Brief Definitive Report

DISSECTION OF THE PROLIFERATIVE AND DIFFERENTIATIVE SIGNALS CONTROLLING MURINE CYTOTOXIC T LYMPHOCYTE RESPONSES*

By HERMANN WAGNER, CONNY HARDT, BARRY T. ROUSE,‡ MARTIN RÖLLINGHOFF, PETER SCHEURICH, and KLAUS PFIZENMAIER

From the Institut für Medizinische Mikrobiologie der Johannes Gutenberg-Universität, D-6500 Mainz, Federal Republic of Germany

The induction of antigen-specific primary cytotoxic T lymphocyte (CTL) responses in vitro clearly requires interactions between several cell types and soluble mediators (1–5). One essential interaction occurs between the Lyt-2^+ CTL precursor (CTL-P) and an antigen-activated Lyt-1^+ helper T cell (6–8). This interaction is considered to occur by way of soluble mediator such as interleukin 2 (IL-2), a lymphokine produced by antigen-triggered helper cells that binds to IL-2 receptors expressed on CTL-P as a sequel to their preactivation by antigen or mitogen (9, 10). The expression of IL-2 receptors probably occurs as antigen- or mitogen-triggered CTL-P move from G_0 to G_1 in the cell cycle (11). Further progress through the cell cycle, then, only occurs if IL-2 is present. However, whereas IL-2 may indeed drive an antigen- or mitogen-preactivated CTL-P into proliferation, it is not clear whether IL-2 is also the stimulus that causes the CTL-P to differentiate into effector CTL. This uncertainty exists because the source of IL-2 used by most investigators to study its role in CTL induction comes from concanavalin A (Con A) supernatants, which contains other biologically active molecules as well. We realized that IL-2 alone may not be sufficient to mature cells into CTL in preliminary experiments in which certain sources of IL-2 were shown capable of driving Con A-preactivated Lyt-2^+ cells to proliferation but not differentiation into CTL (12). However, as we show in this communication, IL-2 preparations unable to cause CTL differentiation can be reconstituted with Con A supernatants that have been selectively depleted of their IL-2 content. As such, our results are the first to provide evidence for the existence of a cytotoxic T cell differentiation factor (CTDF) that functions to convert IL-2-driven proliferating CTL-P into CTL.

Materials and Methods

Mice. CBA/Ca (H-2^k) mice were obtained from OLAC Ltd., Shaw's Farm, Blackthorn, England.

IL-2-producing T Lymphoma Cells. The IL-2-producing EL_4 variant (13) was kindly provided by Dr. Farrar (National Institutes of Health). The IL-2-producing, radiation-induced T cell lymphoma LBRM-33 (14) was a kind gift of Dr. G. Cudkowicz, Buffalo, N. Y.

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Preparation of IL-2 Batches. To produce LBRM-33 cell-derived IL-2, LBRM cells were grown in culture medium (a 50% vol/vol mixture of Click's and RPMI 1640 media supplemented with 10 mM Hepes, fresh glutamine, 5×10^{-5} M 2-mercaptoethanol, and 5% fetal calf serum (FCS)) and were washed and replated in serum-free medium containing 1% phytohemagglutinin (PHA-M) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) for 6 h. After exchanging PHA-containing medium with normal medium, the cells were incubated for an additional 24 h, and the supernatant was recovered. EL_4-derived IL-2 was produced by seeding cells in serum-free medium (density of 1×10^6/ml) and stimulating for 36 h with 10 ng/ml phorbyl myristic acetate (Sigma Inc., Munich, Federal Republic of Germany [FRG]). IL-2 from CBA mouse spleen cells (10^7 cells/ml) was produced under serum-free conditions by stimulating with 1 μg/ml Con A (Pharmacia Fine Chemicals, Uppsala, Sweden) for 24 h as described previously (6). The supernatants were harvested and are termed "crude supernatant."

Semipurification of IL-2. The various culture supernatants were concentrated by ultrafiltration with an Amicon YM-10 membrane (Amicon Corp., Scientific Sys. Div., Lexington, MA) and subjected to fractionated $(NH_4)_2SO_4$ precipitation. Proteins precipitating between 40 and 80% $(NH_4)_2SO_4$ saturation were dialyzed against 0.02 M phosphate-buffered saline (PBS) (pH 7.4), concentrated, and applied to a previously calibrated Sephadex G-100 column. Effluent fractions were tested for IL-2 activity.

Assay for IL-2 Activity. IL-2 was assayed by its ability to sustain in vitro growth of Cl-3 cells, a cloned continuous IL-2-dependent line of $H\text{-}2^k$ anti-$H\text{-}2^d$ CTL kindly provided by Dr. Th. Hünig, Institut für Virologie, Würzburg, FRG. In brief, the Cl-3 cells were washed free of growth medium and were seeded at 4×10^3/100 μl into flat-bottomed 96-well microplates (Greiner GmbH, Nürtingen, FRG). After the addition of test samples of IL-2, cultures were incubated at 37°C for 24 h, and pulsed for 4 h with 0.5 μCi of [^{3}H]thymidine (TdR) (NEN Chemicals, Dreieich, FRG) as described (6).

Selective Depletion of IL-2 from Crude Supernatant. IL-2-addicted Cl-3 cells were washed free of growth medium and incubated in fresh medium for an additional 2 h at 37°C. Thrice-washed cells (30×10^6) were resuspended in 3 ml of crude supernatant at 4°C for 30 min, pelleted, and incubated at 37°C for 2 h in fresh medium to allow for shedding of receptor-bound IL-2 (M. Röllinghoff, unpublished observations). The cells were reused for absorption five more times, after which the crude supernatant was tested for remaining IL-2 activity.

Preparation of Lyt-$1^-2,3^+$ Cells Depleted for Macrophages. Splenic lymphocytes from 4–6-wk-old CBA mice were passed over nylon wool to enrich for T lymphocytes and then over Sephadex G-10 to further deplete for adherent cells. Subsequently, 10^7 cells were treated with a 1:200 dilution of a monoclonal anti Lyt-1.1 antibody (NEN Chemicals) and a nontoxic rabbit complement diluted 1:12. Dead cells were removed by centrifugation over Ficoll. Applying the indirect immunofluorescence technique with the appropriate Lyt antisera, virtually all viable T cells proved to express the Lyt-$1^-2,3^+$ phenotye.

Polyclonal Activation of CTL-P. Replicates of Lyt-$1^-2,3^+$ T responder cells (10^5) were seeded into flat-bottomed 96-well microplates in 200 μl medium containing 2.5 μg Con A/ml plus the lymphokine batch indicated. The proliferative response was assayed by pulsing cultures after 42 h for 6 h with 0.5 μCi ^{3}H[TdR]. The polyclonal induction of CTL was measured in a lectin-mediated universal readout (15), and cells of four microcultures were pooled, washed once, and resuspended in 1 ml. Graded numbers of viable cells were incubated with a constant number (2×10^3) ^{51}Cr-labeled P815 tumor cells in the presence of 10 μg/ml PHA-P (Gibco Laboratories). Percent specific lysis was calculated as detailed previously (6).

Preparation of Macrophage Culture Supernatant Rich in IL-1. Thioglycolate-induced peritoneal macrophages (4×10^6/ml) were plated in 2 ml culture medium containing 0.5% FCS and incubated with 5 μg/ml lipopolysaccharide (Difco Laboratories, Detroit, Michigan) for 24 h. The culture supernatants were harvested and dialyzed against culture medium. These supernatants, which lacked detectable IL-2 activity, were rich in IL-1 activity as assayed by their ability to replace macrophages required for the Con A-induced production of IL-2 from purified T cells (9). The activity of IL-1 was compared with a standard preparation arbitrarily set as 100 U/ml (16). The IL-1 preparation used contained 250 U.

Results and Discussion

To evaluate our notion that IL-2 alone is not sufficient to cause the differentiation

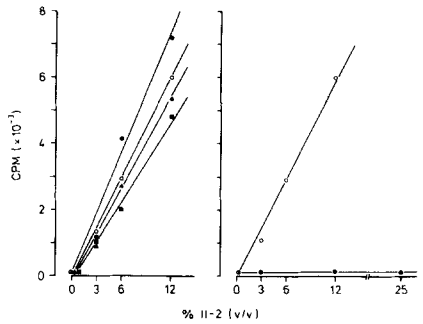

FIG. 1. Left: comparison of the IL-2 activity of various lymphokines. In preliminary experiments, the IL-2 activity of the lymphokine batches used were adjusted to comparable levels. Thereafter, the IL-2 activity of crude supernatant (○), semipurified IL-2 from the crude supernatant (■), from EL_4 tumor cells (●), and from RBRM tumor cells (▲) were titrated on IL-2-addicted Cl-3 cells. Right: lack of IL-2 activity within crude supernatant after repeated absorption with Il-2-addicted Cl-3 cells.

of primary CTL in vitro, our experimental protocol included the following rationale. First, it was essential to avoid interference by regulatory Lyt-1^+ T cells during CTL induction because such cells would provide IL-2 as well as other putative helper factors endogeneously. To this end, negatively selected, macrophage-depleted Lyt-2^+ cells were used as the responder population. Second, we needed to produce a source of factor that retained differentiation activity but lacked demonstrable IL-2. This was achieved using an IL-2-addicted cell line to absorb out IL-2 from crude Con A-activated supernatants. As shown in Fig. 1 (right panel), absorption of Con A supernatants five times with Cl-3 cells completely removed IL-2, as measured by its ability to cause proliferation of Cl-3 cells. That this factor still had CTDF activity is described subsequently.

Four sources of IL-2 were investigated for their ability to cause proliferation of Lyt-2^+ T cells as well their differentiation into CTL. As shown in Fig. 1 (left panel), the four factors EL_4, LBRM, crude Con A supernatant, and semipurified Con A supernatant exhibited comparable IL-2 activity, as measured by their ability to cause proliferation of the IL-2-addicted cell line Cl-3. In addition, as shown in Table I, all factors caused Con A preactivated Lyt-2^+ T cells to proliferate to a similar extent. However, in contrast to their comparable proliferation activities (i.e., IL-2), it was only the crude Con A supernatant that caused the induction of high levels of cytolytic activity (Fig. 2 A). These data indicate that IL-2-driven proliferation (growth) of preactivated Lyt-2^+ cells does not necessarily result in CTL generation. Furthermore, they infer that a second activity present in crude supernatants causes proliferating CTL-P to differentiate into CTL.

Further evidence for this notion was obtained in experiments in which Con A supernatants depleted of IL-2 by absorption with clone 3 cells and lacking proliferation activity (Fig. 1 B) were shown able to cause CTL induction (Fig. 2 A, B, C). However, far more striking was the observation that the IL-2-depleted Con A supernatant could restore the CTL-inducing activity of those sources of IL-2 that alone caused only Con A-preactivated CTL-P to proliferate (Fig. 2 A, B, C). In these combination experiments strong cytolytic responses were induced.

Taken together our results lead us to conclude that the role of IL-2 in CTL induction is to cause the clonal expansion of those CTL-P that have been preactivated

TABLE I

Proliferative Responses of Con A-preactivated Lyt-1⁻2,3⁺ T Responder Cells in the Presence of IL-2 and CTDF

Lyt-1⁻2,3⁺ T responder cells plus Con A		
Lymphokines added		[³H]TdR incorporation
IL-2 (10% vol/vol)	Absorbed crude Con A supernatant (5% vol/vol)	
		cpm × 10^{-3}
—	—	3.9 ± 0.3
—	+	5.3 ± 0.2
Crude Con A sup	—	16.2 ± 0.6
LBRM*	—	13.5 ± 0.3
LBRM*	+	10.7 ± 0.3
EL_4*	—	18.1 ± 0.6
EL_4*	+	17.9 ± 1.7
Crude Con A supernatant*	—	10.8 ± 0.4
Crude Con A supernatant*	+	10.4 ± 0.6
—	IL-1‡	7.2 ± 0.7

* Semipurified. See Materials and Methods.

‡ Culture supernatant of activated macrophages containing 250 U of IL-1.

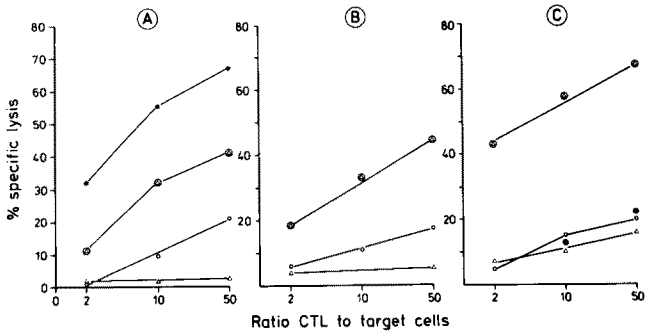

FIG. 2. Crude Con A supernatant depleted for IL-2 contains CTDF. Macrophage-depleted Lyt-1⁻2,3⁺ responder T cells were cultured for 48 h in the presence of 0.5 μg/ml Con A plus the lymphokine batches indicated below. Thereafter the lytic activity induced was tested in the presence of PHA (18) towards P815 target cells. The background lysis of P815 target cell during the 4-h assay time did not exceed 22%. (A) △, 10% (vol/vol) IL-2 semipurified from crude supernatant; ●, 10% (vol/vol) crude supernatant; ○, 10% (vol/vol) IL-2-depleted (absorbed) crude supernatant; ⓐ, 5% (vol/vol) of 1:1 mixture of IL-2 semipurified crude supernatant and IL-2-depleted crude supernatant. (B) △, 10% (vol/vol) LBRM-derived semipurified IL-2; ○, 10% (vol/vol) IL-2-depleted crude supernatant; ⓐ, 5% (vol/vol) of 1:1 mixture of both. (C) △, 10% (vol/vol) EL_4-derived semipurified IL-2; ○, 10% (vol/vol) IL-2-depleted crude supernatant; ⓐ, 5% (vol/vol) of 1:1 mixture of both; ⓘ, 5% (vol/vol) of 1:1 mixture of EL_4-derived semipurified IL-2 plus IL-1 (see Materials and Methods).

by antigen or mitogen. However, such proliferating CTL-P do not differentiate into cytolytic effector cells. For this to occur, they must be stimulated by a second factor, distinct from IL-2, termed here CTDF.

Although our data implicate the Lyt-2⁺ cell as the cell that responds to CTDF, at this stage we can only speculate as to the nature of the cell that produces the factor. Presumably it is not the Lyt-2⁺ cell itself, since such cells, driven to proliferate by IL-2, fail to differentiate into CTL. The data depicted in Fig. 2 C also argue against

the macrophage as the producer cell, because the supernatants of activated macrophage were devoid of CTDF activity. Because these supernatants were rich in IL-1, these data also serve to indicate that IL-1 is not the differentiation factor. Clearly further investigations are necessary to define the origin of CTDF and its relationship to other known lymphokines (17, 18). We do not favor the idea that CTDF is identical to γ interferon (IF), because Lyt-2^+ cytotoxic T cells themselves produce γ-IF and should therefore be expected to provide the differentiation signal endogenously (19). Biochemical and functional investigations are required before a clearer picture will emerge.

The dissection of proliferative and differentiative signals controlling cytolytic responses of CTL-P in a lectin-mediated universal readout resembles that recently observed for IgM responses in unprimed B cells (20, 21). Could it be that the rules governing growth and differentiation of effector cells of both the T cell and B cell lineage parallel each other?

Summary

Evidence is presented that interleukin 2 (IL-2) is not sufficient to cause the differentiation of primary cytotoxic T lymphocytes (CTL). Sources of IL-2 were compared for their ability to cause proliferation as well as differentiation into CTL. Whereas all factors caused proliferation, only the crude Con A supernatant had cytotoxic T cell differentiation factor (CTDF) activity. Furthermore, factors absorbed with an IL-2-dependent cell line to remove IL-2 still retained CTDF activity. Thus, IL-2 functions to cause clonal expansion of CTL precursors preactivated by antigen or mitogen, but for their differentiation into CTL, an additional factor is required, here called CTDF.

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