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Immunology

Culture supernatants of a stimulated T-cell line have helper activity that acts synergistically with interleukin 2 in the response of B cells to antigen

(T cell factors/lymphokines/B cell activation/regulation of the immune response/B cell differentiation)

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ABSTRACT **Culture supernatants of an antigen-stimulated long-term alloreactive T cell line, C.C3.11.75, contain a T-cell-replacing factor (TRF) activity for the B-cell response to antigen. These same supernatants show little activity in the T-cell growth assay or the costimulator assay. TRF activity was assayed by using spleen cells that were rigorously depleted of both T cells and macrophages. In this assay preparations containing interleukin 2 and supernatants from stimulated C.C3.11.75 cells are relatively inactive if added alone but show marked synergy when added together. We conclude that the C.C3.11.75 TRF activity is not due to interleukin 1 or to interleukin 2 but to a third factor provisionally designated as (DL)TRF. This activity may be equivalent to the (late-acting) TRF described by Schimpl and Wecker. Evidence is presented suggesting that the helper activity (DL)TRF is a product of the T-cell line.**

Many of the interactions of T cells with other cells are mediated by factors, and it is possible that all such interactions may take place via soluble products. These products can be divided into the antigen-specific and the non-antigen-specific, and it seems possible that these may represent two distinct classes of molecules that have quite different physiological roles.

One of the first non-antigen-specific T-replacing helper factors was described in 1971 (1) and was obtained from culture supernatant of mixtures of spleen cells disparate in antigens of the major histocompatibility complex (MHC). It was shown that such helper activity could also be obtained from spleen cell suspensions cultured with concanavalin A (2) and in a variety of other experimental models (see ref. 3). The non-antigen-specific factors do not contain Ia antigens and show no MHC or immunoglobulin restriction for the target cell.

An increasingly large number of other lymphokines or activities were added to the list in the years after the initial demonstration of the nonspecific helper and suppression factors.

In most cases the characterization consisted of a description of the experimental protocol in which the activity was generated and the measurement of the activity in a complex assay system. It is probable that many of the culture supernatants that were used contained a mixture of two or more factors and that the assays could not distinguish between factors in that several different factors could each be measured by the same assay system.

Two recent developments have served to dispel much of the confusion that these problems had generated. First, a number of cloned cell lines have been demonstrated to produce culture supernatants with a more limited range of activities. The macrophage line $P388D_1$ has been shown to produce a 15,000-dalton protein with lymphocyte-activating activity (4). The T-cell hybridoma FS6 14.13 (5), the irradiation-induced leukemia LBRM 33 (6), and others (7, 8) have been shown to produce a 30,000-dalton protein with T-cell growth activity. Second, an unequivocal assay for the T-cell growth factor (TCGF), which is required for the growth of various cloned T-cell lines, has been reported (9).

These and other developments have made it possible to group a number of hitherto different activities in one of two classes, which have been designated interleukin 1 and interleukin 2 (IL1 and IL2) (10). IL1 is a macrophage product; it is a differentiation signal that, together with antigen (or mitogen), leads to the activation of the resting T cell to an active state in which receptors for IL2 are expressed. The activated T cell then proliferates in response to the T cell growth factor IL2.

The additional details of this scheme are still unclear and the questions such as whether IL1 and IL2 act also on B cells are not yet resolved (for a discussion, see ref. 3).

It now seems likely that both IL1 and IL2 are components of many of the mitogen- and alloantigen-stimulated "helper" factors (1, 2, 11, 12) originally described. They act directly, or indirectly through residual T cells, on antigen-stimulated B cells, which proliferate extensively (13). A third factor (14), currently designated as T-cell-replacing factor (TRF), is believed to act as a final differentiation signal and promote immunoglobulin secretion. It has been suggested that this factor is the "late acting" TRF of Schimpl and Wecker (11), which has been chromatographically separated from IL2. It has a molecular weight of 30,000 and has T-cell-replacing activity but no T-cell-growth activity (15).

In this paper we show that a T-cell-replacing activity with similar functional properties can be obtained from a T-cell line cultured (16) with Ia-positive stimulator cells (17, 18). The culture supernatants have little or no costimulator or TCGF activity. They have moderate levels of TRF activity in a conventional TRF assay; the levels fall to low values when the responding B cells are more rigorously depleted of T cells and macrophages. The culture supernatants, however, show very high levels of TRF activity when IL2-containing culture supernatants from FS6 14.13 are also added. IL2 alone has little or no activity in this assay. Thus it seems likely that the C. C3.11.75 TRF activity is due to a differentiation factor that can drive activated B cells to immunoglobulin secretion.

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Abbreviations: ATS, anti-thymocyte serum; Con A, concanavalin A; (DL)TRF, T-cell-replacing factor from Dennert line C.C3.11.75; IL1, interleukin 1; IL2, interleukin 2; MHC, major histocompatibility complex; PFCs, plaque-forming cells; PMA, phorbol 12-myristate 13-acetate; SRBC, sheep erythrocytes; TCGF, T-cell growth factor (= IL2); TRF, T-cell-replacing factor.

MATERIALS AND METHODS

Animals. All strains of inbred mice were bred in our colony at the University of California, San Diego. B cells were obtained from (C57BL/6 × DBA/2)F_1 (BDF_1) mice. BALB.K mice were used for stimulating factor production.

Production of Factors. *IL1.* An IL1-containing preparation was produced from the $P388D_1$ cell line kindly provided by Steven Mizel (4). Cells of this line were stimulated with 5 μM phorbol 12-myristate 13-acetate (PMA; Sigma). Supernatants were collected after 6 days of culture and $(NH_4)_2SO_4$, was added to give a final saturation of 80%. The precipitate was collected, dialyzed against RPMI medium, spun at 20,000 × *g*, and stored at −20°C. IL1 activity was determined by the ability of this supernatant to costimulate with concanavalin A (Con A) the proliferation of thymocytes (see below). The supernatant also had activity in restoring the ability of macrophage-depleted spleen cells to produce Con A-induced T-cell factors.

IL2. An IL2-containing culture supernatant was produced from the FS6 14.13 cell line kindly provided by John Kappler and Philippa Marrack (5). Cells in serum-free RPMI medium were stimulated with Con A at 2 μg/ml and supernatants were collected after 16–24 hr. IL2 activity was determined by the ability of the supernatant to support the growth of a line of NK cells developed by one of us (19). In some cases we used a preparation of IL2 purified by Sephadex column chromatography and isoelectric focusing (15), kindly provided by James Watson.

Dennert line TRF [(DL)TRF]. This factor was usually produced by incubating 1.25 × 10^5 cells from the BALB/c-derived cell line C.C3.11.75 (4), 7–9 days after the last stimulation, with 2.5 × 10^6 anti-Thy-1.2-treated BALB.K spleen cells. Culture supernatants were recovered after 18–24 hr, centrifuged at 20,000 × *g*, and kept frozen at −20°C. In some cases cells of a golden hamster–mouse (C3H) hybridoma, GHL10, kindly provided by William Raschke, were used as stimulators. This cell line expresses both Ia^k and surface Ig (W. Raschke, personal communication). Preliminary screening of these factors was done by assessing their ability to replace T helper cells in the primary response of T-cell-depleted spleen cells to sheep erythrocytes (SRBC) (20) (TRF assay).

Con A factor. This factor was produced by incubating spleen cells of BALB/c mice at 5 × 10^6 cells per ml with Con A at 2 μg/ml. Culture supernatants were collected after 18–24 hr and 10 mM methyl α-D-mannopyranoside was added to inactivate any residual Con A (20); supernatants were kept frozen at −20°C. These supernatants were screened for activity in the TRF assay.

TCGF (IL2). This activity was measured by incubating 10^4 cells of a TCGF-dependent NK line (19) in 0.1-ml cultures. From each of several 1:2 dilutions of supernatant, 10-μl samples were added to triplicate wells. ^{125}I-Labeled uridine [0.1 μCi (3700 becquerels)] was added to all cultures on day 1. Two days after culture initiation cells were harvested and washed on a multiple automatic sample harvester and the incorporation of ^{125}I-labeled uridine was determined with a Searle model 1197 gamma counter. Geometric means of cpm were determined.

Costimulator activity. This activity was determined essentially as reported by Paetkau *et al.* (21). Thymocytes from BALB/c mice were incubated at 10^5 cells in 0.1-ml triplicate cultures with Con A at 2 μg/ml. After 72 hr ^{125}I-labeled uridine was added to each culture. The cultures were harvested 6 hr later and ^{125}I incorporation was measured as described for the T-cell growth assay.

TRF. This activity was determined by the ability of supernatants to provide help for B cells in the primary antibody response to SRBC. In the conventional TRF assay, spleen cells from BDF_1 mice were treated with anti-Thy-1.2 plus guinea pig complement. This treatment is usually effective in abolishing the response of spleen cells to SRBC, and residual spleen cells do not proliferate or produce TRF in response to Con A. However, we postulate that some residual T cells remain under these conditions (see data in this paper). To more rigorously deplete T cells, BDF_1 mice were injected with rabbit anti-mouse-thymocyte serum (ATS; Microbiological Associates, Wakesfield, MD) at 0.06 ml per mouse 48 hr before sacrifice. This procedure is effective in removing many of the functions ascribed to mature T cells (22). Spleen cells of ATS-treated mice were then treated with anti-Thy-1.2 antiserum plus complement and passed over a Sephadex G-10 column (23). This series of treatments gives a population of B cells that require both IL2 and (DL)TRF in order to fully restore plaque formation. To assay TRF activity, supernatants were added to cultures of 6 × 10^5 B cells per well with 0.1% SRBC as antigen. Cells that formed direct (IgM) plaques (PFCs) in SRBC layers were determined 4 days later. Geometric means and SEMs of triplicate cultures were determined.

RESULTS

Cells from the C.C3.11.75 T cell line were cultured with a 20-fold excess of T-cell-depleted spleen cells from a strain of mice carrying the IA^k allele.

Large volumes were prepared so that a variety of assays could be carried out on the same batch of material. Culture supernatants from FS6 14.13 were used as a source of IL2 and supernatants from PMA-stimulated $P388D_1$ were used as a source of IL1. Culture supernatants were also prepared from Con A-stimulated spleen cells.

The four supernatants were each assayed by titration in the costimulator assay, the TCGF assay, and a conventional TRF assay. The results are shown in Figs. 1–3. In Fig. 1 it can be seen that preparations containing IL1, IL2, or Con A super-

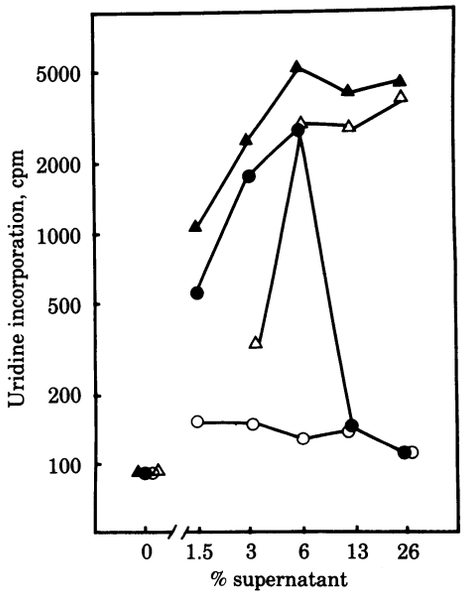

FIG. 1. Costimulator activity of different factors. Four supernatants were titrated in 1:3 dilutions by addition to 0.1-ml cultures containing 10^5 BALB/c thymocytes and Con A at 2 μg/ml. ▲, Supernatant of Con A-stimulated spleen cells; △, supernatant of the $P388D_1$ cell line stimulated with PMA (IL1); ●, supernatant from the FS6-14.13 cell line stimulated with Con A (IL2); and ○, supernatant from the cell line C.C3.11.75 stimulated with BALB.K anti-Thy-1.2-treated spleen cells [(DL)TRF]. ^{125}I-Labeled uridine was added after 72 hr and incorporation was determined 6 hr later.

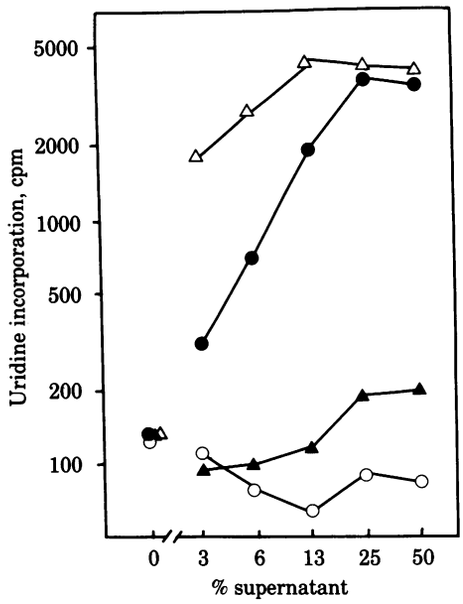

FIG. 2. TCGF activity of different factors. The same four supernatants used in Fig. 1 were added to 10^4 cells per well of a TCGF-dependent cell line. △, Con A supernatant; ▲, P388D$_1$ supernatant; ●, FS6-14.13 supernatant; and ○, C.C3.11.75 supernatant. ^{125}I-Labeled uridine was added at 24 hr and incorporation was determined at 48 hr.

natants all had the expected costimulator activity. The suppression at higher concentrations of FS6 supernatant was seen in some but not all experiments. The culture supernatants from IA^k-stimulated C.C3.11.75 had little or no activity at any of the concentrations tested. In Fig. 2 it can be seen that only the IL2-containing supernatant and the Con A supernatant were active in the growth factor assay. The IL1 preparation from P388D$_1$ had no growth activity, as expected, and the C.C3.11.75 supernatants were also inactive. In Fig. 3 all the supernatants except IL1 had some activity in the conventional TRF assay in which no special steps were taken to deplete the culture of residual T cells or macrophages, although Con A supernatants (which contain a mixture of IL1, IL2, and TRF) were able to induce higher levels of PFCs.

The conventional TRF assay responds to IL2 as well as the Schimpl and Wecker TRF and cannot distinguish between the two activities (15). We postulate that the residual T cells can respond to antigen, proliferate in the presence of IL2, and produce significant amounts of the third factor, TRF. Conversely in the absence of added IL2, significant amounts of IL2 are still produced and the system responds to added TRF. We therefore devised a more rigorous TRF assay (see *Materials and Methods*) in which the preparations of both the IL2 and the (DL)TRF alone had minimal activity (Fig. 4). With this assay we were able to show a very marked enhancement (greater than 20-fold) of the response to C.C3.11.75 supernatant in the presence of added IL2 (Fig. 4). This enhancement was dependent on the concentration of added IL2, with higher concentrations giving larger total responses. These enhanced responses were comparable to those seen in the presence of optimal concentrations of Con A supernatants. In additional experiments not shown, exactly comparable synergy was seen between the C.C3.11.75 supernatants and the column-purified isoelectric-focused preparation of IL2 provided by Watson.

These studies demonstrate that the supernatants of stimulated cultures of C.C3.11.75 contain a factor that has little costimulator activity, little TCGF activity, and a TRF activity that is markedly enhanced in the presence of IL2. We therefore conclude that this activity is not IL1, is not IL2, and has some of the properties of the TRF described by Schimpl, Wecker, and colleagues (11, 14) as elaborated by Watson and his colleagues (15). We have given this factor the provisional designation (DL)TRF because it is obtained from a cell line developed by Dennert (Dennert line).

The C.C3.11.75 supernatants used in most of these studies were prepared from cultures stimulated with T-cell-depleted spleen cells. In separate experiments we showed that (DL)TRF activity was also generated when a hamster–mouse hybridoma line GHL10 was the stimulating cell. This cell is surface immunoglobulin positive and carries *IA*^{k}. These supernatants had

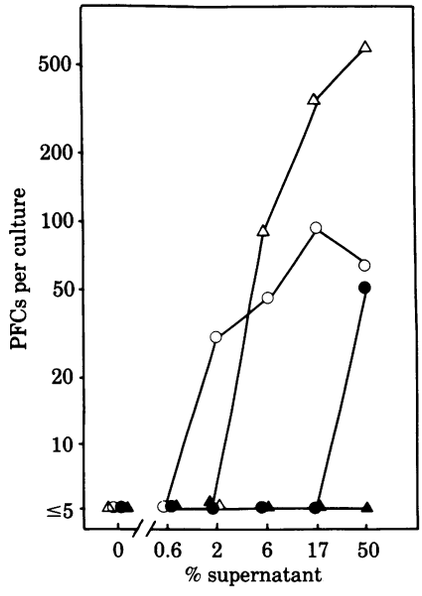

FIG. 3. TRF activity of different factors. The same four supernatants in Figs. 1 and 2 were titrated into anti-Thy-1.2 plus complement-treated spleen cells with SRBC. △, Con A supernatant; ▲, P388D$_1$ supernatant; ●, FS6-14.13 supernatant; and ○, C.C3.11.75 supernatant. After 4 days PFCs to SRBC were determined.

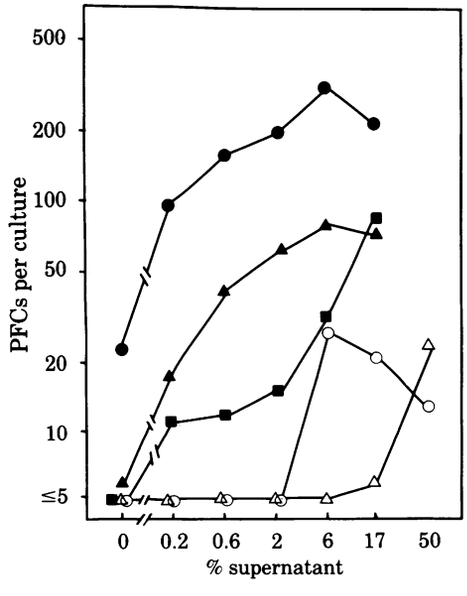

FIG. 4. Synergy of factors in T-cell-replacing assay. Supernatants from FS6-14.13 (△) and C.C3.11.75 (○) were titrated into rigorously T-cell-depleted spleen cells. In addition, titrations of the C.C3.11.75 were repeated with three concentrations of the FS6-14.13 supernatant: 50% (●), 17% (▲), and 6% (■).

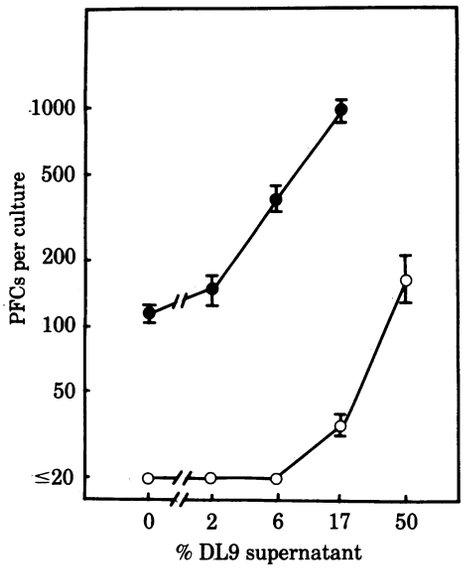

FIG. 5. Synergy of C.C3.11.75 factor after stimulation with the GHL10 hybridoma. Supernatant was produced by incubating C.C3.11.75 (DL9) with the golden hamster–mouse hybridoma GHL10. Supernatants alone (○) or with 17% FS6-14.13 (●) were titrated into rigorously T-cell-depleted spleen cells. Bars indicate SEM.

the same characteristics as those prepared previously and again showed marked synergy with IL2 in the rigorous TRF assay, as seen in Fig. 5.

DISCUSSION

Recent studies by Watson *et al.* (15), Parker (13), and others have suggested that "helper factor" activity found in Con A supernatant is in fact a mixture of factors, IL2, TRF, and perhaps IL1. It is therefore necessary to determine which factor is present in our supernatants that show helper activity.

The data presented show that cultures of the long-term alloreactive T-cell line C.C3.11.75 produce a helper activity, (DL)TRF, when stimulated with cells from a mouse carrying the IA^k allele. The culture supernatants have little activity in the costimulator and TCGF assays. The helper activity is thus not due to IL1 or IL2. The conventional TRF assay will respond to a number of interleukins. We have used a more discriminating TRF assay in which the B cells rigorously depleted of T cells and macrophages do not respond significantly to IL1 or IL2. This kind of result has previously been reported by Harwell *et al.* (5). In this assay the T-cell line culture supernatants have little activity but show marked activity in the presence of IL2.

We conclude that B cells responding to antigen stimulation (SRBC) require at least two factors in order to proliferate and secrete immunoglobulin. One of these factors is supplied by the addition of IL2.

The simplest interpretation is that the IL2 acts directly on the B cell as a B-cell growth factor, although the possibility that it acts through residual T cells is not excluded by our data.

The second factor is present in the supernatants of stimulated cultures of C.C3.11.75. The experiments described here do not define the role of this second factor, but it is likely that the (DL)TRF provides the signal for immunoglobulin secretion and its role is thus similar to that claimed for the TRF found in Con A supernatants (13) described in Schimpl and Wecker (11, 14).

IL1 may also play an important role in B-cell activation. An earlier study of Hoffmann and Watson (24) found synergy in a PFC response between a Con A factor (TRF-T) and a lipopolysaccharide-activated macrophage factor (TRF-M). This synergy may have been due to the IL2 and IL1 activities of the two factors, though other factors in these preparations could be responsible.

Two additional points need to be discussed. First, the C.C3.11.75 line used in these studies was not a cloned line. It is thus possible that more than one cell is involved in the production of the helper activity. A number of clones, however, have been obtained from C.C3.11.75 (unpublished) and it has been shown that some of these still produce TRF activity when stimulated.

Second, the culture supernatants are obtained from cultures containing 1.25×10^5 C.C3.11.75 and 2.5×10^6 stimulator cells per ml. It is possible that the helper activity is secreted by the stimulator cell and not C.C3.11.75 or that components of the activity come from both cell sources. The supernatants, however, do not contain appreciable quantities of IL1 or IL2, the products of a normal mixed lymphocyte reaction. Moreover, helper activity can also be obtained when the C.C3.11.75 is stimulated with the IA^k-positive hamster–mouse B-cell line GHL10, and this activity also shows strong synergy with IL2 (Fig. 5). The simplest explanation is that the helper activity is a product of the T cell line, but again more complex possibilities are not rigorously excluded.

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