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ACTIVATION OF CYTOTOXIC T LYMPHOCYTES REQUIRES AT LEAST TWO SPLEEN CELL-DERIVED HELPER FACTORS BESIDES INTERLEUKIN 2

WERNER FALK, DANIELA N. MÄNNEL, AND WULF DRÖGE

From the Institut für Immunologie und Genetik, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-6900 Heidelberg, West Germany

The dependency of induction of T cell cytotoxicity on lymphokines was studied. 1 × 10^5 nylon wool-purified thymic lymphocytes or 10^4 spleen cells were cultured with TNP-haptenated syngeneic UV-irradiated spleen cells in the presence of a variety of lymphokine preparations. Concanavalin A-induced spleen cell supernatants mediated strong cytotoxic responses in this system. Three other preparations, namely, a partially purified IL 2 preparation from PMA-stimulated EL-4 thymoma cells, a Con A-induced spleen cell supernatant that was absorbed with an IL 2-dependent cell line, and a Con A-induced supernatant that was dialyzed at pH 2 were all ineffective in mediating a cytotoxic response. In reconstitution experiments, cytotoxic responses were only obtained when either the absorbed preparation or the pH 2-treated preparation was mixed with the IL 2 preparation from EL-4 cells. No reconstitution occurred after mixing of the absorbed with the pH 2-treated preparation. pH 2 treatment of the absorbed preparation did not abolish its synergistic effect when added to the IL 2 preparation from EL-4 cells. These results led to the conclusion that activation of cytotoxic lymphocyte precursors requires at least two other lymphokines in addition to IL 2. One T cell cytotoxicity-inducing factor (TCF1) remained in Con A-induced supernatants after absorption with IL 2 receptor-bearing T cell line cells. It was pH 2-resistant and was not found in EL-4 supernatants. A second T cell cytotoxicity-inducing factor (TCF2) was pH 2-sensitive and was found in Con A-induced spleen cell supernatants as well as in interferon-free supernatants of PMA-stimulated EL-4 cells. This activity co-purified with IL 2. It was absorbed by the IL 2-dependent T cell line together with IL 2. IL 2 differs from TCF2 since it is pH 2-resistant.

The induction of cytotoxic T lymphocytes (CTL) is the result of interactions between different cell types. Ia-positive macrophage-like accessory cells as well as T helper cells (1–17) are required to induce the maturation and proliferation of CTL precursor cells into effector CTL. There is evidence that during these interactions antigen-nonspecific lymphokines are produced, which participate in these processes. It is possible to overcome the requirement for accessory cells with factors present in concanavalin A- (Con A) induced supernatants of spleen cells (18). The involvement of the interleukins IL 1 and IL 2 (11, 19–25) in CTL activation has been shown. IL 1 is required for the activation of the IL 2-producing helper T cells and thus participates only indirectly in the activation of CTL, whereas IL 2 is required for the proliferation of CTL precursor cells. Recently, we found evidence for a late-acting helper factor that is different from IL 2 (26). In addition, two other reports showed the ineffectiveness of IL 2 alone in converting mitogen-induced CTL precursor cells into effector CTL (27, 28). Both groups presented evidence that the response was reconstituted by differentiation factors present in Con A-induced supernatants. In this paper evidence is shown that in an antigen-driven activation of cytotoxic T cells, at least two factors other than IL 2 are required. One of these factors may be identical with the reported differentiation factors.

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MATERIALS AND METHODS

Mice. C3H/TIF and DBA/2J were purchased from Bomholtgard, Ry, Denmark, and had food and water *ad libidum*. They were used at 6 to 10 wk of age.

Culture medium. If not indicated otherwise, the culture medium used was RPMI 1640 (GIBCO, powdered media) with L-glutamine (2×10^{-3} M) and supplemented with 10% heat-inactivated fetal calf serum, HEPES[1] (0.02 M), 2-mercaptoethanol (4×10^{-5} M), and gentamicin (50 μg/ml).

Lymphokine preparations. Con A- (Pharmacia) induced supernatants were prepared by conventional methods. Splenic single cell suspensions were adjusted to 10^7 cells/ml in culture medium and were incubated for 24 hr with 5 μg Con A/ml in moist air containing 5% CO_2. To cell-free supernatants, α-methyl-D-mannoside (Sigma) was added to a final concentration of 1 mM. All preparations were stored at 4°C.

T cell cytotoxicity assay. The microcultures were prepared as described previously (29), based on the procedures of Shearer (30) and Teh *et al.* (15). In short: 10^4 to 10^5 spleen or thymus responder cells were co-cultured with 3×10^5 UV-irradiated TNP-haptenated syngeneic spleen cells or allogeneic cells as stimulators. Varying amounts of lymphokine preparations were added so that the total volume per well was 0.16 ml. After 5 days of culture in humidified air with 5% CO_2, 2×10^4 ^{51}Cr-labeled TNP-haptenated syngeneic or allogeneic target cells were added in 0.04 ml of culture medium. Target cells were prepared by culturing spleen cells at a density of 3×10^6/ml of culture medium and were incubated for 16 hr with 5 μg Con A/ml. After 3 to 4 hr, the plates were centrifuged, and 0.1 ml was removed and counted in a gamma-counter. Maximal release was determined by lysis after addition of sodium dodecyl sulfate. Data are presented as specific ^{51}Cr-release (experimental count − spontaneous release/SDS count − spontaneous release) × 100. Spontaneous release (SR) was determined in supernatants of wells without added lymphokines. Data are means of triplicate cultures. The standard error of the mean was always ≤ 2.3%.

Assay for IL 2. The amount of IL 2 in supernatants was measured by their ability to support the growth of the T cell clone W-2 (originally M-2, cloned by Michael Stöck, Hannover, West Germany). The culture supernatants were titrated by serial 2-fold or 3-fold dilutions in flat-bottom tissue culture plates (Flow Laboratories) in a volume of 0.1 ml of culture medium without FCS. One hundred microliters of W-2 cell suspension (10^5/ml) in culture medium were added per well, and the plates were incubated at 37°C in moist air with 5% CO_2 for 20 hr. One microcurie of ^{3}H-TdR in 25 μl of RPMI 1640 was added per well, and the plates were incubated for another 4 to 6 hr. The cells were then harvested with a Skatron Cell Harvester (Flow Laboratories), and the filters were counted in a liquid scintillation counter. Each point was assayed in duplicate. Data are given as IL 2 activity units, based on the method described by Farrar *et al.* (19). Units were calculated with an HP 9815 computer using linear regression analysis of the linear part of the titration curve.

Interferon assay. Interferon (IFN) titrations were performed in microtiter wells. L929 cells in 0.2 ml of RPMI 1640 + 5% FCS per well were challenged

[1] Abbreviations used in this paper: IL 2, interleukin 2; IFN, interferon; TCF1 and 2, T cell cytotoxicity-inducing factors 1 and 2; SR, spontaneous release; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

with vesicular stomatitis virus (Indiana strain). One unit was defined as the minimal amount of interferon capable of conferring protection to 50% of the cells. One microtiter unit per 0.2 ml corresponds to 2 NIH reference units. All titers are expressed in laboratory units.

Nylon wool treatment. In some experiments the responder cells were purified by one passage through a nylon wool column according to Julius *et al.* (31).

Preparation of partially purified IL 2. An IL 2 preparation was prepared as described by Hilfiker *et al.* (32). EL-4 thymoma cells (generously supplied by J. J. Farrar, NIDR, NIH, Bethdsda, MD) were suspended at 10^6/ml in RPMI 1640 containing no serum or 0.5% heat-inactivated horse serum. Ten nanograms per milliliter of phorbol myristic acetate (PMA) were added, and the cells were incubated for 48 hr. The cell-free supernatant was concentrated on an Amicon PM 10 membrane. Proteins were then precipitated with ammonium sulfate (80% saturation). A small amount of FCS was added to preparations made without serum to prevent loss of activity during purification. The precipitate was washed once and dissolved in water. The dissolved material was equilibrated with 0.8 M ammonium sulfate in 0.01 M phosphate buffer, pH 7, and was applied on a Penylsepharose column (Pharmacia Fine Chemicals). The column was then washed with 2 volumes of 0.64 M ammonium sulfate + 10% ethanediol. The IL 2-containing peak was finally eluted with 40% ethanediol in 0.16 M ammonium sulphate. The IL 2 fractions were concentrated or an Amicon PM 10 membrane and were equilibrated with column buffer (0.05 M Tris-hydroxymethylacetate, 0.1 M sodium chloride, pH 7.2, containing 0.05% polyethleneglycol 6000). The material was then applied to a Sephadex G100 column, and the activity was eluted with an apparent molecular weight of approximately 32,000. The peak fractions were pooled and dialyzed against culture medium after adding FCS to a final concentration of 10%.

Absorption of IL 2. Con A-induced supernatants were depleted of IL 2 by absorption with an IL 2 receptor-bearing T cell line. One milliliter of Con A-induced supernatant was mixed with approximately 10^5 W-2 cells and was incubated for 48 hr. The cells were then centrifuged, and the supernatant was dialyzed against culture medium and then again incubated for 48 hr with 10^5 W-2 cells per ml. The supernatant was again dialyzed against culture medium and α-methyl-mannoside was added to a final concentration of 1 mM. These supernatants contained less than 0.01 units/ml of IL 2 activity.

Dialysis at pH 2. Con A-induced supernatants were dialyzed overnight in 50 mM glycine buffer, pH 2, and were readjusted to pH 7 by dialysis in culture medium.

RESULTS

The aim of the study was to identify activities that play a role in the activation of T cells for cytotoxicity. A microsystem was chosen to minimize the number of helper cells in the responder population. The stimulator cells were UV-irradiated to destroy any metabolic and proliferative activity. Gamma-irradiated stimulator cells have been shown to provide helper activity and were therefore not suitable (26). Under these conditions, the cultures produced no cytotoxic activity after 5 days even with up to 12 $\times$ 10^4 responder cells unless lymphokine preparations were added (Fig. 1). Experiments with either syngeneic or allogeneic stimulator cells gave similar results.

Failure of partially purified IL 2 to induce cytotoxicity. IL 2-containing supernatants were prepared by PMA stimulation of a clone of EL-4 thymoma cells (32). Partial purification was achieved by consecutive ammonium sulfate precipitation, Phenylsepharose chromatography, and Sephadex G100 chromatography. The final pool of IL 2-containing fractions had up to 1000 laboratory units of IL 2 (i.e., about 10 times the titer of normal Con A-induced supernatants) and was diluted in culture medium to the indicated concentrations. As shown in Figure 2, these IL 2 preparations were incapable of inducing a cytotoxic response under the conditions of the system used. In addition, they did not inhibit activation of CTL precursors in the presence of a standard Con A-induced supernatant (Fig. 2). The IL 2 titers in both preparations were comparable. The conclusion from these experiments was that IL 2 was not sufficient to induce a cytotoxic response and that this IL 2 preparation was lacking a lymphokine that is necessary for induction of cytotoxicity.

Reconstitution of the cytotoxic response with an IL 2-deficient Con A-induced supernatant. A standard Con A-induced supernatant was absorbed with an IL 2-dependent T cell line until no trace of IL 2 activity (less than 0.01 units/ml) was detectable in the supernatant. The titer of interferon-γ (IFN-γ) was measured to test for nonspecific loss of activity and was found slightly decreased (1100 units to 600 units), but this decrease was independent of the incubation time. The IL 2-free Con A-induced spleen cell supernatant by itself was not able to support a cytotoxic response (Fig. 3), but it was found to reconstitute the deficient IL 2 preparation from EL-4 cells utilizing either splenic (Fig. 3*A* or thymic responder cells (Fig. 3*B*). When graded amounts of the absorbed preparation were added to a constant amount of the IL 2 preparation from EL-4 cells, cytotoxicity was induced, and the dose-response curve was comparable to that of a normal Con A-induced spleen cell supernatant with the same titer of IL 2. The following controls were made but are not included in the figures: Neither the IL 2 preparation from EL-4 cells nor the absorbed preparation inhibited the response to a normal Con A-induced supernatant. Also, comparable dose-response curves were obtained when graded amounts of the IL 2 preparation from EL-4 cells were added to a constant amount of the absorbed preparation. The strong synergistic effect seen in these experiments suggested that there must be at least one lymphokine in a standard Con A-induced spleen cell supernatant that is different from IL 2 and is required for induction of cytotoxicity. This lymphokine was termed T cell cytotoxicity-inducing factor 1 (TCF1).

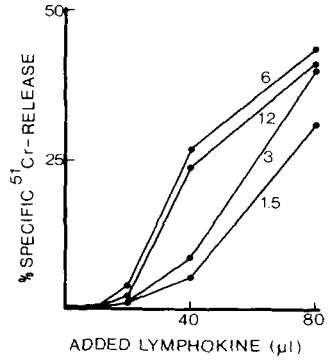

Figure 1. Cytotoxic responses at various lymphokine and responder cell concentrations. 12, 6, 3, and 1.5 $\times$ 10^4 C3H spleen cells were cultured with 3 $\times$ 10^5 TNP-haptenated, UV-irradiated C3H spleen cells and with graded amounts of Con A-induced spleen cell supernatants in a total volume of 0.16 ml. On day 5 of culture, 2 $\times$ 10^4 TNP-haptenated, ^{51}Cr-labeled C3H spleen cell blasts were added in 0.04 ml. ^{51}Cr-release was measured after 4 hr. High control: 3580 cpm; SR = 15%.

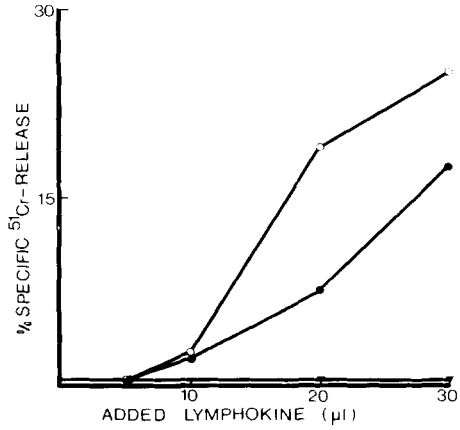

Figure 2. IL 2 alone does not support a cytotoxic response. 1 $\times$ 10^4 C3H spleen cells were cultured with 3 $\times$ 10^5 UV-irradiated DBA spleen cells and with different lymphokine preparations. On day 5, 2 $\times$ 10^4 ^{51}Cr-labeled DBA/2 spleen cell blasts were added as targets, and ^{51}Cr-release was measured after 4 hr. (●) Con A-induced spleen cell supernatant (80 U/ml IL 2); (▼) IL 2 preparation from EL-4 cells (75 U/ml IL 2); (○) Con A-induced spleen cell supernatant + 30 μl IL 2 preparation from EL-4 cells.

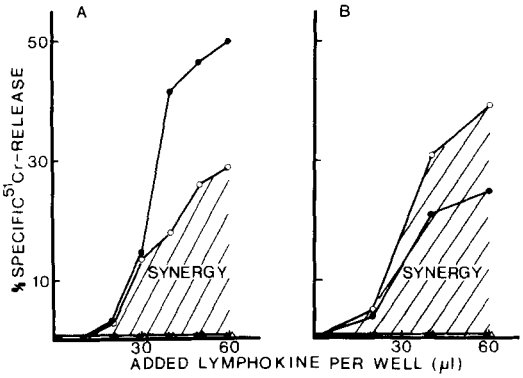

Figure 3. Synergistic effect after mixing the IL 2 preparation from EL-4 cells with the absorbed Con A-induced spleen cell supernatant. 3 × 10^4 C3H spleen cells (*A*) or 6 × 10^4 nylon wool-nonadherent C3H thymocytes (*B*) were cultured with TNP-haptenated, UV-treated stimulator cells and the following lymphokines: (●) Con A-induced spleen cell supernatant (100 U/ml IL 2 in *A* and 80 U/ml IL 2 in *B*); (▲) IL 2 preparation from EL-4 cells (75 U/ml IL 2 in *A* and 950 U/ml IL 2 in *B*); (△) absorbed Con A-induced spleen cell supernatant (< 0.01 U/ml IL 2); (○) absorbed Con A-induced spleen cell supernatant + 20 μl (*A*) or 60 μl (*B*) of the IL 2 preparation from EL-4 cells. The high controls were 2273 cpm (*A*) and 2130 cpm (*B*); SR = 22% (*A*) and 13% (*B*). The shaded areas represent the synergistic effect. For other details see legend to Figure 1.

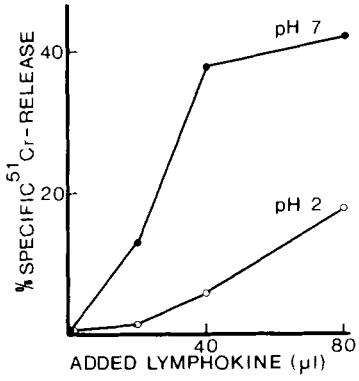

Figure 4. Decrease of cytotoxicity-inducing activity of Con A-induced spleen cell supernatants after pH 2 dialysis. Two portions of the same Con A-induced spleen cell supernatant were dialyzed overnight at either pH 2 or pH 7 and then assayed in a microcytotoxicity assay. 1 × 10^5 nylon wool-nonadherent C3H thymocytes were cultured with TNP-haptenated, UV-irradiated C3H stimulator cells and the indicated lymphokines. High control: 1650 cpm; SR = 22%.

Reconstitution of the cytotoxic response with a pH 2-treated Con A-induced spleen cell supernatant. In order to further characterize this lymphokine, a Con A-induced spleen cell supernatant was dialyzed overnight at pH 2. A control sample of the supernatant was dialyzed at pH 7 by an otherwise identical protocol. IL 2 activity was unchanged or was only slightly decreased after pH 2 dialysis, whereas IFN activity was abolished (data not shown). On the other hand, helper activity of the two preparations for induction of cytotoxicity was decreased by about 75% after pH 2 treatment (Fig. 4).

A strong synergy was seen when this pH 2-treated Con A-induced spleen cell supernatant was mixed with the TCF1-free IL 2 preparation from EL-4 cells (Fig. 5). Taken together, these two experiments led to the conclusion that the pH 2-treated lymphokine still contained IL 2 and TCF1, and that TCF1 was not destroyed by pH 2 treatment. Therefore, for induction of cytotoxicity, there must be at least one other activity present that is pH 2-sensitive and is found in supernatants of Con A-stimulated spleen cells, as well as in supernatants of PMA-stimulated EL-4 cells. This activity is not interferon-γ since our EL-4 supernatants were devoid of any antiviral activity (data not shown). This pH 2-sensitive factor was termed T cell cytotoxicity-inducing factor 2 (TCF2). When an absorbed Con A-induced spleen cell supernatant (containing TCF1) was mixed with a pH

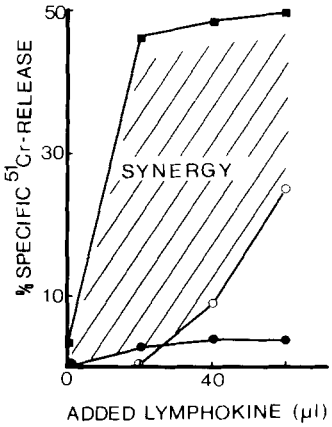

Figure 5. Synergism between the IL 2 preparation from EL-4 cells and the pH 2-treated, Con A-induced spleen cell supernatant. 1 × 10^5 nylon wool-nonadherent C3H thymocytes were cultured with 3 × 10^5 TNP-haptenated, UV-irradiated C3H stimulator cells and with the following lymphokines: (○) Con A-induced supernatant dialyzed in pH 2 buffer overnight (30 U/ml IL 2); (●) IL 2 preparation from EL-4 cells (950 U/ml); (■) Con A-induced spleen cell supernatant dialyzed at pH 2 + 20 μl of the IL 2 preparation from EL-4 cells. Similar results were obtained when graded amounts of IL 2 preparations from EL-4 cells were added to a constant amount of pH 2-treated supernatant. High control: 1920 cpm; SR = 23%. The shaded area depicts the synergistic effect.

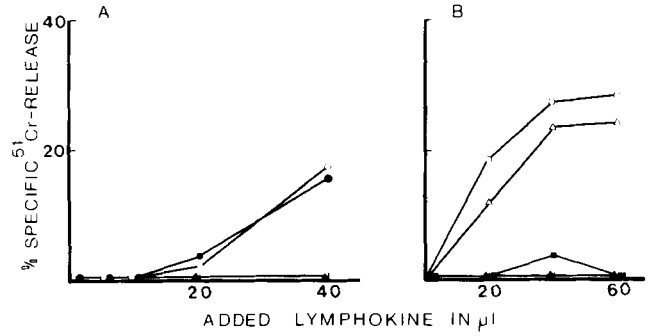

Figure 6. Demonstration that the pH 2-sensitive lymphokine is not TCF1. 1 × 10^5 nylon wool-nonadherent C3H thymocytes were cultured with 3 × 10^5 TNP-haptenated, UV-irradiated C3H stimulator cells and with various lymphokine preparations: (▲) Con A-induced spleen cell supernatant absorbed with the IL 2-dependent W-2 T cell line and same absorbed supernatant dialyzed at pH 2 (containing TCF1 and < 0.01 U/ml IL 2); (■) IL 2 preparation from EL-4 cells (950 U/ml IL 2); (○) pH 2-treated, Con A-induced spleen cell supernatant (TCF2 deficient, 60 U/ml IL 2); (●) pH 2-treated, Con A-induced spleen cell supernatant + 20 μl of the absorbed Con A-induced spleen cell supernatant (TCF1); (□) absorbed Con A-induced spleen cell supernatant + 20 μl of the IL 2 preparation from EL-4 cells; (△) pH 2-treated *and* absorbed Con A-induced spleen cell supernatant + 20 μl of the IL 2 preparation from EL-4 cells. *A*, Lack of reconstitution of absorbed Con A-induced spleen cell supernatant by pH 2-treated, Con A-induced spleen cell supernatant. In contrast, a mixture of 20 μl of the IL 2 preparation from EL-4 cells (70 U/ml IL 2) and 20 μl of the Con A-induced, pH 2-dialyzed spleen cell supernatant used in this assay mediated 35% specific ^{51}Cr-release. High control: 2001 cpm; SR = 11%. *B*, TCF1 in absorbed Con A-induced spleen cell supernatant is not pH 2-sensitive. The TCF1-containing absorbed Con A-induced spleen cell supernatant was dialyzed overnight at pH 2 and brought back to pH 7 by dialysis in culture medium. High control: 1921 cpm; SR = 23%.

2-treated Con A-induced supernatant, there was no detectable reconstitution of the cytotoxic response, whereas the synergy of pH 2-treated supernatant with the IL 2 preparation from EL-4 cells was expectedly strong (Fig. 6*A*). This indicated that the absorption procedures on the IL 2-dependent W-2 T cell line removed not only the IL 2 but also the TCF2 activity.

To demonstrate the pH 2-stability of TCF1 directly, a TCF1 preparation was dialyzed at pH 2 and assayed for synergy. Figure 6*B* shows that the absorbed *and* pH 2-treated Con A-induced spleen cell supernatant was still able to complement the IL 2 preparations from EL-4 cells.

TCF1 and TCF2 were found to be produced by live spleen cells upon stimulation with Con A. Neither component was found in supernatants of unstimulated spleen cells or in supernatants of Con A-stimulated, UV-treated spleen cells (data not shown).

This observation also excluded the possibility that the TCF1 or TCF2 activities were due to Con A. Neither activity was detected in nonstimulated supernatants of EL-4 cells or in supernatants of PMA-stimulated, UV-killed EL-4 cells (data not shown).

Taken together, the following conclusions can be drawn from these experiments. Two factors besides IL 2 are necessary for induction of cytotoxicity. Con A-induced spleen cell supernatants contained all three activities. The IL 2 preparation from EL-4 cells contained IL 2 and TCF2. The pH 2-treated Con A-induced spleen cell supernatant contained IL 2 and TCF1; and the W-2-absorbed lymphokine preparation contained only TCF1 and no TCF2.

DISCUSSION

We show in this report that maturation and differentiation of cytotoxic T lymphocyte precursor cells into effector cells involves at least three different lymphokines: IL 2 and the T cell cytotoxicity-inducing factors 1 and 2 (TCF1 and TCF2). Their requirement was shown by reconstitution experiments in cultures with numbers of accessory and helper cells that were insufficient to provide enough help. In this restricted system three different preparations of lymphokine-containing supernatants were used. The partially purified IL 2 preparation from EL-4 cells and the Con A-induced spleen cell supernatant that was absorbed with the IL 2-dependent W-2 T cell line did not mediate any response. The pH 2-treated, Con A-induced spleen cell supernatant mediated only weak cytotoxic activity, although its IL 2 titer was unchanged compared to a pH 7-treated control sample. In mixing experiments, synergy was only observed when the IL 2 preparation from EL-4 cells was mixed with either the absorbed preparation or the pH 2-treated, Con A-induced spleen cell supernatant. No synergy was seen after mixing of absorbed and pH 2-treated preparations. On the other hand, only a slight loss of synergistic activity was observed when the absorbed lymphokine preparation was pH 2-treated. These data only be explained by postulating a minimum of three different lymphokines: IL 2, which is pH 2-resistant and absorbed by the T cell line; TCF1, which is not absorbed by the T cell line and is pH 2-resistant; and TCF2, which is also absorbed by the T cell line but is pH 2-sensitive.

All three activities are different from interferon-γ, which is sensitive to pH 2 but not present in the stimulated EL-4 supernatant. Con A-induced spleen cell supernatants contain all three factors. The absorbed Con A-induced preparations contain only TCF1; the pH 2-treated supernatants contain TCF1 and IL 2; and the IL 2 preparation from EL-4 supernatants contains IL 2 and TCF2. TCF2 co-purified with IL 2. The two assay systems may detect different active sites on the molecule. One of these sites may be pH 2-sensitive.

During the course of this work other groups have reported on factors different from IL 2 present in Con A-induced supernatants (25–28, 33). Raulet and Bevan (27) were the first to describe a differentiation factor that is needed for induction of T cell cytotoxicity. Like TCF1, it was obtained by absorbing IL 2 from Con A-induced spleen cell supernatants. Wagner *et al.* (28) also described a differentiation factor found in IL 2-depleted, Con A-induced spleen cell supernatants. It is therefore very likely that the TCF1 activity described in this report is similar or identical with these activities, despite differences in the assay systems. Raulet and Bevan (27) also showed that the helper activity of their differentiation factor was destroyed by dialysis at pH 2, but their experimental system did not allow them to distinguish between the pH 2-labile TCF2 activity described in this report and the differentiation factor that was defined by absorption.

TCF1 and TCF2 are products produced by live spleen cells upon stimulation with Con A. Neither component was found in supernatants of unstimulated spleen cells; in supernatants of Con A-induced, UV-treated spleen cells; in supernatants of unstimulated EL-4 cells; or in supernatants of PMA-stimulated, UV-killed EL-4 cells (data not shown). The inability of supernatants of Con A-induced, UV-treated spleen cells to complement the IL 2 preparation also excluded the possibility that the TCF1 activity was due to Con A.

In preliminary experiments, evidence was obtained that TCF1 and TCF2 are required at different times during the five days of activation (submitted for publication). TCF1 was required early, whereas TCF2 was active if added as late as day 3 of culture. TCF2 may be identical with the late-acting helper factor that was described by our laboratory (26) and that was shown to be different from IL 2.

The findings in this study suggest that the activation of CTL is as complex as the B cell activation and differentiation for antibody production (34; K. Nakanishi, personal communication). It is an intriguing question whether one or more of the lymphokines participating in these two processes are identical. The function of the two cytotoxicity-inducing lymphokines, their biochemical nature, their cellular origin, and their targets are objects of current investigation.

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