

ROLE OF
B LYMPHOCYTES IN CELL-MEDIATED IMMUNITY
I. Requirement for T Cells or T-Cell Products for Antigen-Induced
B-Cell Activation

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Bone marrow-derived (B) lymphocytes are antibody-forming cell precursors which when appropriately activated differentiate into antibody-secreting cells. Activation of B lymphocytes by mitogens and certain thymic-independent antigens is thought to occur directly without cooperation of other cells (1, 2). In contrast, the antibody response to most soluble protein antigens is defective in the absence of stimulated T cells (3-5). In addition to differentiation into antibody-secreting cells, B cells have also been shown to be capable of producing the biologically active mediators (lymphokines) associated with cell-mediated immune responses once thought to be the exclusive function of T cells. While B cells can be activated nonspecifically by B-cell mitogens and by triggering at their C3 and Fc surface receptors (6), investigations in our laboratory (6, 7) and in others (8) suggest that B cells cannot be triggered by soluble protein antigens to produce lymphokines. In view of the failure of B cells to differentiate into antibody-producing cells in the absence of T cells, we considered it likely that the defect in lymphokine production might be due to a requirement for T cells.

In the present study, we have investigated the mechanism of antigen-specific lymphokine synthesis by B cells. We have found that this B-cell function requires T-cell help and that this help is mediated by a soluble factor produced by antigen-activated T cells.

Materials and Methods

Animals and Immunization. 400-500 g male, Hartley guinea pigs (Buckberg Lab Animals, Tomkins Cove, N. Y.) were immunized with dinitrophenylated ovalbumin (DNP₁₄-OA)¹ in complete Freund's adjuvant (CFA) (7) or with 3 µg tetanus toxoid (T.T) (Department of Public Health, Boston, Mass.) in CFA. Spleens and/or oil-induced peritoneal exudate cells were obtained from these animals 2-4 wk later. Nonimmunized guinea pigs (>500 g) were used as a source of glycogen-induced peritoneal macrophages for chemotaxis assays.

Separation of T and B Lymphocytes. T and B lymphocytes were separated from single cell

¹ *Abbreviations used in this paper:* BCAF, B-cell-activating factor; CFA, complete Freund's adjuvant; CTX, chemotactic factor; DNP-OA, dinitrophenylated ovalbumin; E, rabbit erythrocytes; [³H]TdR, tritiated thymidine; LPS, lipopolysaccharide; MNL, monocyte or macrophage; PELs, peritoneal exudate lymphocytes; POL, polymerized flagellin; T.T., tetanus toxoid.

suspensions of the spleens of immunized guinea pigs as previously described (6). Briefly, T lymphocytes were eluted off a nylon wool column and further purified by repassage over a second nylon column. B lymphocytes were isolated by removing on Ficoll-Hypaque gradients T cells which had rosetted with rabbit erythrocytes (E). Repeating this procedure with the nonrosetted cells resulted in a population of cells representing 10–25% of the total spleen cells which were >95% Ig positive (6) and responded to B, but not T, cell mitogens. Lymphocytes were suspended in RPMI 1640 media (NIH Media Unit, Bethesda, Md.) containing 2 mM glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin, but without serum and cultured as reported (6).

Proliferation Assay. Cultures of T and B cells were pulsed with 1 µCi/ml tritiated thymidine (³H]TdR) (sp. act 6.0 Ci/mmol; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) 4 h before harvest. The cultures were harvested by means of a modified Skatron automated harvester (Flow Laboratories, Inc., Rockville, Md.) onto glass fiber filters. The filters were placed in scintillation vials with 5 ml scintillation fluid for determination of incorporated [³H]TdR in a Beckman Scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Data are expressed as mean counts per min per culture (1×10^6 cells) for triplicate cultures.

Chemotaxis Assay. Cell-free supernates from these cultures were assayed for their ability to induce macrophage migration across polycarbonate (Nuclepore, Neuroprobe, Inc., Bethesda, Md.) filters with 5-µm pores. The chemotaxis assays were carried out in modified Boyden chambers (6). Triplicate filters were run for each sample and migration quantitated by counting 20 oil immersion fields per filter. Data are expressed as the mean number of cells per oil immersion field \pm 1 SE for the triplicate filters.

Production of Supernates from T Lymphocytes. Peritoneal exudate cells were obtained 4 days after injection of sterile mineral oil (Drakeol 6-VR; Penreco Inc., Butler, Pa.) into the peritoneal cavities of immunized guinea pigs. The harvested cells were pooled, washed several times, suspended in warm RPMI 1640 containing 10% fetal calf serum, and applied to prewashed glass bead columns (9). The loaded columns were incubated 45 min at 37°C, in 5% CO₂, and the nonadherent cells eluted with 100 ml of warm medium. These nonadherent peritoneal exudate lymphocytes (PELs) which are enriched in antigen-reactive T cells (10) were washed several times in serum-free medium and resuspended at 1×10^6 /ml with 2 µg/ml antigen or medium only and incubated for 20 h unless otherwise indicated. The cultures were centrifuged and the cell-free supernates frozen (–20°C) until used. These supernates were added to B-lymphocyte cultures (0.5 ml at 2×10^6 /ml) in varying amounts and the total volume of each culture adjusted to 1 ml with serum-free RPMI 1640.

Mitogens, Antigens, and Drugs. Antigens used in these experiments included DNP-OA and T.T. Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5, (Difco Laboratories, Detroit, Mich.) and polymerized flagellin (POL) prepared from *Salmonella adelaide* (11) were utilized as B-cell mitogens. Cycloheximide and actinomycin D were obtained from Sigma Chemical Co., St. Louis, Mo.

Results

T-Lymphocyte Requirement for Antigen-Induced Lymphokine Production by B Cells. B cells obtained by the selective removal of E-rosette forming T cells from the spleens of DNP-OA-sensitized guinea pigs were incapable of responding to the thymic-dependent antigen DNP-OA as measured by proliferation and production of a lymphokine chemotactic for macrophages (MNL CTX) (Table I). However, these same B cells proliferated and produced MNL CTX when stimulated with the B-cell mitogens, LPS and POL (Table I). Previous studies have shown that addition of macrophages does not enable B cells to respond to DNP-OA (7), however, a B-cell response could require T cells. In order to determine whether the B cells could be induced to respond to DNP-OA in the presence of T cells, the purified B lymphocytes were reconstituted with increasing numbers of DNP-OA-primed T cells. As seen in Fig. 1, B cells alone did not respond to DNP-OA, but when reconstituted with as few as 10% T cells a significant amount of mediator production became evident in the cultures. Further addition of T cells

TABLE I
Proliferation and Lymphokine Production by B Cells in Response to Mitogens and Antigens

Stimulant	[³ H]TdR incorporation (E/C)	Chemotactic activity
None*	1.0‡	9 ± 3
DNP-OA	1.1	3 ± 1
LPS	3.5	69 ± 4
POL	7.1	87 ± 4

* 1×10^6 B lymphocytes/ml serum-free medium were cultured with 0.1 ml medium only, 2 μ g/ml DNP-OA, 10 μ g/ml LPS, or POL for 48 h. The cell-free supernates were then assayed for chemotactic activity (mean number of macrophages per oil immersion field \pm 1 SE of triplicate filters).

‡ Parallel cultures were pulsed with 1 μ Ci [³H]TdR 4 h before harvest at 48 h and assayed for incorporation of [³H]TdR. Ratio of mean counts per minute of experimental to control cultures (E/C) is shown.

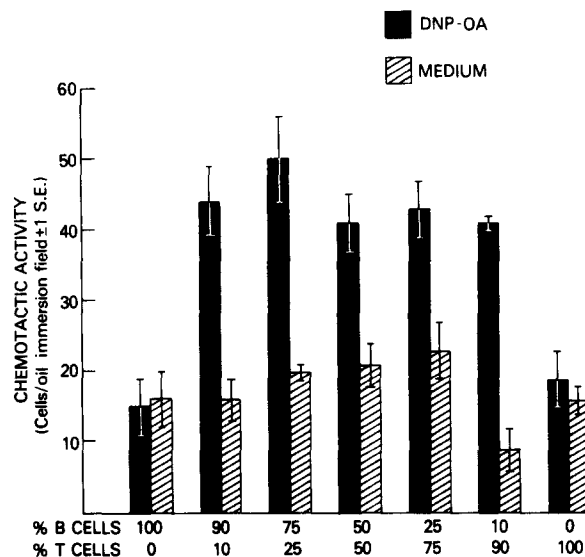


FIG. 1. Production of MNL CTX by B cells in the presence of T cells. Chemotactic activity measured in 48-h supernates of cultures containing a total of 1×10^6 B and/or T lymphocytes in a vol of 1 ml medium. Increasing numbers of T cells were added to decreasing numbers of B cells and these cultures were stimulated with 2 μ g/ml DNP-OA or with medium only.

with coincident decrease in total B cells resulted in little enhancement of MNL CTX. T cells alone did not respond to DNP-OA due to the absence of macrophages in these preparations (Fig. 1) (7).

Replacement of T Cells by a Cell-Free Supernate from Antigen-Activated T Cells. Although suggestive that in the presence of small numbers of T cells (10%), the B cells were producing MNL CTX, it could not be excluded that the T cells themselves were responsible for production of the MNL CTX found in these cultures. However, if the T cells were triggering B cells to undergo mediator synthesis, this might be accomplished by a T-cell product capable of interacting with B cells in the presence of antigen. To test this possibility a population of

highly enriched sensitized T cells (PELs) containing macrophages were cultured with or without DNP-OA for 20 h. The cell-free supernates from these short-term cultures were then added at varying concentrations to B cells in the presence of 2 $\mu\text{g/ml}$ DNP-OA. After 48 h incubation of the B cells under these conditions, the supernates of the B cells were assayed for the presence of chemotactic activity. Identical cultures were prepared of the varying concentrations of PEL supernates but in the absence of B cells to assess the chemotactic activity produced by the T cells during the 20 h culture period. As is evident in Fig. 2, the addition of supernates from DNP-OA-activated PELs to the B-cell cultures resulted in significant levels of MNL CTX in the B-cell cultures as compared to the chemotactic activity found in the PEL supernates alone. Dilution of the T-cell supernate to 1:20 still resulted in significant activation of the B lymphocytes. Moreover, the supernates of nonstimulated T cells were unable to induce B-cell production of the chemotactic lymphokine. These data suggested that the antigen-stimulated T cells were producing a soluble mediator with the ability to stimulate B-cell lymphokine synthesis in the presence of additional antigen.

Specificity of the T-Cell Factor for B Cells. Supernates from antigen-stimulated PELs were added to B cells and to T cells with DNP-OA to determine whether the T-cell factor(s) could induce mediator synthesis by T cells as well as B cells. Whereas B cells cultured in varying concentrations of the T-cell factor produced highly significant levels of MNL CTX, the macrophage-depleted T cells incubated with identical concentrations of the factor were not activated to produce MNL CTX (Fig. 3). Rather, loss of inherent chemotactic activity in the PEL supernates occurred when added to T-cell cultures. Thus it appeared that the factor(s) produced by antigen-activated T cells was B-cell specific.

Kinetics of Production of the B-Cell Activating Factor (BCAF). PEL cultures were established with and without antigen and incubated for varying times from 1 to 48 h. At each interval the supernates were harvested and frozen until the final cultures were terminated. These T-cell supernates were then added to B-cell cultures and to cultures lacking B cells for 48 h. The supernates from these secondary B-cell cultures were then assayed for chemotactic activity. After 1 h incubation of both antigen-stimulated and nonstimulated PELs their supernates were consistently capable of inducing significant levels of mediator production by the B cells (Fig. 4) as compared to the levels of chemotactic activity found in the supernates alone. A decline in BCAF activity from 4-h PEL supernates followed by a rise in the BCAF of supernates from 8-h antigen-exposed T cells, but not in nonstimulated T-cell cultures, suggested an antigen-induced synthesis of this T-cell factor which activated B cells. The stimulated cells produced high levels of BCAF peaking at about 20 h with no significant increase at later intervals. In contrast, nonantigen-stimulated T cells did not demonstrate release of BCAF during the 48 h period with the exception of the 1st h culture supernates. Furthermore, the intrinsic chemotactic activity of an equivalent amount of antigen-stimulated PEL supernate was low at 8 h and because it was diluted when added to the B cells showed only a slight increase during the 48 h culture period.

Requirement for Specific Antigen and BCAF in Initiation of B-Cell Mediator

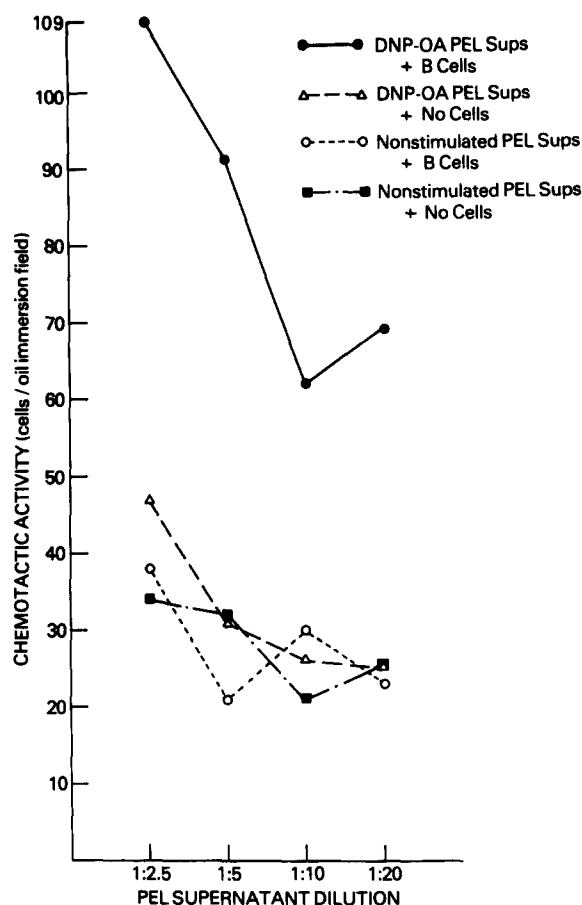


FIG. 2. Production of MNL CTX by B cells in the presence of T-cell supernate. Chemotactic activity measured in the 48-h supernates (Sups) of B cells which had been cultured with DNP-OA-activated PEL supernates or nonstimulated PEL supernates. Additionally, the PEL supernates were diluted and cultured under identical conditions without B cells as a control assay for PEL-produced chemotactic activity. All cultures received 2 μ g/ml DNP-OA. Data are presented as the mean of triplicate samples with standard errors less than 10% of the mean.

Production. Experiments were initiated to determine whether the BCAF was antigen specific or a nonspecific factor capable of triggering the B cells. Supernates were prepared from DNP-OA-stimulated PELs of DNP-OA-immunized guinea pigs and also from T.T.-stimulated PELs of T.T.-immunized animals. These supernates were then added along with the appropriate antigen or the nonspecific antigen to sensitized B-cell cultures. Addition of DNP-OA plus DNP-OA PEL supernates to DNP-OA-sensitized B cells resulted in marked production of MNL CTX by the B cells as already described and as shown in Fig. 5. Similarly, DNP-OA plus supernates from T.T.-stimulated T cells when added to DNP-OA-sensitized B cells also resulted in production of MNL CTX by the B cells. It does not appear necessary for the T cells to be triggered by the same antigen as the B cells since T.T.-activated T cells produce a factor capable of

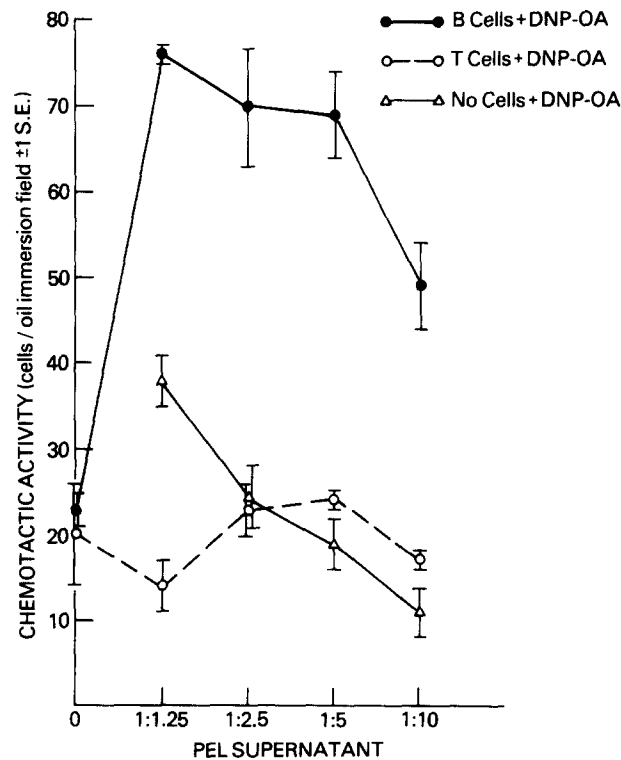


FIG. 3. Specificity of the T-cell factor for B cells. Supernates from DNP-OA-stimulated PELs were added in varying dilutions (1:2.5-1:10) to 1×10^6 B cells or T cells from spleens. The same dilutions were incubated in the absence of cells. The 48-h supernates from these cultures were assayed for chemotactic activity.

stimulating DNP-OA-sensitized B cells. A requirement for specific antigen in addition to BCAF, however, was demonstrated since DNP-OA-sensitized B cells were not activated in the absence of DNP-OA. T.T. in concert with T.T.-induced PEL supernates was not sufficient to trigger DNP-OA sensitized B cells (Fig. 5, left panel) although the same supernates in the presence of DNP-OA did trigger B-cell mediator production. Identical results were obtained when T.T.-sensitized B cells were used as the test population (Fig. 5, right panel). T.T. in combination with supernates from either DNP-OA- or T.T.-induced PELs resulted in B-cell triggering. Thus in this system, specificity resides in both the T cell and the B cell, in that each cell must be triggered by the appropriate antigen. However, the T-cell factor is nonantigen specific in its action on the B lymphocyte.

Kinetics of BCAF Antigen-Induced Release of MNL CTX by B Cells. Addition of BCAF from 20 h T-cell cultures and antigen to B cells resulted in lymphokine production consistent with the kinetics of a cellular immune response. Significant MNL CTX activity above controls in these B-cell cultures was observed at 12 h after the addition of antigen and BCAF (Fig. 6). BCAF-induced B-cell activation continued to increase from 12 h to 48-72 h, declining thereafter.

Proliferation of B Cells Induced by BCAF. In parallel cultures to those

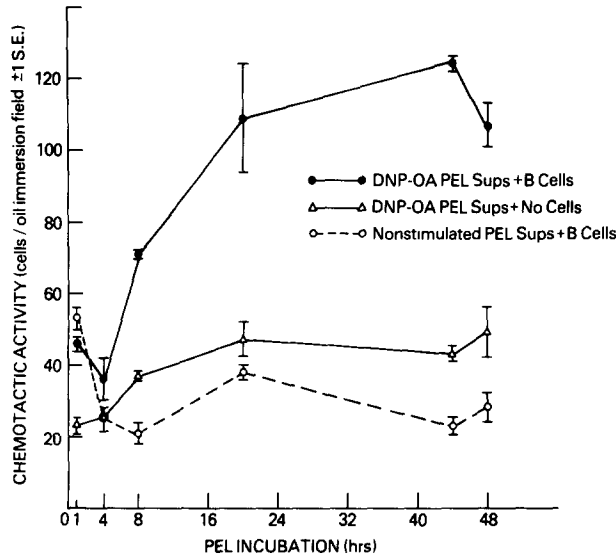


FIG. 4. Kinetics of BCAF production. Chemotactic activity in 48-h B-cell supernates (Supers) which were cultured with antigen and 1- to 48-h supernates (1:2.5) from DNP-OA and nonstimulated PELs. DNP-OA PEL supernates (1:2.5) were also cultured without B cells to determine intrinsic T-cell-derived MNL CTX.

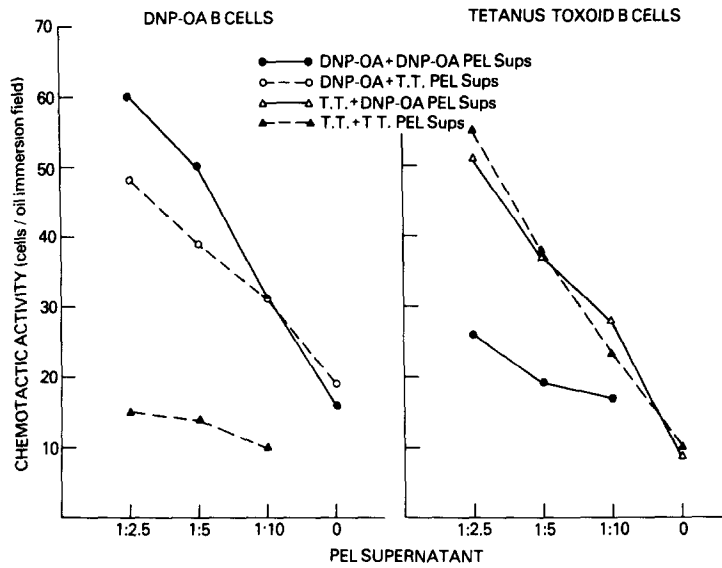


FIG. 5. Requirement for BCAF and specific antigen in B-cell activation. Chemotactic activity in 48-h supernates for B cells stimulated with specific or nonspecific antigen and BCAF. Left panel: DNP-OA-sensitized B cells incubated with DNP-OA or T.T. and DNP-OA-stimulated supernates (Supers) from DNP-OA-sensitized T cells or T.T.-stimulated supernates from T.T.-sensitized T cells. Right panel: T.T.-sensitized B cells incubated with the same combinations of antigen and T-cell supernates as indicated for the DNP-OA-immunized B cells.

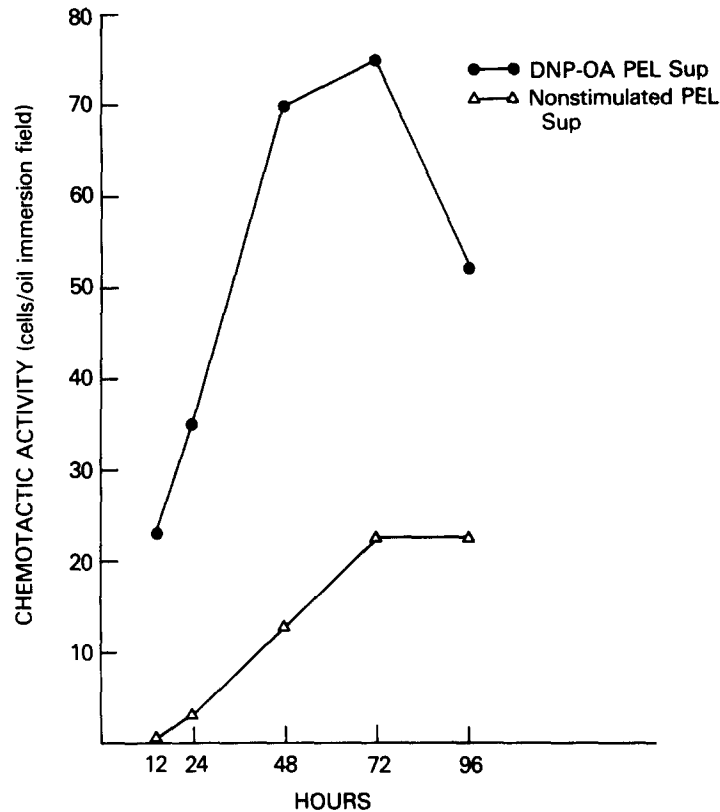


FIG. 6. Kinetics of B-cell production of MNL CTX. B cells were incubated with DNP-OA and antigen-stimulated or nonstimulated T-cell (PEL) supernates (Sup) for various intervals from 12 to 96 h. The cell-free supernates from the B-cell cultures were then assayed for chemotactic activity (mean of triplicate experiments with SE less than 10% of mean).

established for analysis of the kinetics of B-cell MNL CTX production triggered by BCAF and antigen, the degree of [^3H]TdR incorporated by the B cells under these conditions was also assessed (Fig. 7). High background incorporation of [^3H]TdR was recorded at 24 h, precluding detection of significant elevation of proliferation by BCAF. However, by 48 h, nonstimulated cultures had little incorporation of [^3H]TdR, while cultures stimulated with BCAF and DNP-OA had elevated levels of proliferation. By 72-96 h in these serum-free cultures, blastogenic responses were subsiding. Thus, in addition to triggering B cells to synthesize lymphokines, the T-cell factor(s) also induced a B-cell proliferative response to this T-dependent antigen.

Discussion

The inability of isolated populations of sensitized B cells to produce lymphokines when stimulated with DNP-OA and the restoration of this function with small numbers of T cells is suggestive that B-cell triggering by antigen is T-cell dependent. While it has been reported that B cells alone can produce lymphokines in response to antigen (12), our studies indicate a T-cell requirement. These findings are consistent with the recent report of Bloom and Shevach (8) in

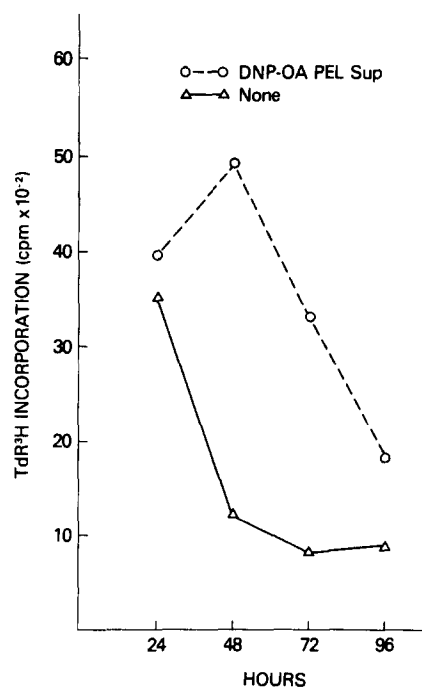


FIG. 7. Kinetics of B-cell proliferation induced by BCAF and antigen. B cells were incubated with DNP-OA and antigen-stimulated T-cell (PEL) supernate (Sup) or no supernate for 24-96 h. The cultures were pulsed 4 h before harvest with 1 μ Ci [³H]TdR and assayed for incorporation of [³H]TdR in triplicate (mean of triplicate cultures with SE less than 10% of mean).

which migration inhibition factor was not produced by either T or B lymphocytes in a mixed population when nonresponsiveness to an antigen was a T-cell defect, suggesting a T-cell dependence for B-cell lymphokine synthesis. However, since unseparated populations of spleen cells were used it was unclear whether the B cells were triggered to produce migration inhibitory factor even in the responder strain bearing functional T cells. Similarly, in the T- and B-cell mixing experiments reported here, the data is not conclusive that the T cells are inducing B-cell mediator production in response to DNP-OA. It is possible, although unlikely, that these low levels of T cells and not the B cells were responsible for the MNL CTX found in the supernates. Therefore, our attention was focused on the possible identification of a biologically active substance(s) derived from T cells which could enhance or regulate B-lymphocyte function subsequent to exposure to antigen as measured by lymphokine production in an *in vitro* system of cell-mediated immunity. Since intact T cells could be replaced by supernates of DNP-OA-stimulated T cells, T-B-cell contact did not appear to be essential for B-cell triggering. Furthermore, this demonstrated that the MNL CTX was indeed a B-cell product and that the T-cell help could be mediated by a soluble factor. The absence of BCAF in the supernates of nonstimulated 20 h PEL cultures indicated that the production of BCAF was antigen specific, requiring activation of the T cells for its elaboration. This is consistent with the inhibition of BCAF production by the protein synthesis inhibitor, cyclohexi-

mide, and by actinomycin (data not shown). Although some MNL CTX was produced by the antigen-activated T cells and was therefore present in these secondary supernates, the T-cell factor responsible for triggering B cells could be readily separated from T-cell-produced MNL CTX by molecular weight,² indicating a distinct mediator of B-cell reactivity. Also when added to pure T cells, the PEL supernate factor did not stimulate these cells to produce lymphokines.

Specifically activated T lymphocytes then release a factor(s) that can trigger B-cell lymphokine production. The factor released from either T.T.- or DNP-OA-sensitized T cells by the appropriate antigen could stimulate DNP-OA-sensitized B cells in the presence of DNP-OA to undergo lymphokine synthesis and proliferation. Similarly, either T-cell factor would trigger T.T.-sensitized B cells provided T.T. was available to the B cells in addition to the T-cell factor. Although the T-cell factor is nonspecific in its action, specificity occurs both at the level of the T cell and at the B cell. Apparently, the nonspecific T-cell product can synergize in some fashion with the antigen to trigger B cells and the specificity of the response is ensured since the mediator will only effectively trigger those B cells which are responding to specific antigen. It appears then that two signals are required for B-cell participation in cellular immune phenomena. The first signal is a consequence of the interaction of antigen and B-cell receptor, with the second signal being the nonspecific T-cell mediator. The B-cell encounter with antigen is not sufficient to trigger a response, but the antigen is converted to an inductive signal by means of BCAF which can replace T cells themselves.

How this BCAF with the potential to recruit B cells into active participants of a cell-mediated reaction relates to the numerous T-cell factors (13-17) described for activating B cells to undergo antibody synthesis in response to thymic-dependent antigens is unclear. Both nonantigen-specific (13-16) and antigen-specific (16, 17) T-cell helper factors have been identified which enable T-B-cell collaboration in humoral immune responses. Recent evidence in the mouse suggests that the T-cell mediator in antibody production may be a cell surface antigen coded for in the Ia region of the *H-2* locus which binds to a homologous acceptor site on B-cell membranes (18, 19), thus requiring compatible T and B cells. Whether the B-cell receptor is related to Ia antigen and whether the T-cell product which functions to enable T-B-cell collaboration in cellular immunity is identical or distinct from the factors described in humoral responses is presently being investigated.

However, in contrast to the antibody response which requires 4-5 days for T-cell-mediated B-lymphocyte differentiation culminating in antibody production, the B-cell response in this cell-mediated system resulting in lymphokine production is of much shorter duration, peaking by 48 h. Possibly, lymphokine synthesis is an early response of the B cells, occurring before B-cell differentiation and thus controlled by an early inductive T-cell stimulus (20), whereas a late-acting T-cell component (15) effective after 48 h may be responsible for B-cell differentiation into antibody-secreting cells. Multiple factors may be involved in the control of the total B-cell response. Perhaps different T-cell-derived mediators

² Wahl, S. M., and J. B. McCarthy. Manuscript in preparation.

are responsible for the diverse responses of the B lymphocyte including lymphokine production, proliferation, and antibody secretion. Alternatively, B cells may respond differently at different stages of their life history (21). A less mature B cell may be one involved in lymphokine production and therefore in cellular immune reactions rather than a more mature cell which may differentiate into an antibody-producing cell. Whether subpopulations of B cells are responsible for lymphokine synthesis and involvement in cell-mediated immunity and for antibody production cannot be determined at this stage. Such a distinction might serve as a possible explanation for the different responses of B cells. At any rate, since the fundamental function of T cells is cellular immunity and since nonspecific lymphokines are instrumental in many T-cell-mediated responses it is reasonable that similar nonspecific factors influence B-cell responsiveness not only in humoral but also in cellular immune responses.

It thus appears that B cells may play an important function in cell-mediated reactions. On recognizing antigen, T cells release soluble lymphokines, among them BCAF with which B cells must interact in addition to their own recognition of antigen. Upon interaction of both BCAF and an antigen with the B cells, these cells respond by proliferating and elaborating MNL CTX and presumably other lymphokines associated with cell-mediated phenomena. The T-cell response occurs early after antigen contact, thereby effecting a recruitment of B cells into a cell-mediated reaction in the early stages resulting in amplification of the response. Indeed, Turk and Parker (22) have shown that cyclophosphamide-treated animals which are B-cell depleted have diminished delayed hypersensitivity responses to OA. Thus, cell-mediated reactions which were considered a T-cell function may only be T-cell dependent, but a function of both T lymphocytes and B lymphocytes. The BCAF produced by antigen-stimulated T cells may provide the major pathway for recruitment of B cells into cell-mediated immune reactions.

Summary

Although B lymphocytes can be triggered by B-cell mitogens and by certain other molecules to produce lymphokines, they do not produce lymphokines when stimulated with specific soluble protein antigens. We have investigated whether T-cell help would enable B cells to produce lymphokines when activated by antigens. Addition of small numbers of T cells to B-cell cultures resulted in significant production of a monocyte chemotactic factor. T cells could be replaced by supernates of antigen-stimulated T cells, demonstrating both that the chemotactic factor was B-cell-derived and that T-cell help was mediated by a soluble factor. Although the T-cell factor was nonantigen specific, B-cell activation required the presence of both antigen and T-cell factor. Thus, it appears that although dependent upon T cells, B lymphocytes may play an important role in amplification of cell-mediated immune responses.

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