mAb directed specifically against the TCR. The responses observed were comparable to those obtained when the cloned cells were stimulated with alloantigen. Using this approach, we have been able to show that at least some conventional CTL cells can be stimulated through the TCR to proliferate, as evidenced by incorporation of thymidine, entry into cell cycle, and increase in cell number via an IL-2-independent pathway.

Several recent refinements in culture conditions account for our current ability to detect thymidine incorporation in cloned CTL cells in the absence of exogenous IL-2. In the present study, the magnitude of thymidine incorporation was influenced both by the number of CTL L3 cells and by the number of allogeneic stimulating cells (Fig. 1). The thymidine incorporation detected was found to be directly proportional to the concentration of CTL L3 cells (e.g., a threefold increase in CTL L3 cell concentration resulted in a threefold increase in thymidine incorporation). In previous studies, thymidine incorporation by relatively small numbers of CTL L3 cells usually was measured after 5 d of culture, a time that was found to be optimal when alloantigen and IL-2 were used together (4). Since the previous experiments used assays that were usually harvested after 3–5 d of culture and used fewer cloned T cells per culture well, significant thymidine incorporation was not readily observed. In contrast, the assays used for this report used at least 5 × 10⁴ cloned T cells per microwell and were harvested after 48 h of culture.

Several lines of evidence argue against a role for IL-2 in the proliferation of CTL stimulated via the TCR. First, although high levels of IFN-γ, as reflected by MAF activity, were found in culture supernatants from CTL clones that were proliferating in response to stimulation with T cell–depleted allogeneic spleen cells, allogeneic tumor cells, or immobilized mAb reactive with the TCR, IL-2 was not detected. It could be argued that CTL L3 and CTL dB45 cells, since they have IL-2 receptors, may have absorbed all the IL-2 that they might have produced. However, when the numbers of CTL L3 and CTL dB45 cells used in these experiments were cultured in 1 U/ml IL-2, small but detectable amounts of IL-2 remained at 24 h. Therefore, had the CTL cells produced 1 U/ml of IL-2 over a period of 24 h, it is likely that it would have been detected in our IL-2 assay.

Second, in repeated experiments, mAbs reactive with the cell surface receptor for IL-2 did not inhibit proliferative responses of these CTL clones in response to stimulation of the TCR, whereas these mAbs inhibited IL-2-induced proliferation of the same CTL clones. In contrast, the cloned Th L2 cells secreted large amounts of IL-2 into the medium after stimulation of the TCR. The anti–IL-2-R antibodies profoundly inhibited both TCR-driven and IL-2-driven proliferation of the IL-2-producing Th L2 clone. Apparently, Th L2 cells that are stimulated via the TCR proliferate predominantly via an IL-2-mediated, autocrine pathway.
Third, although we repeatedly detected IFN-γ mRNA in all the T cell clones tested, we consistently were unable to show the presence of IL-2 mRNA in cloned CTL L3 and dB45 cells, whereas it was readily detectable in cloned Th L2 cells. These findings appear to exclude the possibility that unsecreted IL-2 could act intracellularly to mediate the activation of the cloned CTL cells.

Although our data show an IL-2-independent pathway for CTL activation, IL-2 appears also to have an important role in many situations. IL-2 alone can induce sustained proliferation in our CTL clones (37). Furthermore, a profound synergistic effect is observed when L3 cells are stimulated with alloantigen together with IL-2 (38), and T cell-depleted syngeneic splenic adherent cells appear to enhance L3 proliferation induced by P-815 mastocytes (data not shown). Thus, IL-2 and accessory cells may influence the magnitude of the response initiated by and dependent upon stimulation of the TCR.

The importance of the IL-2-independent pathway for immune responses in vivo remains to be determined. This pathway could be important for CTL proliferation in microenvironments where T cell help may not be present. It may be important in the first few days after antigenic challenge, or it may operate intermittently only after a repeated challenge. It may operate in immune responses where only differences in class I MHC antigens are present. In fact, it has recently been reported that T lymphocytes bearing surface Lyt-2 but not L3T4 can respond to class I MHC antigen in the absence of L3T4+ lymphocytes (39). However, the role of IL-2 in such responses was not evaluated.

In addition to these questions, it remains unclear whether the TCR-driven IL-2-independent pathway shown here for CTL also operates in Th. This route of T cell activation in CTL may also involve different biochemical pathways than the IL-2-dependent pathway used by Th. CTL clones that can be activated via the TCR through an IL-2-independent pathway for proliferation and lymphokine production provide a model system for investigating these questions.

Summary

Proliferation of T lymphocytes can be induced by IL-2, either through an autocrine pathway in which the responding cell produces its own IL-2 or through an exocrine pathway in which IL-2 secreted by Th stimulates proliferation of IL-2-dependent CTL. However, proliferation of at least some CTL clones, such as CTL L3 and CTL dB45, also can be induced by stimulation of the antigen receptor in the absence of IL-2. Stimulation of these cloned CTL with T cell-depleted allogeneic spleen cells, allogeneic tumor cells, or immobilized mAb reactive with the T cell antigen receptor (TCR) induced thymidine incorporation, entry into cell cycle, and secretion of macrophage activating factor, but these stimuli did not induce the secretion of IL-2. Several observations indicated that such proliferation of cloned CTL induced by stimulation of the TCR was independent of IL-2; IL-2 could not be detected in supernatants from stimulated CTL cells. mAbs reactive with the murine IL-2-R efficiently blocked IL-2-mediated thymidine incorporation in cloned CTL and Th, but had no inhibitory effect on TCR-driven thymidine incorporation in the CTL clones. TCR-driven thymidine incorporation in cloned Th L2 cells was profoundly inhibited by these antibodies, indicating the operation of an IL-2-mediated autocrine pathway for
proliferation in this cloned Th. When antibodies to the TCR were used to stimulate cloned CTL and Th, IFN-γ mRNA was easily shown in the cloned CTL and Th. Although IL-2 mRNA could be detected in the cloned Th, it was never observed in the cloned CTL. These findings provide evidence for the existence of a TCR-mediated, IL-2-independent pathway for induction of cellular proliferation in cloned murine CTL.

We thank Drs. Thomas Malek, Marcus Nabholz, Philippa Marrack, and Michael Bevan for generously providing some of the mAb-producing hybridoma cell lines used in this study and Dr. K. Smith for providing other cell lines. We also wish to acknowledge the excellent technical assistance of Daisy Freeman, Yukio Hamada, Brigitta Clinchy, and Larry Kavanaugh, and the secretarial assistance of Frances Mills. We are indebted to Gillis Otten for invaluable help with cell cycle analysis and IL-2 assays.

Received for publication 23 September 1985 and in revised form 20 February 1986.

References

1. Cantrell, D., and K. Smith. 1983. Transient expression of interleukin 2 receptors. Consequences for T cell growth. J. Exp. Med. 158:1895.
2. Hemler, M., M. Brenner, J. McLean, and J. Strominger. 1984. Antigenic stimulation regulates the level of expression of interleukin 2 receptor on human T cells. Proc. Natl. Acad. Sci. USA. 81:2172.
3. Meuer, S., R. Hussey, D. Cantrell, J. Hodgdon, S. Schlossman, K. Smith, and E. Reinherz. 1984. Triggering of the T3-Ti antigen-receptor complex results in clonal T-cell proliferation through an interleukin 2-dependent autocrine pathway. Proc. Natl. Acad. Sci. USA. 81:1509.
4. Glasebrook, A., and F. Fitch. 1980. Alloreactive cloned T cell lines. I. Interactions between cloned amplifier and cytolytic T cell lines. J. Exp. Med. 151:876.
5. Glasebrook, A., and F. Fitch. 1979. T cell lines which cooperate in generation of specific cytolytic activity. Nature (Lond.). 278:171.
6. Widmer, M., and F. Bach. 1981. Antigen driven helper cell-independent cloned cytolytic T lymphocytes. Nature (Lond.). 294:750.
7. Bluestone, J. 1983. Characterization of cytotoxic T cell (CTL) clones derived from mutant H-2Kbα10 anti-H-2Kb mixed lymphocyte culture populations. In Interleukin Communication in Leucocyte Function. J. W. Parker and R. L. O'Brien, editors. John Wiley & Sons Ltd., Chichester, United Kingdom. 149.
8. Kelso, A., and A. Glasebrook. 1984. Secretion of interleukin 2, macrophage-activating factor, interferon, and colony-stimulating factor by alloreactive T lymphocyte clones. J. Immunol. 132:2924.
9. Lancki, D., D. Ma, W. Havran, and F. Fitch. 1984. Cell surface structures involved in T cell activation. Immunol. Rev. 81:65.
10. Ryser, J., J. Cerottini, and K. 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.
11. Farrar, J., S. Mizel, J. Fuller-Farrar, W. Farrar, and M. Hilfiker. 1980. Macrophage-independent activation of helper T cells. 1. Production of interleukin 2. J. Immunol. 125:793.
12. Davidson, W., and C. Parish. 1975. A procedure for removing red cells and dead cells from lymphoid cell suspensions. J. Immunol. Methods. 7:291.
13. Cerottini, J. -C., H. Engers, H. MacDonald, and K. Brunner. 1974. Generation of
cytotoxic T lymphocytes in vitro. I. Response of normal and immune mouse spleen cells in mixed leukocyte cultures. *J. Exp. Med.* 140:703.

14. Baker, P., S. Gillis, and K. Smith. 1979. Monoclonal cytolytic T cell lines. *J. Exp. Med.* 149:273.

15. Lancki, D., M. Lorber, M. Loken, and F. Fitch. 1983. A clone-specific monoclonal antibody that inhibits cytolysis of a cytolytic T cell clone. *J. Exp. Med.* 157:921–935.

16. Ortega-R, G., R. Robb, E. Shevach, and T. Malek. 1984. The murine IL 2 receptor. I. Monoclonal antibodies that define distinct functional epitopes on activated T cells and react with activated B cells. *J. Immunol.* 133:1970.

17. Malek, T., G. Ortega-R, J. Jakway, C. Chan, and E. Shevach. 1984. The murine IL 2 receptor. II. Monoclonal anti-IL 2 receptor antibodies as specific inhibitors of T cell function in vitro. *J. Immunol.* 133:1976.

18. Malek, T., R. Robb, and E. Shevach. 1983. Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin 2 receptor-ligand complex. *Proc. Nat. Acad. Sci. USA.* 80:5694.

19. Lowenthal, J., R. Zubler, M. Nabholz, and H. MacDonald. 1985. Similarities between interleukin-2 receptor number and affinity on activated B and T lymphocytes. *Nature (Lond.)* 315:669.

20. Haskins, K., C. Hannum, J. White, N. Roehm, R. Kubo, J. Kappler, and P. Marrack. 1984. The antigen-specific major histocompatibility complex–restricted receptor on T cells. VI. An antibody to a receptor allotype. *J. Exp. Med.* 160:452.

21. Staerz, U. D., H. G. Rammensee, J. D. Benedetto, and M. J. Bevan. 1985. Characterization of a murine monoclonal antibody specific for an allotypic determinant on T cell antigen receptor. *J. Immunol.* 134:3994.

22. Sarmiento, M., M. Loken, and F. Fitch. 1981. Structural differences in cell surface T25 polypeptides from thymocytes and cloned T cells. *Hybridoma.* 1:13.

23. Sarmiento, M., A. Glasebrook, and F. Fitch. 1980. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt-2 antigen block T cell-mediated cytolysis in absence of complement. *J. Immunol.* 125:2665.

24. Lancki, D., M. Prystowsky, S. Vogel, D. Beller, D. Dialynas, and F. Fitch. 1984. Effects of monoclonal antibodies directed against murine T lymphocyte cell surface antigens on lymphokine production by cloned T lymphocytes reactive with class I MHC or Mls allospecificities. *J. Immunol.* 133:2051.

25. Glasebrook, A., J. Quintans, L. Eisenberg, and F. Fitch. 1981. Alloreactive cloned T cell lines. II. Polyclonal stimulation of B cells by a cloned helper T cell line. *J. Immunol.* 126:240.

26. Krishan, A. 1975. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. *J. Cell Biol.* 66:188.

27. Gillis, S., M. M. Fern, W. Ou, and K. A. Smith. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120:2027.

28. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65:55.

29. Kelso, A., A. Glasebrook, D. Kanagawa, and K. Brunner. 1982. Production of macrophage activating factor by T lymphocyte clones and correlation with other lymphokine activities. *J. Immunol.* 129:550.

30. Thomas, P. 1983. Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. *Methods Enzymol.* 100:255.

31. Yokota, T., N. Arai, F. Lee, D. Rennick, T. Mosmann, and K.-I. Arai. 1985. Use of a cDNA expression vector for isolation of mouse interleukin 2 cDNA clones: Expression of T-cell growth-factor activity after transfection of monkey cells. *Proc. Nat. Acad. Sci. USA.* 82:68.
32. Glasebrook, A., M. Sarmiento, M. Loken, D. Dialynas, J. Quintans, L. Eisenberg, C. Lutz, D. Wilde, and F. Fitch. 1981. Murine T lymphocyte clones with distinct immunological functions. *Immunol. Rev.* 54:225.

33. Davidson, W. F. 1977. Cellular requirements for the induction of cytolytic T cells in vitro. *Immunol. Rev.* 35:263.

34. Pimlott, N., and R. Miller. 1984. A glycopeptide extract can inhibit cytotoxic T lymphocytes-target cell conjugation in an H-2 restricted manner. *J. Immunol.* 133:1763.

35. Bach, F., S. Wee, Roopenian, D. C., Chen, L.-K., C. Orosz, and M. Widmer. 1983. Helper cell-independent cytotoxic T lymphocytes (HITc) in mouse and man. In *Intercellular Communication in Leucocyte Function.* J. W. Parker and R. L. O'Brien, editors. John Wiley & Sons, Ltd. Chichester, United Kingdom. 109.

36. Roopenian, D., M. Widmer, C. Orosz, and F. Bach. 1983. Helper cell-independent cytolytic T lymphocytes specific for a minor histocompatibility antigen. *J. Immunol.* 130:542.

37. Havran, W., D. -K. Kim, R. Moldwin, D. Lancki, and F. Fitch. 1986. IL-2 causes increased expression of IL-2 receptors on cloned murine T cells. *Clin. Immunol. Immunopathol.* In press.

38. Lutz, C., A. Glasebrook, and F. Fitch. 1981. Alloreactive cloned T cell lines. IV. Interaction of alloantigen and T cell growth factors (TCGF) to stimulate cloned cytolytic T lymphocytes. *J. Immunol.* 127:391.

39. Sprent, J., and M. Schaefer. 1985. Properties of purified T cell subsets. 1. In vitro responses to class I vs. class II H-2 alloantigens. *J. Exp. Med.* 162:2068.