Stimulation of B-cell proliferation by membrane-associated molecules from activated T cells

(lymphokines/interleukin 4/interleukin 5/CD4 antigen/T-cell antigen receptor)

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ABSTRACT

Activation of B cells to proliferation and antibody secretion is dependent on soluble lymphokines secreted by activated T cells. Activation of T cells results from physical contact between B cells and T cells through binding of the T-cell antigen receptor to a complex of antigen and class II major histocompatibility complex (MHC) molecules. To determine whether this interaction also contributes to B-cell activation by mechanisms other than those mediated by soluble T cell-derived lymphokines, I examined the ability of isolated T-cell plasma membranes to stimulate proliferation in cultures of unfractionated B cells. Membranes prepared from a cloned antigen-specific helper T-cell line induced substantial proliferation provided that the T cells had been mitogen-activated before isolation of membranes. Membranes from splenic Con A-treated blasts also stimulated B-cell proliferation, suggesting that this activity may be a common property of some subsets of activated T cells. Induction of B-cell proliferation was not found to be antigen-dependent or MHC-restricted, indicating no significant contribution by the T-cell receptor for antigen. The presence of interleukins 4 and 5 in membrane fractions was indicated by proliferation of lymphokine-sensitive cell lines, although culture supernatants from mitogen-activated T cells proved to be far more potent sources of these activities. The combined effect of membranes and lymphokine-containing culture supernatants in B-cell cultures was greater than their added effects in separate cultures. This observation suggests that lymphokines or molecules with mitogenic activity for B cells other than those found in abundance in culture supernatants may be present on activated T-cell membranes.

Despite the isolation and characterization of a number of T cell-derived lymphokines that act on B cells, the role of major histocompatibility complex (MHC)-restricted recognition in activation of B cells remains in question. Binding of Ia and antigen by the T-cell antigen receptor leads to T-cell activation and subsequent lymphokine production. It is not known whether MHC-restricted interactions between T and B cells contribute to activation of B cells. Evidence for transduction of an activating signal by B-cell Ia molecules has come from a number of experimental systems. Differentiation of the murine B-cell lymphoma cell line CH12 to secretion of IgM was reported to require both (i) antigen reactive with CH12 cell surface immunoglobulin and (ii) T cells (1) or alloantibody reactive with I-E-encoded Ia molecules (2). Anti-class II MHC antibodies have been reported to stimulate proliferation and immunoglobulin secretion by pokeweed mitogen-treated B cells (3). On the other hand, the requirement for a signal through Ia seems doubtful in the light of reports of MHC unrestricted proliferation and immunoglobulin secretion by small resting third-party B cells in mixed cultures of antigen-specific T cells and antigen-presenting cells (4, 5). From these latter observations, it would appear that, once activated, T cells induce B-cell activation regardless of MHC haplotype. In another report (6), B cells were stimulated to proliferate in cultures of mitogen-activated T cells even though supernatants from these cultures were ineffective in the same assay, suggesting that the direct action of T cells on B cells, MHC-restricted or not, differs qualitatively from the action of lymphokines recoverable from culture supernatants.

This issue is illustrated in the experiment summarized in Table 1. Cells of the cloned murine helper T-cell line D10.G4.1 (D10) were γ-irradiated and combined with unprimed T cell-depleted, MHC-matched murine spleen cells. Proliferation in these cultures is compared with that in parallel cultures supplemented with culture supernatants from Con A-stimulated D10 cells, which we shall refer to as “Con A supernatants.” Much less proliferation occurred in cultures supplemented with supernatants than in cultures containing T cells and the antigen for which they are specific. One explanation for this observation is that physical contact with T cells provides a signal, in the absence of which B cells are unresponsive to secreted lymphokines. Alternatively, recognition by T cells may serve primarily to activate the T cell, leading to the expression of membrane-associated molecules or lymphokines secreted at such low levels that they are effective over only very short distances.

In this report I address the question of T-cell contact-mediated stimulation of B cells by using plasma membrane fragments isolated from D10 cells. This line is a CD4− (L3T4+), CD8− (Lyt-2−), type 2 helper T cell (7) specific for conalbumin presented by I-Aα cells (8). It secretes interleukin 4 (IL-4) (B-cell-stimulatory factor 1), IL-3, granulocyte-macrophage colony-stimulating factor, and IL-5 (B-cell growth factor II), but not IL-2, and stimulates B-cell proliferation and immunoglobulin secretion. I found that isolated D10 membranes were able to stimulate proliferation of unfractionated B cells, but only if the D10 cells had been previously activated. The antigen receptor made no more than a minor contribution to the observed activity. The lymphokines IL-4 and IL-5 were detected in membrane preparations; however, the ability of isolated membranes to stimulate proliferation is not accounted for by the presence of these lymphokines.

MATERIALS AND METHODS

Cell Culture. All cell culture was carried out in minimal essential Eagle’s medium with Hanks’ salts and nonessential amino acids (GIBCO) supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and 100 units of penicillin and 100 μg of streptomycin sulfate per

Abbreviations: IL, interleukin; LPS/DxSO₄, bacterial lipopolysaccharide and dextran sulfate; MHC, major histocompatibility complex; D10 cell line, D10.G4.1 cloned cell line.

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Table 1. T cell-dependent induction of B-cell proliferation

<table>
<thead>
<tr>
<th>Addition to culture</th>
<th>[3H]Thymidine incorporation, cpm</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Without antigen</td>
</tr>
<tr>
<td>D10 cells</td>
<td></td>
</tr>
<tr>
<td>2 × 10⁴</td>
<td>7,766 ± 805</td>
</tr>
<tr>
<td>2 × 10⁵</td>
<td>13,142 ± 493</td>
</tr>
<tr>
<td>SN</td>
<td></td>
</tr>
<tr>
<td>1:1</td>
<td>6,601 ± 557</td>
</tr>
<tr>
<td>1:2</td>
<td>8,623 ± 546</td>
</tr>
<tr>
<td>1:9</td>
<td>7,177 ± 200</td>
</tr>
<tr>
<td>1:3</td>
<td>5,192 ± 1184</td>
</tr>
<tr>
<td>No dilution</td>
<td>2,176 ± 174</td>
</tr>
<tr>
<td>LPS/DxSO₄</td>
<td>89,383 ± 9177</td>
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</tbody>
</table>

T cell-depleted spleen cells (2 × 10⁵) from B10.BR mice were cultured with the indicated numbers of irradiated (2000 rad) D10 cells in the presence or absence of conalbumin (200 µg/ml) or with culture supernatants (SN) from Con A-activated D10 cells, diluted as shown. Thymidine incorporation was measured at 48 hr after a 10-hr pulse. LPS/DxSO₄ bacterial lipopolysaccharide and dextran sulfate.

ml at 37°C in 5% CO₂ in air. The D10 cell line was maintained by biweekly stimulation with antigen (conalbumin, 200 µg/ml) and spleen cells from I-A<sup>k</sup> mice. Three days after antigen stimulation, D10 cells were expanded and rest in medium containing 5% mutant supernatant from Con A-stimulated rat spleen cells. For activation, Con A (2.5 µg/ml) was added to 150-cm² tissue culture flasks containing rested D10 cells at ~1 × 10⁶ per ml. In assays that compare activities associated with culture supernatants and cell membranes, supernatants and cells used for membrane preparation were collected from the same cultures. In this report, "resting" D10 cells refer to cells rested in mutant supernatant from Con A-stimulated rat spleen cells for at least 7 days. "Activated" D10S refers to cells that were activated by culture with Con A after a period of ~7–10 days of rest.

Membrane Preparation. The procedure used is based on that of Maeda et al. (9). After overnight activation, α-methyl-D-mannoside was added to the cultures to a concentration of 100 µg/ml, and cells were incubated for an additional hour at 37°C. Cells were then harvested, washed twice in phosphate-buffered saline containing α-methyl-D-mannoside, and finally resuspended at a density of 1–5 × 10⁶ cells per ml in cold homogenization buffer (100 µg of α-methyl-D-mannoside per ml/20 mM Tris chloride/10 mM NaCl/0.1 mM MgCl₂/0.1 mM phenylmethylsulfonyl fluoride/0.5 µg of DNase I per ml). Cells were disrupted by a Polytron (Brinkman) homogenizer at a speed of 5–7. One to three 5-sec homogenizations were usually found to be sufficient to break >90% of the cells. The homogenate was transferred to an ultracentrifuge tube and underlaid with cold 41% (wt/vol) sucrose in homogenization buffer. Membranes were separated from soluble proteins and dense subcellular debris by centrifugation at 95,000 × g for 1 hr (SW 28 rotor at 26,000 rpm). The fluffy white interfacial band consisting of plasma membrane fragments was collected and diluted 1:2 to 1:3 in serum-free medium and washed twice by centrifugation (Beckman 50 Ti rotor at 35,000 rpm for 30 min) in serum-free medium. The membrane pellet was then resuspended at a final concentration of 1–2 × 10⁷ cell equivalents per ml based on the starting cell number. All operations were performed aseptically.

B-Cell Proliferation. Mice were 6–12 wk old and were obtained from The Jackson Laboratory or from a mouse colony maintained at the University of California at San Diego by Richard Dutton. T cells were removed from spleen cell preparations by incubation in an antibody cocktail containing HO-13-4 (anti-Lyt-2) (10), AD4(15) (anti-Lyt-2) (11), and F7DSK6 (anti-Thy-1.2) (12); or T24/31.7 (rat anti-

### RESULTS

**B-Cell Response to T-Cell Membranes.** T cell-depleted spleen cells (B cells) were cultured with membranes prepared from resting or Con A-activated D10 cells. Significant proliferation occurred in cultures containing membranes from activated but not resting D10 cells (Fig. 1). The response to membranes from activated T cells was dose-dependent. No increase in the ability of inactive membranes to stimulate B-cell proliferation occurred when Con A (0.8 µg/ml) was added to cultures. Thus, the effect of activated membranes is not due to residual Con A. The effect of conalbumin on B-cell proliferation induced by activated D10 membranes (Fig. 1) varied from experiment to experiment, from an infrequently observed enhancement of ~30% to the more usual undiscernible increase in proliferation. Proliferation in cultures containing membranes from resting D10

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**Fig. 1.** Effects of antigen [conalbumin (conalb.), 200 µg/ml] and previous activation (Act. D10, activated D10) on the ability of D10 membrane preparations to induce B-cell proliferation. The maximal concentration shown is 8 × 10⁷ cell equivalents of membrane preparation per well. In this experiment, the duration of the [3H]thymidine pulse was 72 hr. Responding cells were from B10.BR mice. Control values were: background, 6919 ± 1108; with conalbumin, 9397 ± 1056; with LPS/DxSO₄, 78339 ± 1861.
cells was never higher than in control cultures, even in the presence of conalbumin. Conalbumin itself increased background proliferation, even with B cells from the endotoxin-resistant strain C3H/HeJ.

Proliferation also occurred in response to supernatants from activated D10 cells. The levels seen were generally lower than proliferation induced by membrane preparations at high concentrations of membranes. Fig. 2 shows dose-response curves for membranes from activated D10 cells and the corresponding Con A supernatants (see also Table 1). The most notable characteristic of these titrations is the comparatively flat curve obtained for supernatants compared to membranes.

As might be expected from the insensitivity to antigen, induction of B-cell proliferation was not found to be MHC-restricted (Fig. 3). The response of B cells from MHC-matched B10.BR mice was not greater than the response of B cells from BALB/c mice. To determine whether stimulation of B-cell proliferation was an unusual property of membrane preparations from the D10 cell line, these experiments were repeated with membranes prepared from Con A-stimulated blasts. Membranes prepared from 72-hr blasts also stimulated B-cell proliferation (Fig. 4). As with D10 membranes, no difference was seen in the level of stimulation or in the dose-response curves for syngeneic and nongeneic B cells. Although the level of stimulation was not as high as that usually mediated by D10 membranes, it was reproducibly several fold above background.

Membrane-Associated Lymphokines. Experiments repeatedly showed that membrane preparations from activated and resting D10 cells were vastly different in their ability to stimulate B-cell proliferation. A possible explanation for this difference is that lymphokines secreted into the medium by activated cells partition or adsorb onto the membrane and in this form are more effective. However, the ability of membranes from activated D10 cells to stimulate B-cell proliferation was not reconstituted by combining D10 Con A supernatants with membranes from resting D10 cells (not shown). Even in B-cell cultures containing supernatants concentrated 10-fold, addition of membranes from resting D10 cells caused no increase in the level of proliferation. Thus, it seems unlikely that the effect of membranes from activated D10 cells is simply an enhanced delivery of the complement of secreted lymphokines via membrane adsorption. To investigate further the possibility that membranes carry secreted lymphokines, culture supernatants and membranes from activated D10 cells were titrated into cultures of the IL-4- and IL-2-sensitive cell line NK. This cell line was exquisitely sensitive to lymphokines secreted by the D10 cell line (Fig. 5a). Even at dilutions of supernatant in the order of 1:1000, the level of proliferation was substantial. Membranes from activated D10 cells also caused NK proliferation, but the dose-response curves were very different. Membranes were consistently found to be 2-4 orders of magnitude less potent than supernatants for stimulation of NK proliferation. The rat monoclonal antibody 11B11 (17) against murine IL-4 blocks 70% of the membrane-associated activity in this assay (not shown). Membranes from Con A-stimulated blasts also stimulated NK proliferation (not shown). The levels seen were very low and were substantially below those found in supernatants. Similar titrations were carried out in the presence of the IL-5-sensitive B cell lymphoma BCL1 (Fig. 5 b and c). Proliferation of this cell line was stimulated by membrane preparations from activated D10 cells, but the membranes were again less potent by orders of magnitude than the corresponding Con A supernatants.

Interaction Between Membrane-Associated and Secreted Activities. As another approach to determining whether the
membrane-associated activity merely reflects the profile of lymphokines contained in supernatants. B-cell proliferation assays were carried out in which membrane preparations were titrated in the presence of a fixed concentration of supernatant from Con A-activated D10 cells. In Table 2, the last column shows the difference between the observed effect of combining supernatant and membranes and the expected effect based on the levels seen with each variable alone. The effect of combining membranes and supernatants was not additive at high concentrations of membranes. The proliferation seen in cultures containing both membranes and supernatants was greater than the value obtained by taking the sum of proliferation by B cells cultured with membranes only and supernatant only. At the high end of the range tested—i.e., at membrane concentrations of $4 \times 10^6$ cell equivalents per well and greater, the difference was statistically significant ($P < 0.005$ in Student’s $t$ test). At low concentrations of membranes, the effect of combining membranes and Con A supernatants was sometimes less than additive, though the difference usually was not found to be statistically significant in the range tested.

**Table 2. Interaction between membranes and Con A supernatants in the stimulation of B-cell proliferation**

<table>
<thead>
<tr>
<th>Murine responder cells</th>
<th>Cell equivalents of membranes $\times 10^{-5}$</th>
<th>$[^{3}H]$Thymidine incorporation, cpm</th>
<th>Membranes</th>
<th>Membranes + SN (1:8)*</th>
<th>Observed - expected†</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.BR (72 hr)</td>
<td>17</td>
<td>10,373 ± 1281</td>
<td>32,331 ± 926</td>
<td>18,311</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5,968 ± 946</td>
<td>16,463 ± 1165</td>
<td>6,848</td>
<td></td>
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<tr>
<td></td>
<td>4</td>
<td>4,351 ± 793</td>
<td>10,831 ± 842</td>
<td>2,833</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2,542 ± 579</td>
<td>7,289 ± 1211</td>
<td>1,000</td>
<td></td>
</tr>
<tr>
<td>C3H/HeJ (48 hr)</td>
<td>17</td>
<td>12,251 ± 1291</td>
<td>29,528 ± 2872</td>
<td>10,730</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8,628 ± 350</td>
<td>21,320 ± 1216</td>
<td>6,645</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6,067 ± 326</td>
<td>15,396 ± 858</td>
<td>2,782</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4,317 ± 278</td>
<td>10,660 ± 1780</td>
<td>-204</td>
<td></td>
</tr>
</tbody>
</table>

*At a 1:8 dilution of supernatant (SN) from Con A-activated D10 cells, the level of proliferation was $5008 \pm 214$ cpm in B10.BR cells and $8933 \pm 785$ cpm in C3H/HeJ cells; background proliferation was $1361 \pm 124$ cpm and $2386 \pm 335$ cpm, respectively; and levels induced by LPS/DxSO_{4} were $87,394 \pm 12,392$ cpm and $24,586 \pm 670$ cpm, respectively.

†Experimentally observed values (column 3) – expected values. Expected values were calculated by adding the value for $[^{3}H]$thymidine incorporation observed in the presence of a 1:8 dilution of supernatant to the value observed for membranes alone and subtracting the value for background proliferation.

**DISCUSSION**

To resolve contributions of soluble factors from those of physical contact during interactions between T and B cells, I have chosen the simple approach of assessing the ability of plasma membrane fractions from T cells to stimulate B-cell proliferation. Isolated membranes stimulated proliferation provided that the T cells had been activated prior to preparation of membranes. The observation that isolated T-cell membrane fractions can induce B-cell proliferation is unusual and implies a role for membrane receptors or lymphokines in B-cell activation. Some obvious sources of artifact in these experiments are residual Con A or endotoxin contamination in the membrane preparations. To neutralize and remove Con A, cells were washed and homogenized in buffer solutions containing α-methyl-D-mannoside. Addition of Con A to cultures containing inactive membrane fractions did not cause proliferation of B cells. Membranes from resting and Con A-activated D10 cells were prepared in parallel from parallel cultures. The consistent inability of membrane fragments from resting D10 cells to stimulate B-cell proliferation argues against the presence of significant levels of endotoxin contamination. Levels of proliferation of cells from the endotoxin-resistant strain C3H/HeJ were comparable to levels obtained in cultures of cells from other strains, even though LPS/DxSO_{4}-induced proliferation is substantially reduced. Therefore, Con A and endotoxin contamination are highly unlikely to be sources of membrane-associated activity described in this report.

Although the presence of IL-4 and IL-5 on activated T-cell membranes was indicated by the proliferation of lymphokine-sensitive cell lines, the B-cell proliferation-inducing activity of activated T-cell membranes does not appear to be ascribable solely to membrane-bound forms of these two lymphokines for two reasons. First, dose–response curves for Con A supernatants and activated T-cell membranes in B-cell cultures did not reflect those found in assays for lymphokines. Although membranes were nearly always more effective in stimulating B-cell proliferation, supernatants were consistently far more potent in lymphokine assays using indicator cell lines. Furthermore, the effect of combining membranes and supernatants from activated D10 cells in B-cell cultures was more than additive, implying a qualitative difference in membrane- and supernatant-associated activities.

The addition of conalbumin, the antigen for which D10 cells are specific, to cultures containing D10 membranes had little or no effect on B-cell proliferation beyond an increase in background levels. This observation supports the view that the primary role of antigen-specific MHC-restricted recognition of B cells by T cells is activation of the T cell,
with no necessary contribution to B-cell activation. A number of reports have suggested a signal-transducing role for class II MHC on B cells during activation. Antibodies against Ia have been reported to augment (3) or to inhibit (18, 19, *) B-cell activation by B-cell mitogens or mitogen-activated T cells. More recently, ligation of class II MHC has been shown to cause the translocation of protein kinase C from the cytoplasm to a detergent-insoluble nuclear fraction (20). It might be argued that proliferation, as reflected by thymidine incorporation, is too insensitive to observe a T-cell receptor-mediated contribution to B-cell activation. Early experiments using flow cytometry to measure cell cycle progression also failed to show any effect of conalbumin. Although the T-cell receptor for antigen is evidently irrelevant in the assay system used here, CD4 may participate in its capacity as a receptor for class II MHC (21, 22).

Flow cytometric analysis indicated little difference in the level of expression of either the antigen receptor or CD4 after activation of D10 cells by Con A. This argues against the direct involvement of CD4 in induction of B-cell proliferation. In the absence of a quantitative difference in CD4, a qualitative difference might be sought to explain the strong dependence of B-cell proliferation on previous activation of D10 cells. Recently, a physical interaction between antigen receptor and CD4 has been suggested to occur during T-cell recognition (23). A Con A-induced redistribution of CD4 in D10 membranes might furnish the basis for a qualitative difference in the signal delivered by activated and resting T-cell membranes. Alternatively, ligation of Ia by CD4 may provide a signal that is inadequate by itself but able to synergize with signals provided by other molecules on the surface of the activated T-cell.

IL-1 is known to be associated with the membranes of activated macrophages and monocytes (24). The mechanism for this association is an enigma because the genes for IL-1α and IL-1β do not encode leader sequences (25). Similarly, granulocyte-macrophage colony-stimulating factor is compartmentalized by stromal-cell glycosaminoglycans (26). The preferential association of growth factors and hormones with cell membranes or the extracellular matrix may be an important mechanism for the maintenance of specialized microenvironments in vivo. By maintaining locally high concentrations of growth factors at the activated T-cell surface, this mechanism would enhance the specificity of immune responses by ensuring that B cells presenting antigen are preferentially activated. The association of T-cell growth factors, possibly either IL-2 or IL-4, with T-cell membranes has been reported (27). In addition to membrane-associated IL-4 and IL-5 found in the present work, the nonadditive effect of combining supernatants from Con A-stimulated D10 cultures and membranes suggests that other molecules with mitogenic activity for B cells may be membrane-associated but scarce in supernatants. It is not possible to conclude whether any of these lymphokines reside on the membrane through simple adsorption from solution, or whether these are truly membrane forms having a hydrophobic anchor. Regardless of the mechanism for their association with membranes, IL-4 and IL-5 are either present in much lower quantities than in Con A supernatants or they exist in a form that is not efficiently delivered to the indicator cell lines, one of which is a B-cell lymphoma.

It cannot be stated whether the observed effect is directly on B cells, which B-cell population is responding, or whether the proliferating cells differentiate into immunoglobulin-secreting cells. On the balance of the evidence I conclude that T-cell activation leads to the expression of cell-surface molecules with mitogenic activity toward some population of B cells and perhaps other hemopoietic cells. These molecules could be the source of the often observed MHC-unrestricted bystander or transstimulation occurring in mixed cultures of T cells and allogeneic and syngeneic antigen-presenting B cells. Whether the observed proliferation in such cultures is MHC-restricted or not would depend primarily on cell density and the ratio of syngeneic to allogeneic B cells. These factors would influence T-cell activation and the probability of contact between activated T cells and allogeneic B cells. Under physiological conditions, where antigen is limiting, the B cells most likely to activate T cells and thereby be exposed to T-cell membrane-associated lymphokines are those that present antigen trapped by specific surface immunoglobulin.

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