Brief Definitive Report

A MONOCLONAL T CELL-REPLACING ACTIVITY CAN ACT DIRECTLY ON B CELLS TO ENHANCE CLONAL EXPANSION*

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B lymphocytes must interact with T cells for most immune responses. Some of these interactions, however, can be mediated by soluble products. Thus, supernatants of T cells stimulated in mixed lymphocyte reaction (MLR) (1, 2) or by polyclonal activators like concanavalin A (Con A) (3) can replace T cells in an in vitro immune response. This activity was attributed to a single entity and was given the name T cell-replacing factor (TRF). Reports that antigen alone induces B cell proliferation (4, 5) and TRF functions when added after B cell exposure to antigen (2) led to a model in which nonspecific TRF induces antibody secretion by acting on B cells already proliferating in response to antigen (5, 6).

Subsequent experiments have suggested a more complex model. Purified interleukin 2 (IL-2), a T cell growth factor (7), restores in vitro immune responses to sheep erythrocyte (SRBC) antigens in cultures of nude (8) and normal (9) spleen cells pretreated with anti-Thy-1 or anti-theta and complement. With more rigorous T cell depletion, however, IL-2 no longer replaces T cells (9, 10), and a second T cell activity is needed to synergize with IL-2 to restore in vitro B cell antibody secretion. One source of this TRF activity, (DL)TRF, is the monoclonal Dennert alloreactive T cell line C.C3.11.75 (10). A similar activity has been described in the 30,000–40,000 mol wt fraction of Con A supernatants (11).

There are thus at least two component activities in the earlier TRF. It is now necessary to redefine the role and target cell of each. B cell preparations obtained by anti-thymocyte serum (ATS) preinjection and treatment with several anti-T cell reagents may still contain small numbers of T cells or their precursors, which mature during culture (12). Hence, IL-2 or (DL)TRF may act on residual T cells to restore immunocompetence of conventional T cell-depleted B cell preparations. Only in the complete absence of T cell function can unequivocal description of a mediator's target cell be made.

We used a B cell cloning system, described by Wetzel and Kettman (13), in which the response of a single isolated B cell can be followed. (DL)TRF containing supernatants increased the proportion of B cells stimulated to clonal expansion by mitogens. These supernatants did not increase the average B cell clone size. Similar activity was found in Con A and MLR supernatants with no detectable IL-2. These experiments provided unequivocal proof that some monoclonally and polyclonally derived T cell products act directly on B lymphocytes.

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Materials and Methods

Mice. BDF$_1$ (C57BL/6 × DBA/2)F$_1$ male and female mice, 2–4 mo old, were bred in our colony at the University of California, San Diego.

Mitogens. *Escherichia coli* 0127:B8 lipopolysaccharide (LPS) was obtained from Difco Laboratories (Detroit, MI). Dextran sulfate (DXS) was obtained from Gibco Laboratories (Grand Island Biological Co., Grand Island, NY) as a sodium salt, 17% sulfur, 500,000 mol wt.

Lymphocyte Cultures. Spleen cells from mice injected with 0.06 ml rabbit ATS (Microbiological Associates, Walkersville, MD) 48 h before killing were treated with anti-Thy-1.2 plus complement as described previously (10). LPS and DXS were used at final concentrations of 25 and 20 μg/ml, respectively. The visual assay of clonal proliferation is described elsewhere (13).

DL-TRF. Supernatants from C.C3.11.75 stimulated with anti-Thy-1.2 and mitomycin C-treated BALB.K spleen cells for 24 h were prepared as described elsewhere (10).

Data Analysis. A minimum of two cell inputs (cells per well) with at least 120 cultures/point were used for each experimental condition tested. Poisson analyses were performed, and frequencies of responding cells, i.e., cells induced to clonal expansion, were calculated according to the weighted mean method described by Taswell (14). 95% confidence intervals were calculated for each frequency reported and are included in the data. Differences between two frequencies are significant at the 0.05 level when the confidence intervals do not overlap.

Results

(DL)TRF Increases B Cell Clonal Expansion. T cell-depleted spleen cells were cultured at limiting dilutions with LPS and DXS. Under optimal conditions, ~80% of input B cells yield clones 4 d later, and cultures receiving a single input cell exhibit clonal expansion (13). The absolute magnitude of the B cell growth response depends on the batch of fetal bovine serum (FBS) used (13, 15, 16). FBS with suboptimal growth support was chosen to examine effects of T cell products on clonal B cell growth. Medium or (DL)TRF supernatant were diluted in BSS and added to culture. The results of several experiments are given in Table I. In every experiment, the presence of monoclonal T cell-derived mediator significantly increased clonal B cell growth. The proportion of input B cells stimulated to clonal expansion was increased up to about sevenfold by the presence of supernatants containing (DL)TRF.

Monoclonal and Polyclonal Sources of TRF Increase B Cell Clonal Expansion. Monoclonal (DL)TRF was compared to two other standard sources of TRF activity, Con A, and MLR supernatants. Con A and MLR TRF were harvested after 4 d of culture and were devoid of IL-2 by the T cell growth factor assay (data not shown). Comparison of these sources of TRF with (DL)TRF for clonal T cell growth enhancement is shown in Table II. All sources of TRF increased clonal B cell growth at the appropriate concentration. These results confirm that B cell growth-enhancing activity is found in TRF preparations from both a monoclonal T cell line and normal splenic T cells.

(DL)TRF Acts Directly on Single B Cells. The experiments described in the previous sections were performed at input cell numbers 0.8–4 cells per culture. This leaves a remote possibility that (DL)TRF exerted its B cell clonal growth-promoting activity indirectly by acting on rare T cells that had escaped treatment with ATS and anti-Thy-1.2 plus complement. If these cells were present at the level of 1%, then <1 of 25 cultures should have contained a T cell, and this level of contamination would not significantly affect the frequencies of B cell clonal precursors shown. Nevertheless, it seemed worthwhile to provide a formal proof. Cultures were initiated, and wells were examined to determine those that received a single input cell; these were marked and followed. Poisson analysis of all cultures gave frequencies of all input cells yielding B

TABLE I
(DL)TRF Enhancement of B Cell Clonal Expansion

Experiment	Percent input cells yielding B cell clones	
	Control	1% (vol/vol) (DL)TRF supernatant
1	7.8 ± 2.5*	57.5 ± 14.0‡
2	16.2 ± 4.8	46.5 ± 10.5‡
3	9.5 ± 3.0	47.0 ± 5.5‡
4	10.0 ± 3.5	26.3 ± 6.3‡
5	13.5 ± 4.5	63.2 ± 13.2‡

Limiting dilution analyses of T cell-depleted spleen cells induced to clonal expansion by LPS + DXS. Cultures received 1% (vol/vol) medium (control) or (DL)TRF supernatant at culture initiation.
* Frequency of responding cells and 95% confidence interval determined from a minimum of 240 cultures.
‡ Significantly different from control; $P < 0.05$.

TABLE II
Comparison of (DL)TRF with Con A and MLR TRF Supernatants Devoid of IL-2

Percent (vol/vol)	Percent input cells yielding B cell clones		Percent (vol/vol)	Percent input cells yielding B cell clones	
	4-d C57Bl/6 anti-DBA/2 supernatant	C.C3.11.75 anti-BALB.K supernatant		4-day Con A supernatant	C.C3.11.75 anti-BALB.K supernatant
0	16.4 ± 5.0	16.4 ± 5.0	0	29.0 ± 8.5	29.0 ± 8.5
8.5	14.8 ± 5.2	47.5 ± 10.5*	4	39.0 ± 10.5	—
1.7	40.5 ± 9.5*	30.5 ± 7.5*	1	57.0 ± 14.0*	—
0.3	23.5 ± 7.0	28.8 ± 7.5	0.25	31.5 ± 9.0	65.0 ± 17.5*
			0.06	—	47.0 ± 12.5

Frequencies of input cells yielding B cell clones are given with 95% confidence intervals. MLR and Con A supernatants were harvested on day 4 and were devoid of IL-2, presumably due to exhaustion by T cells. (DL)TRF supernatant was harvested on day 1 and was devoid of IL-2.
* Significantly different from control; $P < 0.05$.

cell clones. This was compared with the frequency of cultures with one initial cell yielding B cell clones. The results are presented in Table III. The frequency of single cell (DL)TRF cultures stimulated to clonal expansion was not significantly different from that calculated by limiting dilution analysis. This confirmed the previous results and provided unequivocal evidence that (DL)TRF did not act through accessory cells to enhance B cell growth.

(DL)TRF Does Not Increase Average B Cell Clone Sizes. It is possible that growth-promoting activities in (DL)TRF could act to increase the clone size of B cells stimulated to grow. This was tested by calculating average B cell clone sizes in the presence and absence of (DL)TRF. These are shown in Table IV. The total number of clones was calculated and divided into the total number of cells in all wells on day 4. Average mitogen-induced B cell clone sizes were comparable whether or not (DL)TRF was present. This suggests that average rates of entry into cycle and division were similar in the presence and absence of (DL)TRF. Experiments with purified factors may, however, yield different results. Hence, clonal B cell growth-promoting

TABLE III
(DL)TRF Acts on Single B Cells

Experiment	Additions	Average input/well	Percent input cells yielding B cell clones	
			Poisson analysis of all cultures	Cultures with only one input cell
1	Medium	0.8	8.7 ± 3.3	—
	1% (DL)TRF	0.8	24.2 ± 7.0*	28.1 (41/146)‡
2	Medium	3.0	9.9 ± 3.5	—
	1% (DL)TRF	3.0	26.7 ± 6.7*	27.5 (65/236)

* Significantly different from control; $P < 0.05$.
‡ Numbers in parentheses indicate the number of wells with one input cell positive for growth over the total number of wells receiving one input cell.

TABLE IV
B Cell Clone Size Analyses in the Presence and Absence of (DL)TRF

Experiment	Additions	Percent input cells yielding B cell clones ± 95% CI*	Average clone size (95% CI)
1	Medium	7.7 ± 2.6	7.6 (5.7–11.5)
	1% (DL)TRF	57.6 ± 14.0‡	7.8 (6.3–10.4)
2	Medium	16.3 ± 4.7	7.1 (5.5–9.9)
	1% (DL)TRF	46.6 ± 10.4‡	8.5 (7.0–11.0)
3	Medium	9.4 ± 3.4	7.5 (5.5–11.8)
	1% (DL)TRF	47.0 ± 10.5‡	9.1 (7.4–11.7)
4	Medium	13.5 ± 4.7	9.3 (6.9–14.2)
	1% (DL)TRF	63.3 ± 14.1‡	7.2 (5.9–9.3)
5	Medium	9.9 ± 3.5	7.7 (5.6–11.9)
	1% (DL)TRF	26.7 ± 6.7‡	7.1 (5.7–9.5)

* 95% confidence interval.
‡ Significantly different from control; $P < 0.05$.

activity of (DL)TRF appeared to act by increasing the number of precursors that undergo clonal expansion rather than by altering the kinetics of proliferation.

Discussion

We have demonstrated that a factor (or factors) present in supernatant of stimulated cultures of an alloreactive T cell line can act directly on a single B cell in the absence of any other cell type. The effect of this factor (or factors) is to increase the frequency of B cells that proliferate in response to LPS and DXS. The average clone size of responding B cells is not altered by the presence of the culture supernatants. This provides clear and unequivocal proof of the direct action of a T cell factor on a target B cell.

Several points can be made regarding the significance of this observation. First, the same supernatants of this T cell line contain an activity, designated as (DL)TRF, which, in synergy with IL-2, restores the plaque-forming response of T-depleted spleen cells to SRBC (10). They also promote immunoglobulin secretion in cultures where most cells were from the B cell tumor, BCL_1 (17). The activity in the single B cell assay is to increase the frequency of B cells that proliferate. It is possible that the same factor is active in all three assays, but we have no evidence as yet to prove this.

Although (DL)TRF supernatants can contain interferon (J. Yamamoto, unpublished observations), they are devoid of both IL-1 and IL-2 (10). Purification and isolation of the factors should clarify whether a single entity can mediate these three activities.

Whether the growth enhancement of (DL)TRF reported here represents a signal that also takes place in responses to antigen is not known. Results from another laboratory (18) suggest, however, that this is the case.

We report only the effect of culture supernatants of the T cell line C.C3.11.75 containing (DL)TRF. We have reason to believe there may be several different factors with different roles that constitute nonspecific TRF activity. Other laboratories have also shown a role for a variety of factors in B cell proliferation and immunoglobulin secretion (19, 20). There is as yet too little information to determine how these activities can be related to one another and to the activity described here. It will be necessary to test each of these in assay systems that measure response initiation, proliferation, and secretion at the single cell level.

Summary

We have used a B cell cloning system in which the response of a single isolated B cell to lipopolysaccharide and dextran sulfide can be followed. We have shown that culture supernatants from the Dennert long-term alloreactive T cell line C.C3.11.75 increase the frequency of B cells stimulated to clonal expansion by mitogens. These culture supernatants are devoid of interleukin 1 and 2 but contain the T cell-replacing factor activity (DL)TRF. These experiments provide unequivocal proof that a T cell-derived factor or factors can act directly on a B lymphocyte in the absence of any other cell.

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