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Induction of Cytotoxic T Cell Function Requires Sequential Action of Three Different Lymphokines

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Activation of resting Ly-2$^+$ precursor T cells to cytotoxic T lymphocytes (CTL)[1] requires stimulation by antigen or mitogen in combination with soluble mediators (1–3). One of these antigen-nonspecific mediators is interleukin 2 (IL 2). IL 2 is released by activated Ly-1$^+$ helper T cells and was distinguished to provide the proliferative signal (4, 5). It was only recently discovered that IL 2 is not the only helper factor required for the cytotoxic response (4–12). In this report, we provide evidence that three different, previously described activities (12) are sequentially required in distinct phases of the cytotoxic response. Absorption of Con A-induced spleen cell supernatants (Con A-Lk) on IL 2-dependent T cell line cells removes the IL 2 activity, but a factor remains that is required through the first 48 hr of culture and is referred to as T cell cytotoxicity-inducing factor 1 (TCF1) in this report. Another factor is only required in the last 48 hr of culture and is called TCF2. Its activity can be destroyed in Con A-Lk by dialysis at pH 2, a treatment that leaves IL 2 titers unchanged (10–12). It is different from TCF1 because it is also present in phorbol myristic acetate-activated EL-4 thymoma cell supernatants that lack TCF1. IL 2 is the third factor and is required at least in the early phase.

MATERIALS AND METHODS

Mice. C3H/TIF and DBA/2J were purchased from Bomholtgard, Ry, Denmark and were used at 6 to 10 wk of age.

Culture medium. The culture medium used was RPMI 1640 (GIBCO, Grand Island, NY; 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. ConA-Lk 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 (Pharmacia, Uppsala, Sweden) in moist air containing 5% CO_2. To cellfree supernatants, α-methyl-D-mannoside (Sigma Chemical, St. Louis, MO) was added to a final concentration of 10 mM. All preparations were stored at 4°C.

T cell cytotoxicity assay. Nylon wool-purified thymocytes (10^5) were cocultured with 3×10^5 glutaraldehyde-fixed TNP-haptenated syngeneic spleen cells as stimulators. Varying amounts of lymphokine preparations were added on day 0 and/or day 3 so that the total volume per well was 0.16 ml. The cytotoxic activity was measured on day 5 in a 3-hr ^{51}Cr-release test with the use of 5×10^3 prelabeled TNP-modified tumor targets (L929, mouse fibrosarcoma).

Assay for IL 2 activity. The amount of IL 2 in supernatants was measured by their ability to support the growth of the IL 2-dependent cell line W-2 (10–12). Thymidine incorporation after a 20-hr incubation and a 4-hr pulse with 0.5 μCi of methyl-[^{3}H]thymidine (specific activity 50 Ci/mol, Amersham International Ltd, Amersham, UK) was determined. Results are expressed in laboratory units as described by Farrar *et al.* (13).

Preparation of partially purified IL 2. An IL 2 preparation was prepared as described by Hilfiker *et al.* (14). EL-4 thymoma cells (generously supplied by J. J. Farrar, NIDR, NIH, Bethesda, 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 were added and the cells were incubated for 48 hr. The cellfree 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 to a phenyl-Sepharose column (Pharmacia). The column was then washed with 2 vol of 0.64 M ammonium sulfate + 10% ethanediol. The IL 2-containing peak was finally eluted with 40% ethanediol in 0.16 M ammonium sulfate. The IL 2 fractions were concentrated on 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% polyethyleneglycol 6000). The material was then applied to a Sephadex G-100 column and the activity was eluted with an apparent m.w. 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 ConA-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 incubated for 48 hr with 10^5 W-2 cells/ml. The supernatant was again dialyzed against culture medium. These supernatants were devoid of any trace 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 AND DISCUSSION

Responder cells (10^5) were co-cultured with TNP-haptenated syngeneic stimulator cells (3×10^5) that had been fixed with glutaraldehyde to prevent proliferation as well as metabolism. Cytotoxicity was determined after 5 days of culture against Con A-induced blasts or tumor targets in a 3-hr ^{51}Cr-release test. Development of CTL activity was detected under these stringent conditions only after the addition of helper factors. Substantial cytotoxicity was obtained only when a large dose of Con A-Lk was added on day 0 or when two doses of Con A-Lk were added to the cultures on day 0 and day 3, which as single doses at day 0 or day 3 were suboptimal (Table I, lines 1–4). This synergistic response was also obtained when the Con A-Lk added on day 3 was replaced by a semipurified IL 2 preparation from EL-4 supernatants (line 5); the response was lost when it was replaced by the pH 2-treated Con A-Lk, which had the same IL 2 titer (line 6). This indicated that the Con A-Lk contained a helper factor that was different from IL 2, sensitive to pH 2 treatment, and present in the IL 2 preparation from EL-4. This factor was called TCF2 in this study. Good CTL responses were measured in cultures that contained only pH 2-treated Con A-Lk in the beginning and received Con A-Lk or EL-4-derived IL 2 preparations on day 3 (lines 10 and 11). This showed that the pH 2-sensitive TCF2 activity was not required in the early phase of culture.

On the other hand, it was not sufficient to furnish the cultures

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[1] Abbreviations used: Con A-Lk, concanavalin A-lymphokine; IL 2, interleukin 2; IL 1, interleukin 1; CTL, cytotoxic T lymphocyte; TCF1 and 2, T cell cytotoxicity-inducing factors 1 and 2; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

TABLE I

*Effect of sequential addition of various lymphokine preparations on the induction of CTL responses**

	Addition of Lymphokines		Percent Specific ^{51}Cr Release
	Day 0	Day 3	
1	—	—	0.0
2	—	Con A-Lk	0.0
3	Con A-Lk	—	6.3
4	Con A-Lk	Con A-Lk	28.5
5	Con A-LK	EL-4-IL 2 preparation	22.0
6	Con A-Lk	pH 2-treated Con A-Lk	0.0
7	—	pH 2-treated Con A-Lk	0.0
8	pH 2-treated Con A-Lk	—	2.0
9	pH 2-treated Con A-Lk	Con A-Lk	23.5
10	pH 2-treated Con A-LK	EL-4-IL 2 preparation	21.8
11	pH 2-treated Con A-Lk	pH 2-treated Con A-LK	0.0
12	—	EL-4-IL 2 preparation	0.0
13	EL-4-IL 2 preparation	—	0.0
14	EL-4-IL 2 preparation	Con A-Lk	0.0
15	EL-4-IL 2 preparation	EL-4-IL 2 preparation	0.0
16	EL-4-IL 2 preparation	pH 2-treated Con A-Lk	0.0

* The cultures were provided on day 0 with 0.04 ml and/or day 3 with 0.04 ml of different lymphokine preparations: *a*) Con A-induced spleen cell supernatant (Con A-Lk). This preparation had an IL 2 titer of 132 U/ml; *b*) same as *a* but dialyzed overnight in pH 2 buffer (pH 2-treated Con A-Lk). This preparation had the same IL 2 titer as *a*; *c*) semipurified IL 2 preparation from phorbol myristic acetate-induced EL-4 thymoma cell supernatant (13, 14) with an IL 2 titer of 400 U/ml (EL-4-IL 2 preparation). (incorporated cpm = 2200; background 8%; SEM of specific cytotoxicity ≤2.3%).

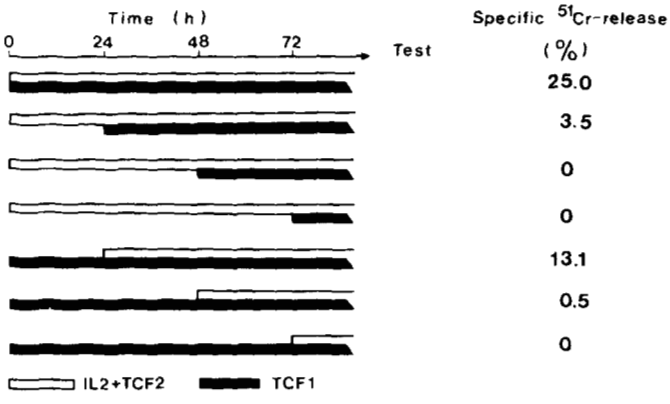

Figure 1. Influence of time of addition of two complementary lymphokine preparations on the induction of CTL responses. The following lymphokine preparations were used: *a*) IL 2 + TCF2: semipurified IL 2 preparation from phorbol myristic acetate-induced EL-4 thymoma supernatant (13, 14); *b*) TCF1: Con A-induced spleen cell supernatant depleted of IL 2 and TCF2 by two absorptions on IL 2 receptor-bearing T cells (W2) (incorporated cpm = 1000; background 13%; SEM of specific cytotoxicity ≤ 2.3%).

with IL 2 and TCF2 by using the IL 2 preparations from EL-4 supernatants in the early phase. In this case, a cytotoxic response was not induced either with a second dose of EL-4-derived IL 2 preparation or with the late addition of Con A-Lk (lines 13–15). The ineffectiveness of this combination was not due to the lack of IL 2 in the early phase because the EL-4-IL 2 preparation had an IL 2 titer of 400 U/ml. Thus, in contrast to the IL 2 preparation from EL-4, the Con A-Lk as well as the pH 2-treated Con A-Lk contained another helper factor that was required in the early phase of culture. This factor was called TCF1.

It was shown previously that absorption of Con A-Lk on IL 2-dependent T cell line cells yields a preparation that contains a factor different from IL 2 that is required for the induction of cytotoxicity (4, 5, 10). Reconstitution experiments have also shown that it synergized with EL-4-derived IL 2 preparations (10–12; Fig. 1). These results indicated the absorbed preparation contained TCF1. Similar experiments established that not only IL 2 was removed by this absorption procedure but also TCF2 (10–12).

The three different lymphokine preparations lacking one or two of the required helper activities were utilized in kinetic studies to determine more precisely the time points of requirement for TCF1, IL 2, and TCF2. Figure 1 shows that TCF1 (the absorbed Con A-Lk) had to be available no later than on day 0 together with IL 2. Because the absorbed preparation was devoid of IL 2, we found as expected that the delayed addition of the EL-4-derived IL 2 preparation reduced the cytotoxic response. Preculture in TCF1 and addition of IL 2 and TCF2 1 day later induced a relatively weak but still significant CTL response on day 5.

Pulse experiments with TCF1 are shown in Figure 2. A 24-hr pulse with TCF1 at any time proved to be insufficient, even when given together with IL 2 at the time of culture initiation. Substantial cytotoxic activity was obtained, however, when TCF1 was present for the first 48 hr. No further increase was observed when the same amount of TCF1 was left in the culture for more than 48 hr. The combination of the EL-4-derived IL 2 preparations plus TCF1 for 48 hr was not sufficient for the induction of the CTL response.

Similar kinetic experiments were performed to determine the exact time span for the requirement for TCF2. It was found that the addition of TCF2 any time between day 0 and day 3 gave essentially the same CTL response, whereas synergism was lost

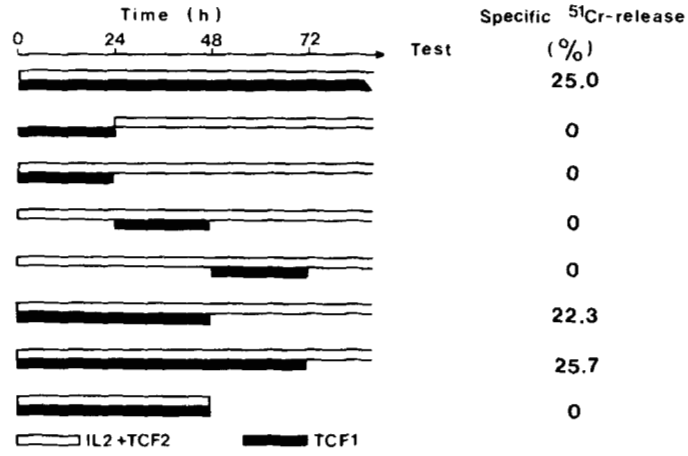

Figure 2. Influence of pulsing with TCF1 on the induction of CTL responses. For details see legend to Figure 1 (incorporated cpm = 1600; background 17%; SEM of specific cytotoxicity ≤ 2.3%).

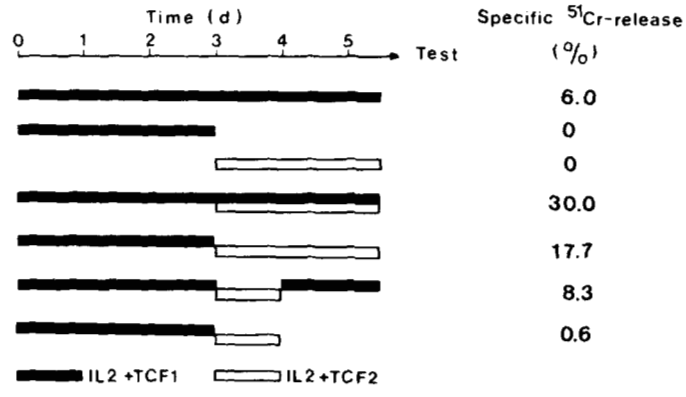

Figure 3. Influence of time of addition of two complementary lymphokine preparations on the induction of CTL responses. The following lymphokine preparations were used: a) IL 2 + TCF1: Con A-induced spleen cell supernatant dialyzed in pH 2 buffer. The IL 2 titer was 130 U/ml; b) IL 2 + TCF2: semipurified IL 2 preparation from phorbol myristic acetate-induced EL-4 thymoma supernatant (13, 14) (IL 2 titer: 400 U/ml). Lymphokine preparations were either added on day 3 or supernatant was taken off the cultures and replaced by another lymphokine (incorporated cpm = 1300; background 12%; SEM of specific cytotoxicity ≤ 2.3%). For other details see legend to Figure 1.

when TCF2 was provided on day 4 or later (data not shown). Figure 3 confirms that the addition of the EL-4-derived IL 2 preparation (IL 2 and TCF2) on day 3 to cultures containing pH 2-treated Con A-Lk (TCF1 and IL 2) produced good CTL responses. Substantial responses were obtained when the pH 2-treated Con A-Lk was present from day 0 through day 5 or day 0 through day 3. The difference in cytotoxic activity in these two situations may be explained by the different IL 2 concentration in the late phase. The cytotoxic activity was markedly reduced, however, when TCF2 was only present from day 3 through day 4, indicating that the TCF2 activity was required until the end of the culture. The remaining weak cytotoxicity could be due to the incomplete inactivation of TCF2 by the pH 2 treatment (12).

Taken together, our experiments demonstrate the requirement for three functionally and biochemically different helper activities in the cytotoxic response. Il 2 is required at least in the early phase of the culture period. TCF1 is required at the initiation of the culture for a minimum of 48 hr. This factor is indistinguishable from the differentiation factor described previously (4, 5). It is possible that it acts on the CTL precursor before IL 2 receptors are expressed. Preliminary experiments in which semipurified IL 1 preparations from Con A-induced spleen cells were used indicated that TCF1 cannot be replaced by IL 1. TCF2, on the other hand, is only required in the final phase and not in the early phase of CTL activation. The requirement for a late-acting soluble helper factor different from IL 2 has been described in a previous report (6). It was shown that this late helper effect was activated by stimulation with a foreign I region determinant. The biochemical characterization of TCF1 and TCF2, their cellular origin, and their mechanism of action are currently under investigation.

Recent publications revealed two distinct phases of the cytotoxic response, i.e., an early antigen-driven phase and a second phase that was dependent on IL 2-containing lymphokines (15–17). The experiments in this paper showed that the cytotoxic response is even more complex and requires the sequential action of at least three different helper activities that are provided by soluble mediators. Mixing experiments excluded inhibitor action (12). So far, TCF2 and IL 2 co-purified, and there is the possibility that the two activities reside on the same molecule. The early helper factor TCF1 is clearly separable from IL 2. It is absent from the EL-4 supernatant and is not absorbed by the IL 2-dependent cell line. The sequential action of several factors in CTL responses was strikingly similar to the conditions of B cell activation and functional differentiation (Reference 18; K. Nakanishi, personal communication). In addition to a B cell growth factor, two more soluble differentiation factors were required, one of which acts at an early stage of activation and the second one at a later stage. It remains to be tested whether these B cell differentiation factors are related to the helper activities necessary for the induction of T cell-mediated cytotoxicity.

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