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Limiting-dilution analysis of human CTL differentiation. Requirement for a lymphokine-mediated differentiation signal

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SUMMARY

The induction of human influenza virus-specific memory cytotoxic T lymphocytes (CTL) from CTL precursors (CTLp) was investigated using limiting-dilution cultures and cell lines. Differentiation of maximal numbers of CTLp in limiting-dilution cultures required at least three signals: antigen stimulation, interleukin-2 (IL-2), and a differentiation factor distinct from IL-2. Antigen-specific CTLp proliferated in response to antigen stimulation and recombinant DNA-derived IL-2, but often failed to acquire cytolytic activity unless conditioned medium (CM) from mitogen-stimulated peripheral blood mononuclear cell (PBMC) cultures was added to the cultures. Temporal analysis of the requirement for CM indicated that it was providing a late signal for CTLp differentiation. This analysis was confirmed by developing CTLp cell lines, which were found to proliferate in response to IL-2 and antigen but not to exhibit influenza virus-specific cytotoxicity until CM was added.

INTRODUCTION

Non-cytotoxic precursors of cytotoxic T lymphocytes (CTLp) are found in various lymphoid tissues and restimulation of these CTLp *in vivo* or *in vitro* with antigen results in the induction of antigen-specific, MHC-restricted cytotoxicity (Zinkernagel & Doherty, 1979). This acquisition of cytolytic function involves several signals provided to the CTLp by various accessory cells or their soluble products (Simon & Eichmann, 1980). Initially, the activation of the CTLp involves an interaction with an appropriate antigen-presenting cell (Kabelitz *et al.*, 1987). Upon activation, clonal expansion of the CTLp requires the presence of IL-2 (Andrus, Granelli-Piperno & Reich, 1984). Under what stimuli these cells acquire cytolytic function remains unclear. IL-2 alone may provide both the proliferation and differentiation signals for these cells (Erard *et al.*, 1985). Alternatively, the acquisition of cytolytic activity may require additional differentiation factors (Reddehase *et al.*, 1982). One reason for this uncertainty is the heterogeneous nature of the lymphocyte cultures frequently employed to generate antigen-specific secondary CTL. In these cultures one is faced with the following considerations: endogenous lymphokine production, indirect effects mediated by the lymphokines on accessory cells, and the possibility of multiple CTLp with different lymphokine requirements. Furthermore it is difficult to determine whether increases in CTL activity resulted from increased activation, proliferation, or differentiation.

In this paper we report the results of our studies of CTLp in limiting-dilution cultures and in CTLp cell lines. Under these conditions we have been able to isolate CTLp whose subsequent development into effector cells is dependent upon the presence of exogenous helper factors. Using this approach we have demonstrated that human CTLp require a differentiation signal that is distinct from IL-2 and interferon (IFN).

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MATERIALS AND METHODS

Preparation of cells

Peripheral blood mononuclear cells (PBMC) were obtained after Ficoll–Pacque (Pharmacia Inc., Piscataway, NJ) gradient separation of either peripheral blood of laboratory volunteers or buffy coats obtained by the NIH Department of Transfusion Medicine, Bethesda, MD. T-cell enriched populations, hereafter referred to as T cells, were obtained by sequential depletion of plastic and nylon-wool-adherent cells; these cells were determined to be >90% Leu 1^+ and Leu 4^+ (Becton-Dickinson, Mountain View, CA) by flow cytometric analysis (Coulter Epics flow cytometer, Hialeah, FL). $CD8^+$ cells were obtained by cytofluorometric sorting of T cells stained with a monoclonal antibody that recognized the CD8 antigen (Leu 2a; Becton-Dickinson).

Lymphokines

Concanavalin A (Con A)-conditioned medium (CM) was prepared by incubating PBMC (10^6/ml) in the presence of Con

A (2 μg/ml; Sigma, St Louis, MO) in RPMI-1640 containing penicillin (100 units per ml), streptomycin (0·1 mg per ml), L-glutamine (2 mM) and 5% human AB serum (Sigma) (RPMI-ABS) at 37° in a CO_2 incubator for 3 days. After pelleting the cells, the supernatant was exhaustively absorbed with Sephadex G-10 (Pharmacia) to remove any remaining mitogen, filter-sterilized (0·22 μm Millex-GS, Millipore Corp., Bedford, MA) and stored at 4° until used. Recombinant DNA-derived IL-2 was provided by Cetus Corporation (Emeryville, CA) and had a specific activity of 3×10^6 units/mg of protein. The IL-2 was cross-standardized to the National Cancer Institute's Biological Response Modifier Program standard. Interferon (IFN)-α_{2a} was provided by Hoffman LaRoche (Nutley, NJ), IFN-α_{2b} was provided by Schering Corp. (Kennerlworth, NJ), and IFN-γ was provided by Biogen (Cambridge, MA). The specific activities of the interferons were 2·0, 2·0, and $0{\cdot}2 \times 10^8$ U/mg of protein, respectively.

Virus stocks

Influenza A/Bangkok (H3Ns) and B/Minnesota (Division of Virology, FDA, Bethesda, MA) were propagated in embryonated hen's eggs. Allantoic fluid containing virus was aliquoted in 1·0 ml volumes and frozen at −80° until used. The titre was 512 haemagglutination units per ml (HAU/ml) for both A/Bangkok and B/Minnesota.

Limiting-dilution cultures

Influenza antigen-presenting cells (stimulator cells) were prepared by infecting autologous PBMC with virus (0·1 ml of virus stock added to 10^7 cells), incubating for 15 min at 37° and then irradiating (2000 rads from a Cesium source). Limiting-dilution cultures consisted of graded numbers of responder T cells incubated with 100,000 autologous stimulator cells in 96-well round-bottomed plates (Costar, Cambridge, MA) in 0·2 ml of RPMI-ABS. In some experiments a single responder cell dose of 20,000 T cells or 16,000 $CD8^+$ cells was used. When lymphokines were added to the cultures 0·1 ml of supernate was removed and the lymphokine was added in a final volume of 0·1 ml of RPMI-ABS. CM, when used, was added at a final concentration of 10% (v/v), IL-2 was added at a final concentration of 25 U/ml (U/ml).

Generation of CTLp cell lines

Primary clones of CTLp were established by incubating 4000 T cells with stimulator cells in the presence of IL-2. Following restimulation with antigen and IL-2, the cultures were split into three replicates. CM was added to one set of replicates and the second set received IL-2; both of these sets of replicate cultures were assayed 3 days later for cytotoxic activity. The third set of cultures received IL-2 and were used as the source plate for cultures selected for subsequent continuous culturing. These cultures were periodically stimulated with stimulator cells or IL-2. A high probability (>90%) of clonality of these lines with respect to CTLp was ensured by selecting a cell concentration in which less than 15% of the wells contained a CTLp.

Cytotoxicity assay

Phytohaemagglutinin A (2·5 μg/ml; PHA; Burroughs Welcome Laboratories, Beckenham, Kent, U.K.)-stimulated (3 days) autologous PBMC blasts were used as targets in the cytotoxicity assay. The cells were washed in RPMI-1640 containing antibiotics, glutamine and 10% fetal calf serum (Grand Island Biological Company, Grand Island, NY) (RPMI-FCS) and $1-3 \times 10^6$ cells were resuspended in 0·1 ml of influenza virus stock in a 15 ml conical centrifuge tube. After incubating for 15 min in a 37° water bath, 0·25 mCi of $Na_2{}^{51}CrO_4$ in saline (New England Nuclear Products, Boston, MA) was added to each tube. The cells were incubated an additional 4 hr at 37° and then washed several times with RPMI-FCS. The cell pellet was then resuspended at a final concentration of 2×10^4 cells/ml in RPMI-FCS and 0·1 ml of the target cell suspension was added to each well being assayed. Identical split wells were assayed for cytotoxic activity against both influenza A- and influenza B-infected targets. The plates were then gently centrifuged (75 *g*, 3 min) and transferred to a 37° CO_2 incubator for a 4-hr incubation. The supernatant from each well was collected using the Skatron SCS harvesting system (Skatron Inc, Sterling, VA) and then counted in a gamma counter. Positive cultures were identified as those in which the ^{51}Cr released exceeded, by at least 2 SD, the mean c.p.m. of the negative control group that contained labelled target cells incubated either alone or with irradiated stimulator cells.

Statistical analysis

Each determination was performed at a minimum of 24 replicate wells. The applicability of the data to the limiting-dilution model was assessed by the method of chi-squared minimization, as described by Taswell (1981). Calculated *P* values of greater than 0·1 were considered to indicate that the data were consistent with the hypothesis that the positivity of a well was solely dependent upon the presence of a cytotoxic cell precursor. Differences between lymphokine-treated and control groups in the proportion of cultures exhibiting influenza A virus-specific cytotoxicity were analysed using Fisher's exact test.

RESULTS

Lymphokine requirement for CTLp maturation in limiting-dilution cultures

When increasing numbers of responder T cells were incubated with autologous influenza virus-infected stimulator cells, we observed a dose-dependent increase in the proportion of cultures exhibiting influenza A virus-specific cytotoxicity (Fig. 1). Under conditions in which the presence of a CTLp in a well is the sole determinant of whether cytotoxicity develops, the proportion of cultures developing cytotoxicity will be determined by the Poisson distribution; therefore the graph of log proportion of wells negative vs. cell concentration will approximate linearity and the *P* value for the minimized chi-square will exceed 0·1 (Taswell, 1981, 1984). However, as the number of responder cells per well was increased the proportion of wells positive for influenza A virus-specific cytotoxicity increased more rapidly than would be predicted by the Poisson model; thus the corresponding *P* value was less than 0·1 and the plot of log proportion of wells negative vs. cell concentration was non-linear (Fig. 2, open circles, dotted line). These results indicated that the presence of an influenza virus-specific CTLp was not the sole determinant of whether cytotoxicity developed. One interpretation of these results is that there was an additional requirement for a source of helper activity in some of the

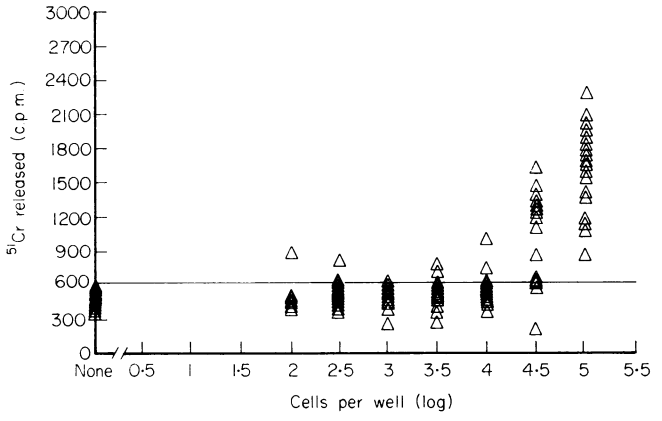

Figure 1. Twenty-four replicate cultures of graded numbers of nylon-wool-purified T cells were incubated with 100,000 influenza A virus-infected, irradiated, autologous PBMC for 10 days. Two thousand ^{51}Cr-labelled, influenza A virus-infected PHA blasts were then added for a 4-hr incubation. In some experiments the cultures were split and assayed for cytotoxicity against uninfected or influenza B virus-infected targets. The supernates were collected and the amount of ^{51}Cr released determined. The results are presented as the amount of ^{51}Cr (c.p.m.) detected in the supernate of each microculture. The total release was 3150 c.p.m. and the background was 250 ± 24 c.p.m.

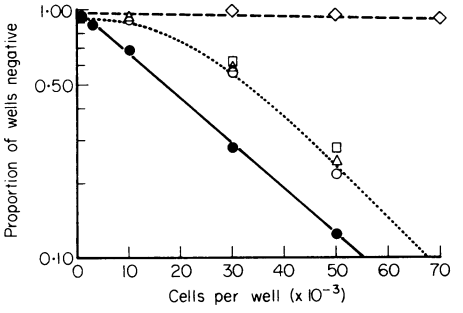

Figure 2. Graded numbers of nylon-wool-purified T cells were incubated with 100,000 influenza A virus-infected, irradiated, autologous PBMC for 7 days (open circles). To some of the cultures were added 25 U IL-2 per ml (triangles) or CM at a final concentration of 10% (v/v) (squares), or both CM and IL-2 (closed circles) 3 days prior to assaying for influenza A virus-specific CTL activity. Wells were scored as positive for influenza A virus-specific cytotoxicity if the amount of ^{51}Cr released exceeded the spontaneous release control by 3 SD. The data is presented as the proportion of wells which failed to develop CTL activity versus number of responder cells per well plotted on semi-log scale. No significant lysis of influenza B virus-infected targets was observed (diamonds).

cultures (Corley, Kindred & Lefkovits, 1978). Addition of conditioned medium (CM) and IL-2 (25 U/ml) to the cultures resulted in an increase in the proportion of wells responding, a linear plot, and a P value $> 0{\cdot}1$ for the minimized chi-square (closed circles). The cytolytic activity was influenza A virus-specific since few of the cultures were cytotoxic for influenza B virus-infected targets (diamonds). Neither IL-2 (triangles) nor CM (squares) alone provided the necessary differentiation signal, even at substantially higher concentrations.

In order to study further the lymphokine requirement for CTLp maturation in limiting dilution cultures, we selected a

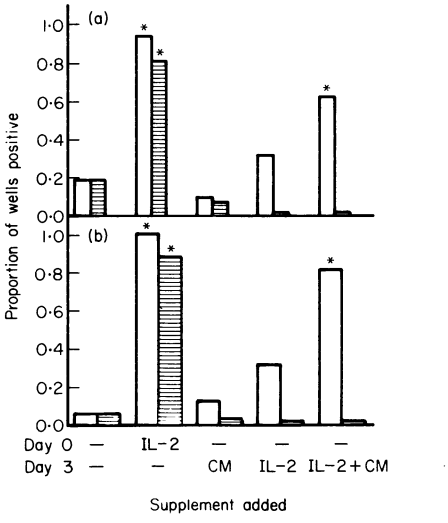

Figure 3. 20,000 T cells (a) or 16,000 CD8$^+$ cells (b) were incubated with 100,000 stimulator cells in 96-well plates. IL-2 and CM were added at the times indicated. The cultures were split and assayed on Day 6 for cytotoxicity against influenza A (open bars) or B (striped bars) virus-infected autologous PHA blast. The results represent the proportion of 32 replicate wells which were cytotoxic against either target. Wells were scored as positive for virus-specific cytotoxicity if the amount of ^{51}Cr released exceeded the spontaneous release control by 3 SD. Those groups significantly different from the medium-only control ($P < 0{\cdot}05$, Fisher's exact test) are indicated with an asterisk (*).

Table 1. Optimal CTLp maturation occurs when CM is added to limiting-dilution cultures after IL-2*

Day IL-2 added	Day CM added	No. of cultures	No. positive
—†	—	32	2
—	5	32	7
2	—	32	8
2	0	32	1
2	5	32	16‡
2	6	32	19§
2	7	32	12
2+5	—	32	6

*20,000 T cells were incubated with 100,000 stimulator cells and IL-2 and CM were added at the times indicated. The cultures were assayed on Day 8 for influenza A virus-specific cytotoxicity.

†—denotes not added.

‡Significantly different from cultures receiving either IL-2 or CM alone, Fisher's exact test, $P < 0{\cdot}07$.

§Significantly different from cultures receiving either IL-2 or CM alone, Fisher's exact test, $P < 0{\cdot}04$.

responder cell concentration of 20,000 T cells to which were added 100,000 stimulator cells and various lymphokines. This responder cell concentration approximates the reciprocal of the frequency of influenza virus-specific CTLp amongst T cells of most healthy individuals (Horohov, Stocks & Siegel, 1986).

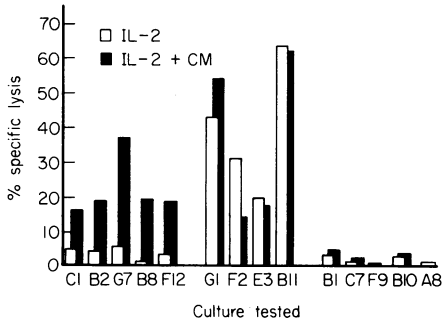

Figure 4. A total of 384 replicate cultures of 4000 T cell and 100,000 stimulator cells were established in 96-well round-bottomed plates. Following restimulation with IL-2 and antigen the plates were split into three replicates. One set of replicate cultures was tested for cytotoxicity 3 days following addition of IL-2 alone (open bars), a second set of cultures was tested following addition of both IL-2 and CM (closed bars). The third set of cultures was restimulated with antigen and IL-2 (no CM) for later use. The percentage specific lysis against influenza A virus-infected targets was calculated for the individual cultures. Total release was 1015 c.p.m., background was 115 c.p.m.

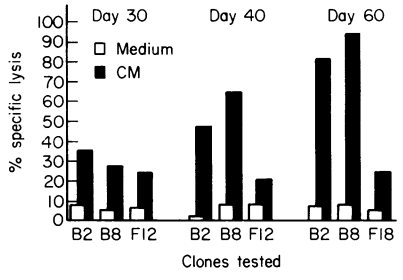

Figure 5. Representative cultures were selected from the source plate described in Fig. 4. These cells were expanded *in vitro* with periodic restimulation with antigen and IL-2. At various times, 20,000 cells were removed from the cultures and placed into 96-well round-bottomed plates. Medium (open bars) or CM (closed bars) was added to the cultures and 3 days later the cultures were assayed for influenza virus-specific cytotoxicity.

Therefore, as predicted by the Poisson distribution, approximately 63% of the cultures would contain one or more CTLp. In the absence of exogenous lymphokines less than 20% of the cultures developed influenza A virus-specific cytotoxicity (Fig. 3a). Addition on Day 0 of IL-2 alone or with CM (not shown) resulted in over 80% of the cultures exhibiting cytotoxicity against both influenza A- and B-infected targets. The addition of either IL-2 or CM on Day 3 did result in an increase in the proportion of wells exhibiting influenza A virus-specific cytotoxicity compared with media only controls; however, maximal generation of specific cytoxicity required the addition of both IL-2 and CM on Day 4. Similar results were obtained when highly purified $CD8^+$ cells were used as responder cells (Fig. 3b). Addition of higher concentrations of IL-2 (> 50 U/ml) resulted in the induction of non-specific cytotoxicity.

CM provides a late signal for CTLp differentiation

The failure of CM alone on Day 3 to provide the necessary signal for maximal CTLp maturation was probably due to the fact that most batches of CM were a poor source of IL-2 (< 2U/ml) and that two signals were required for CTLp maturation, a proliferation signal provided by IL-2 and a later differentiation signal provided by CM. To test this hypothesis we examined the lympho-proliferative responses of the cultures to influenza virus in the presence of medium, IL-2 and CM. When 20,000 T cells were stimulated with 100,000 stimulator cells we observed [^{3}H]thymidine incorporation above background in five of 32 cultures, when IL-2 was added (25 U/ml) an additional 17 cultures (22/32 total) exhibited a lympho-proliferative response; addition of CM did not increase the proportion of cultures responding compared to medium control (6/32), and addition of CM with IL-2 did not increase the proportion of wells responding compared with IL-2 alone (21/32 vs. 22/32). These results indicate that IL-2 was providing the proliferative signal and that addition of CM did not affect this response. In order to test whether CM was providing a post-proliferative signal for CTLp maturation, we next supplemented cultures with IL-2 on Day 2, added CM at various times after culture initiation and assayed cytotoxicity on Day 8 (Table 1). Addition of CM on Day 5 or 6 resulted in a significantly greater increase in the proportion of cultures developing CTL activity than addition at the initiation of the cultures or not at all. Later addition of IL-2 (Day 5) failed to provide the necessary signal for CTLp maturation. Thus it appears likely that IL-2 was providing the necessary signal for CTLp proliferation and that CM was providing a late-acting differentiation signal, but not an earlier one for CTLp activation.

CTLp cell lines require CM in order to acquire cytolytic activity

Further information regarding the nature of the signal provided by CM was derived from the study of CTLp cell lines. CTLp clones were generated by stimulating T cells under conditions (4000 cells/well), which ensured a high probability that each positive culture contained at most 1 CTLp. Three days prior to assaying for cytotoxicity, the cultures were divided into three replicates, IL-2 or IL-2 plus CM was added to two sets of the replicate cultures and the third set of replicates was saved for late subculturing. Figure 4 contains representative examples of the three types of results we observed. The majority of cultures (> 88%) exhibited no cytotoxic activity (e.g. B1, C7, F9, B10 and A8) whether exposed to IL-2 with or without CM; 5% (19/384) of the cultures exhibited influenza A virus-specific cytotoxicity in the presence of IL-2 alone or with CM (G1, F2, E3 and B11); and 7% (27/384) of the cultures exhibited influenza A virus-specific cytotoxicity only when IL-2 and CM were added (C1, B2, G7, B8 and F12). Cultures containing these CM-dependent CTLp were selected from the third set of replicate cultures and continuously propagated with periodic restimulation with antigen and IL-2. These long-term cultures continued to be dependent upon CM for the generation of cytotoxic activity (Fig. 5). Once exposed to CM the cultures remained cytotoxic and independent of CM for continued cytotoxicity.

Recombinant IFNα and IFNγ failed to provide the differentiation signal in limiting-dilution cultures

Some reports (Chen *et al.*, 1986; Simon *et al.*, 1986) have indicated that highly purified interferons exhibited cytotoxic T-

Table 2. Recombinant IFN-α. IFN-γ fail to provide the differentiation signal in limiting-dilution cultures*

Lymphokine tested		No. of cultures	No. positive
IL-2	(25 U/ml)	24	3
CM	(10% v/v)	24	14†
IFN-α_{2a}	(2·5 ng/ml)	16	0
	(0·5 ng/ml)	16	2
	(0·1 ng/ml)	16	2
IFN-α_{2b}	(2·5 ng/ml)	16	0
	(0·5 ng/ml)	16	2
	(0·1 ng/ml)	16	2
IFN-γ	(2·5 ng/ml)	16	0
	(0·5 ng/ml)	16	2
	(0·1 ng/ml)	16	2

*20,000 T cells were incubated with 100,000 stimulator cells. IL-2 was added to all cultures on Day 2 and various lymphokines were added on Day 5. The cultures were assayed for influenza A virus-specific cytotoxicity on Day 8. The data represent one of three experiments involving three different donors. Similar results were obtained with cells from the other two donors.

†Significantly different from cultures receiving IL-2 alone, Fisher's exact test, $P < 0.05$.

cell differentiation activity. However, in our system, addition of either IFN-α or IFNγ on Day 5 of an 8-day culture failed to provide the late signal found in CM (Table 2).

DISCUSSION

In this report we have examined the lymphokine requirement for secondary CTLp differentiation under limiting-dilution conditions. Under these conditions the effect of endogenous lymphokine production is minimized and CTLp expansion and differentiation is dependent upon the addition of exogenously produced helper factors (Skinner & Marbrook, 1976). Thus we observed that at a responder cell concentration where approximately 2/3 of the cultures contained a CTLp, fewer than 1/4 of the cultures contained proliferating cells or developed cytotoxicity in the absence of added lymphokines. This allowed us to then ask questions regarding the role of IL-2 and other lymphokines in CTLp expansion and differentiation. Addition of recombinant DNA-derived IL-2 to these cultures resulted in an increase in the number of cultures containing proliferating lymphocytes; however, the induction of cytotoxic activity required an additional differentiation signal as provided by CM from mitogen-stimulated PBMC cultures. While it was possible that CM could be providing an early activation signal necessary for optimal clonal expansion of the CTLp in response to the IL-2, it seemed more likely that it was providing a late differentiation signal. Thus, addition of IL-2 alone was sufficient for inducing proliferation in the microcultures and addition of CM did not increase the proportion of cultures containing proliferating lymphocytes. By contrast maximal CTLp maturation was found to occur when CM was added after IL-2. And CTLp cell lines were developed which proliferated in response to IL-2 and antigen but remained non-cytotoxic until exposure to CM. These cell lines, together with our results obtained from the limiting-dilution cultures, demonstrate that the maturation of influenza virus-specific secondary CTL required at least three signals: antigen presentation by the stimulator cells, a proliferative signal provided by IL-2, and a late signal for CTLp differentiation mediated by a lymphokine distinct from IL-2 and IFN.

Although we demonstrated a requirement for an additional lymphokine for CTL differentiation, others have reported that IL-2 alone could provide the necessary help in limiting-dilution cultures of polyclonally activated primary CTLp (Erard *et al.*, 1985; Brucker *et al.*, 1987). However, polyclonal CTL induction either by lectin or anti-CD3 stimulation may obscure the requirement for differentiation factor either by stimulating the endogenous production of the differentiation factor or by activating the cells in a manner which bypasses the requirement for a differentiation signal. In another report, IL-2 was sufficient to provide the required help in limiting-dilution cultures of mumps virus-specific secondary CTLp (Enssle, Wagner & Fleischer, 1987). However, their use of T cells purified by rosetting with sheep erythrocytes could have resulted in the polyclonal stimulation of the T cells leading to the endogenous production of lymphokines (Meur *et al.*, 1984). Also, although differentiation factor production is a limiting factor in CTL development in influenza virus limiting-dilution cultures, cells producing differentiation factor in response to some other antigen (e.g. mumps virus) may have a frequency and activity level high enough so as not to be a limiting factor.

In recent studies with influenza virus, Braakman *et al.* (1986) examined the role of IL-2 and $CD4^+$ cells in the generation of secondary cytotoxic $CD8^+$ cells in bulk lymphocyte cultures. Similarly to us, they demonstrated that addition of IL-2 on Day 0 resulted in the induction of non-specific cytotoxicity. However, in contrast with our results they found that recombinant DNA-derived IL-2 added on Day 3 could provide the necessary signal for CTLp induction. A likely explanation for this discrepancy lies in their use of bulk culture conditions, which may lead either to the presence of cells producing differentiation factor or the out-growth of a subpopulation of CTLp not requiring differentiation factor. By generating influenza virus-specific CTL under limiting-dilution conditions, we have reduced the possibility of endogenous lymphokine production and have thus directly demonstrated that IL-2 alone is insufficient for CTLp maturation in most but not all cultures. For those limiting-dilution cultures in which IL-2 appeared to be sufficient, we can offer several possible explanations: the presence of a differentiation factor-producing T-helper cell in the same culture as a CTLp, the possibility of endogenous differentiation factor production by the CTLp itself, or the existence of a subset of CTLp not requiring the factor(s) in CM.

While the existence of a CTL differentiation factor in mice has been under investigation for over 7 years (Finke, Sharma & Scott, 1971), little data has been published regarding a similar factor in humans. Gately, Wilson & Wong (1986) presented data suggesting that a mixed lymphokine supernatant contained a factor that synergized with IL-2 to augment human CTL responses in alloantigen-stimulated bulk lymphocyte cultures.

However, in that system the supernatant also augmented non-specific cytotoxicity, which may have accounted for some or all of the apparent CTL activity. Furthermore, their system did not distinguish between direct effects of the lymphokine on CTL differentiation or those mediated via other accessory cells. By using limiting-dilution cultures and CTLp cell lines we have been able to study CTL differentiation in the absence of non-specific cytotoxicity and, with the clones, in the absence of regulatory cells.

Chen *et al.* (1986) presented data indicating that interferons could induce cytotoxicity in human T-cell lines. However, their cells were cultured in an IL-2 supernatant made with TPA (12-0-tetradecanoyl phorbol-13-acetate), which is known to have a variety of effects on cell differentiation. Gromo *et al.* (1987) recently reported that IL-2 or IFN-γ could stimulate cytotoxicity in $CD8^+$ cells. However, their system differs from ours both in the stimulus used (antibodies to CD2 vs. antigen) and in the cytotoxicity assay (lectin-dependent cytotoxicity vs. antigen-specific lysis of infected targets). Thus the fact that they did not detect a requirement for a differentiation factor other than IL-2 or IFN-γ could result from the possibilities that anti-CD2-activated cells may not require this factor, that anti-CD2-activated cells may produce sufficient quantities of this factor, or that lectin-mediated cytotoxicity may not require a differentiation step required for antigen-specific cytotoxicity. While demonstrating a role for a differentiation factor other than IFN-γ, our results do not preclude a role for interferon in the induction of a CTL response. Indeed, Inghirami *et al.* (1985) reported that IFN-α, IFN-β and IFN-γ enhanced the cytotoxic response in mixed lymphocyte cultures (MLC) when added early to the cultures. Likewise, work in our laboratory has shown that early addition of IFN-γ to MLC augmented both alloantigen-specific cytotoxicity and proliferation and increased early activation antigen expression on $CD8^+$ but not $CD4^+$ cells (Siegel, 1988).

In summary, we have developed two systems, limiting-dilution cultures and CTLp cell lines, which allow for the study of the maturation of antigen-specific CTLp under conditions that minimize the effects of endogenous lymphokine production and non-specific cytotoxicity. In both systems we have found that secondary CTL development depends not only upon IL-2, but also on a novel differentiation factor distinct from IL-2, IFN-α and IFN-γ. The identification and characrerization of the differentiation factor described in this report is currently under investigation.

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