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Synergistic effects of antigen and soluble T-cell factors in B-lymphocyte activation

(B-cell proliferation/B-cell differentiation/lymphokines)

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ABSTRACT **Supernatants from phorbol 12-myristate 13-acetate-activated cultures of the mouse EL4 thymoma, or of several mouse T-cell hybridomas stimulated either by their specific antigen or by concanavalin A, induced primary splenic B cells to proliferate and differentiate to antibody-secreting cells. This effect was not due to interleukin 2 and did not require the presence of macrophages. The antibody response was polyclonal, including antibodies specific for 2,4-dinitrophenyl and pigeon cytochrome *c*, present in amounts of 1% or less of the total immunoglobulin produced. The addition of either of these antigens increased the amount of the corresponding specific antibody. At very high concentrations of dinitrophenyl-hemocyanin the specific response could be depressed. These observations were taken to demonstrate that soluble T-cell factors are sufficient to activate a portion of naive B cells to antibody secretion and that under these conditions *in vitro* the presence of antigen merely enhances the specific response.**

Since the original description of a role for T cells in the antigen-dependent stimulation of B cells (1, 2), a great deal of effort has gone toward elucidating the mechanism by which helper T cells function. The present evidence indicates that B cells require two signals to be stimulated to secrete antibody (3, 4). The first is delivered through the B cell's surface immunoglobulin (Ig) receptor by the binding of antigen, a process that has been proposed to be facilitated by the presentation of antigen by antigen-recognizing T cells (1, 5, 6). The second signal is provided by soluble products synthesized by T cells (7–14) and is not delivered through the Ig receptor but presumably through as-yet-unidentified specific receptors.

Attempts to isolate and characterize soluble helper T-cell factors and to determine how these act in synergy with antigens to stimulate B cells have not yet established their number, their function, or whether these factors act in a definite order. Recent studies suggest that induction of B-cell proliferation requires T-cell factors that are distinct from those inducing B-cell differentiation to antibody-secreting cells (4, 8–10, 12, 15, 16). In certain experimental systems, stimulation to proliferation occurs only in the presence of an additional macrophage-derived cofactor, interleukin 1 (17).

The antigen receptor signal can be provided by the binding of anti-Ig antibodies (10, 12) or of soluble antigen (13, 18–20). While earlier studies indicated that soluble antigens were effective in the absence of T cells (13, 18), more recent reports (19, 20) concluded that the presence of antigen-recognizing T cells of the same major histocompatibility complex (MHC) haplotype were essential. After incubation with either anti-Ig or T cells and antigen, B cells were found to be receptive to soluble T-cell factors (10, 12, 19, 20).

The present study demonstrates that naive B cells can be activated by an appropriate concentration of T-cell factors to both proliferate and secrete antibody of the IgM isotype and that a dose-dependent augmentation of a particular response can be observed with the addition of the corresponding soluble antigen.

MATERIALS AND METHODS

BALB/c female mice 5–6 weeks of age were obtained from Harlan Sprague–Dawley (Madison, WI). CBA/J female mice 5–6 weeks of age were obtained from The Jackson Laboratory (Bar Harbor, ME). The preparation of 2,4-dinitrophenyl-derivatized *Limulus polyphemus* (horseshoe crab) hemocyanin (DNP-HCH), containing approximately 20 mol of DNP per 100,000 g of protein (6), and pigeon cytochrome *c* (pigeon *c*) (21) were as earlier detailed.

Preparation of EL4 Supernatant. Cells of the EL4 mouse thymoma line, kindly provided by Frank W. Fitch (University of Chicago), were cultured at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DME medium; GIBCO), supplemented with folic acid (6 mg/liter), arginine (216 mg/liter), asparagine (36 mg/liter), glutamine (116 mg/liter), sodium pyruvate (110 mg/liter), sodium bicarbonate (2 g/liter), penicillin (1×10^6 units/liter), and streptomycin (1.0 g/liter) (supplemented DME medium) and containing 10% fetal calf serum (Dutchland, Denver, PA). When a density of 1.0×10^6 cells per ml was reached, the EL4 cells were induced by the addition of phorbol 12-myristate 13-acetate (PMA) (Sigma) at a final concentration of 20 ng/ml. After a 4-hr incubation, cells were collected by centrifugation at $750 \times g$, washed three times with DME medium to remove PMA and fetal calf serum, and resuspended to $4–6 \times 10^5$ cells per ml in a serum-free supplemented DME medium. After 24-hr incubation, the supernatants were harvested.

T-Cell Hybridomas. T-cell hybridomas were obtained by the fusion of three cloned T-cell lines, each kindly provided by Frank W. Fitch, with the T-cell thymoma line BW5147 (22). These were J6.19 (23), L2, and L2V, a variant of L2, that does not secrete interleukin 2 (IL-2) (24).

IL-2 Assay. IL-2 was detected by the ability of a supernatant to maintain the growth of the IL-2-dependent cell line HT-2, obtained through the Stanford University Cell Bank (25).

Preparation of B Cells. Single-cell suspensions of spleen cells were treated to remove erythrocytes (26) and depleted of T cells by treatment with complement and the anti-T cell monoclonal antibodies anti-Thy 1.2, anti-Lyt 2.2, and anti-L_3T_4 (23), each kindly provided by Frank W. Fitch. The depletion of functional T cells after this treatment was measured by their response to concanavalin A (Con A) (2

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Abbreviations: DNP-HCH, 2,4-dinitrophenyl-derivatized horseshoe crab hemocyanin; IL-2, interleukin 2; MHC, major histocompatibility complex; PMA, phorbol 12-myristate 13-acetate; pigeon *c*, pigeon cytochrome *c*.

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μg/ml), which was decreased to approximately 1/70th as compared to cells treated with complement alone (1000 cpm/5 × 10^5 cells as compared to 70,000 cpm). Macrophages were removed by passage through two columns of Sephadex G-10 (Pharmacia) (27). After 5 days, the cultures showed none of the macrophage-like cells that were prominent in untreated control cultures. The viable cells were cultured in 96-well tissue culture plates (Nunc) at 5 × 10^5 cells per well in supplemented DME medium with 50 μM 2-mercaptoethanol, 0.01 M 3-(*N*-morpholino)propanesulfonic acid, and 5% fetal calf serum (complete medium) in a final volume of 0.2 ml.

Assay for Cell Proliferation. Twenty-five microliters containing 0.5 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine (2 Ci/mmol, Amersham) were added to 0.2-ml cultures for the last 4 hr of a 24- or 72-hr incubation period. Cells were then harvested onto glass fiber filters (934-AH, Whatman), and the filters were placed in 3 ml of toluene containing 2,5-diphenyloxazole (4 g/liter) and their radioactivities were measured in a liquid scintillation counter.

Antibody Assays. Twenty-four hours after the initiation of the culture, B cells were washed four times to remove antigen, and complete medium containing the appropriate concentration of EL4 supernatant was added back. Culture supernatants were sampled between 3 and 10 days at 3-day intervals and assayed for DNP- and pigeon *c*-specific antibodies and total Ig. The peak antibody response, which usually occurred on day 5 to day 7, is reported. DNP- or pigeon *c*-specific antibody was detected by using a solid-phase radioimmunoassay (28), in which mouse antibodies were detected with rabbit antiserum to mouse F(ab')$_2$ or rabbit antibodies to mouse Ig heavy chains (29), followed by affinity-purified ^{125}I-labeled goat antibodies to rabbit Ig. Total Ig was measured by a modification of this assay using polyvinyl chloride microtiter plates coated with affinity-purified rabbit antibody to mouse F(ab')$_2$ and then affinity-purified ^{125}I-labeled rabbit antibody to mouse F(ab')$_2$ as the detecting reagent.

RESULTS

The 24-hr Proliferative Response of B Cells to Antigen and T-Cell Supernatants. B cells from nonimmune mice were cultured in the presence of graded doses of DNP-HCH, over a range of concentrations of the supernatants from induced T-cell cultures. Twenty-four hours later, the cultures were assayed for cell proliferation. The supernatants examined included those obtained from the EL4 cell line induced by PMA and those from a panel of antigen-specific T-cell hybridomas (listed in Table 1) stimulated by their antigen or Con A. In all cases similar results were obtained, and those obtained with supernatants from PMA-induced EL4 cells are shown. With B-cell cultures from CBA/J mice (Fig. 1), the EL4 supernatant induced a dose-dependent proliferative response, in contrast to supernatants from unactivated EL4 cultures, which had no measurable effect on the B cells.

The addition of DNP-HCH to cultures that contained no EL4 supernatant resulted in a moderately increased thymidine incorporation, while in cultures to which the T-cell factors had also been added, proliferation was increased even further in a dose-dependent fashion.

The factors present in the EL4 supernatant that induced the proliferative response were neither the T-cell growth factor, IL-2, nor PMA. No correlation was observed between the IL-2 titer and the B-cell proliferation-inducing activity of supernatants from a series of T-cell hybridoma cell lines (Table 1). Furthermore, partial purification of the proliferation-inducing factor from the EL4 supernatant by using HPLC resulted in its separation from IL-2 (unpublished data). With regard to possible residual PMA, supernatants of T-cell hybridomas that had been induced with Con A or antigen were as effective as the PMA-induced EL4 supernatant (Table 1), demonstrating that PMA was not responsible for the effects recorded in Fig. 1. Similarly, the presence of macrophages was apparently not needed, since macrophage-depleted and untreated cultures responded in the same fashion, with the corresponding stimulation indices (calculated as in Fig. 1) being 4.1 and 4.4.

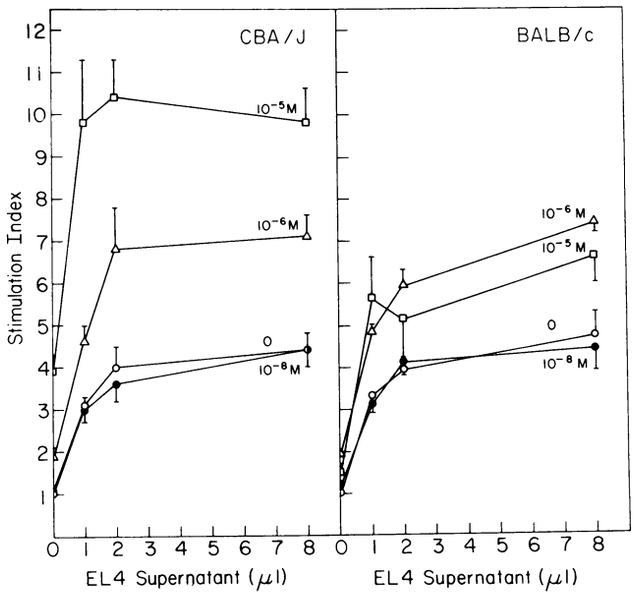

FIG. 1. The 24-hr B-cell proliferative response as a function of the volume of EL4 supernatant at various concentrations of DNP-HCH. The cultures were pulsed with [^{3}H]thymidine for the last 4 hr of a 24-hr incubation period. The ordinate shows the stimulation index, the amount of [^{3}H]thymidine incorporated into stimulated cells divided by that incorporated into unstimulated cells. DNP-HCH is expressed as the molarity of DNP. Unstimulated cultures gave values of approximately 1600 cpm (stimulation index = 1.0). Each point in the figure represents the average of the results of four separate experiments in triplicate and the SEM is shown.

Similar results were obtained with B cells from BALB/c mice (Fig. 1), indicating no strain specificity in the proliferative response to the EL4 supernatant. In general, the responses of BALB/c B cells were lower than those from CBA/J mice, and the BALB/c B cells showed a maximal response at 10^{-6} M DNP, whereas CBA/J B cells increased even further at 10^{-5} M DNP.

Table 1. B-cell proliferation-inducing activity and IL-2 content of T-cell hybridoma supernatants

Hybridoma line	Hybridoma activation*	IL-2 conc.†	B-cell proliferation, cpm‡
L2H.G9	None	<3	1513 ± 226
L2H.G9	Con A	81	7095 ± 922
L2H.DG	Con A	81	3111 ± 279
L2H.G11	Con A	27	6911 ± 760
L2H.B9	Con A	27	1278 ± 111
J6.19H.A10	Con A	9	6708 ± 749
L2VH.B2	Antigen	<3	3026 ± 372
L2VH.F1	Antigen	<3	9052 ± 904

*Hybrid cells (1–3 × 10^6/ml) were treated with Con A at 10 μg/ml for 24 hr and harvested, and methyl α-D-mannoside was added (5-fold molar excess over Con A). Antigen activation was carried out as described by Ely *et al.* (24).

†IL-2 concentration is shown as the reciprocal of the lowest dilution that supported the growth of the IL-2-dependent cell line HT-2.

‡The 24-hr proliferative response was measured in culture media containing 25% activated supernatants. Results are mean ± SEM.

The proliferative response of CBA/J B cells to pigeon *c* was also assessed. The response induced by EL4 supernatant was augmented by the addition of pigeon *c* at 10 μg/ml, the largest stimulation, a 2-fold increase, being observed at the highest concentration of pigeon *c* tested, 160 μg/ml (data not shown).

The 72-hr Proliferative Response of B Cells to Antigen and EL4 Supernatant. The addition of PMA-activated EL4 supernatant in the absence of antigen induced both CBA/J and BALB/c B cells to proliferate in a dose-dependent fashion, as measured 72 hr after the initiation of culture (Fig. 2). The stimulation with the addition of EL4 supernatant alone was approximately the same as the maximal 24-hr response augmented by the addition of DNP-HCH. However, unlike the 24-hr response, antigen only slightly increased the 72-hr proliferative response. A possible explanation of these observations is that the augmentation of the response by antigen, observed at 24 hr, was due to a temporal advantage given to those cells interacting with both antigen and lymphokines, while, 48 hr later, all cells that could respond had already been triggered.

The Antibody Response Stimulated by Antigen and EL4 Supernatant. The addition of EL4 supernatant alone to CBA/J-derived B cell cultures induced DNP-HCH-specific antibody secretion, in a dose-dependent fashion, as measured after 5–7 days of culture (Fig. 3). This specific antibody represented about 1% of the total Ig produced (approximately 15 μg/ml of culture), the vast majority of which was of the μ and not of the γ_1 isotype. The addition of DNP-HCH did not significantly augment the anti-DNP response at any concentration tested, a behavior similar to that of the 72-hr proliferative response.

B cells from BALB/c mice responded to EL4 supernatant in the same fashion (Fig. 3). However, unlike CBA/J B cells, the DNP-specific antibody secretion was slightly augmented by the addition of antigen at one concentration (10^{-6} M DNP), and significantly decreased at a higher concentration (10^{-5} M DNP). These effects appeared to be antigen specific in that the total Ig secreted, approximately 15 μg/ml of culture supernatant, was not significantly different at any dose of DNP-HCH. Thus, it was possible to select sets of conditions under which the specific response to an antigen was increased or decreased, without affecting the overall proliferative response of the culture (see Fig. 2).

The antibody response of B cells from CBA/J mice to pigeon *c* was also found to increase with increasing concentrations of added EL4 supernatant (Fig. 4). This response was augmented by the addition of pigeon *c* to the cultures, with the greatest effect observed at 40 μg/ml, higher or lower concentrations being less effective. The optimal concentration of pigeon *c* for the induction of proliferation (160 μg/ml) was not optimal for antibody secretion, again demonstrating that conditions which favor the proliferative response are not necessarily the same as those which favor antibody secretion.

DISCUSSION

The present experiments demonstrate that a portion of B cells from nonimmune mice can be polyclonally stimulated to proliferate and to differentiate into antibody-secreting cells by exposure to factors contained in supernatants from activated EL4 cells or T-cell hybridomas. The magnitude of specific antibody responses induced by such supernatants alone could be augmented by the addition of the corresponding antigen.

At present, it is not possible to definitively establish if the T-cell supernatant factors act directly on the B cells or indirectly through residual T cells or macrophages present in the culture. While this is likely to be resolved by direct binding studies of pure activating factors to B cells, certain possibilities have been made unlikely by the present results. First, the supernatant factor(s) that induced B cells to proliferate and secrete antibody were clearly not IL-2, as demonstrated by the ability of T-cell supernatants that did not contain measurable IL-2 to activate B cells (Table 1) and the inability of purified IL-2 to trigger B cell responses (data not shown). Thus, these responses could not be due to the indirect effects of T cells activated by IL-2. Second, rigorous depletion of macrophages from the cell preparations had little or no effect on the B-cell response to the T-cell supernatants, indicating that macrophages were unlikely to represent the limiting factor in the B-cell activations observed. However, these results do not rule out the possibility that macrophages may contribute a cofactor for the observed B-cell responses, as

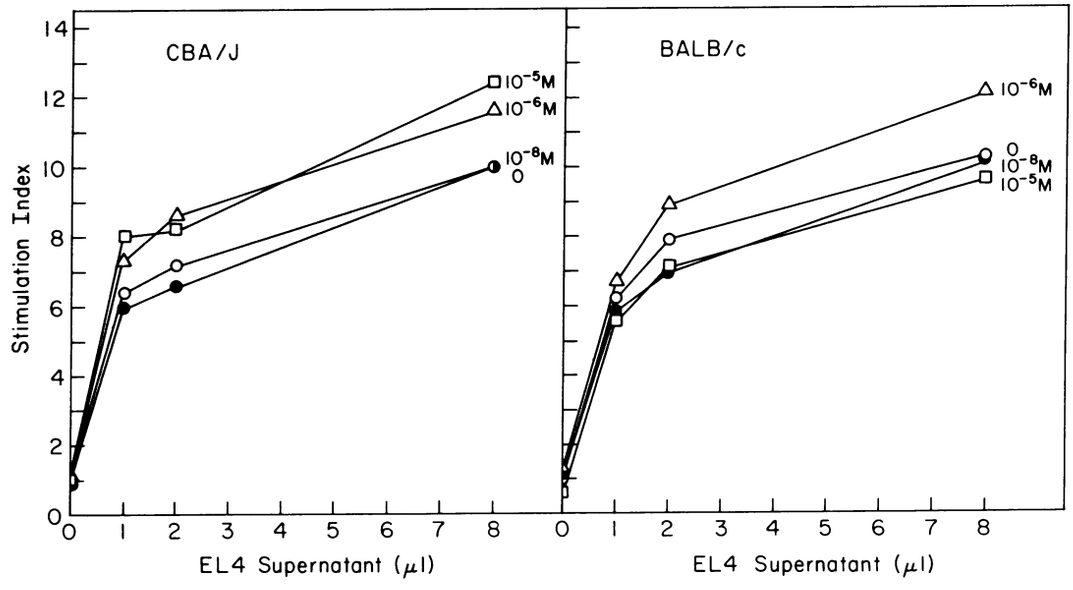

FIG. 2. The 72-hr B cell proliferative response as a function of the volume of EL4 supernatant at various concentrations of DNP-HCH (expressed as molarity of DNP). The cultures were pulsed with [^{3}H]thymidine for the last 4 hr of a 72-hr incubation period. Unstimulated control cultures averaged 1300 cpm (stimulation index = 1.0). Each point in the figure represents the average stimulation index from four experiments in triplicate, for which the SEM was less than 17% for CBA/J B cells and less than 25% for BALB/c B cells.

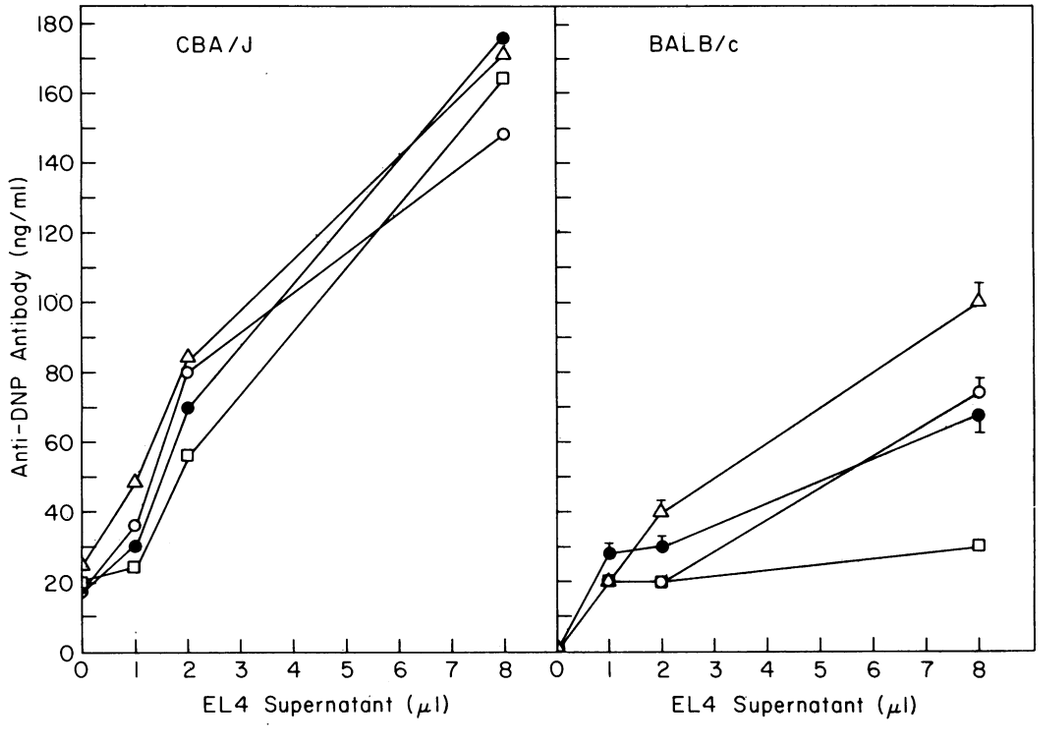

FIG. 3. DNP-specific antibody response as a function of the volume of EL4 supernatant at various concentrations of DNP-HCH. DNP-HCH was added in a final concentration of 10^{-5} M DNP (□), 10^{-6} M DNP (△), 10^{-7} M DNP (●), or not added (○). Each point represents the average of triplicate cultures of a representative experiment. The SEM for CBA/J B cells was less than 20%, and that for BALB/c B cells is shown.

suggested by Howard *et al.* (17), since macrophage depletion may not have been complete.

An important conclusion drawn from the results of the present experiments is that B cells are activated by soluble T-cell factors alone, in the absence of an overt stimulus through the Ig receptor. This is in contrast to the results of other investigators, which suggest that B cells become responsive to growth or differentiation factors only after an antigen or anti-Ig signal (4, 12, 19, 20). However, the phenomenon reported here has been observed before. Eisenberg *et al.* (30) have shown activation of B cells to secrete antibody to phosphocholine after exposure to partially purified factors from cloned T-cell line supernatants. Other investigators, studying T cells specific for proteins present on all cell surfaces, indicated that, when such T cells were cocultured with B cells, a polyclonal antibody response resulted (31, 32), presumably through the action of soluble T-cell factors. Our own studies (33), in which B cells and pigeon *c*-specific T-cell hybridomas were cocultured, demonstrated that the activation of the T cells by their specific antigen leads to a polyclonal activation of the B cells, a finding similar to that very recently reported by DeFranco *et al.* (34).

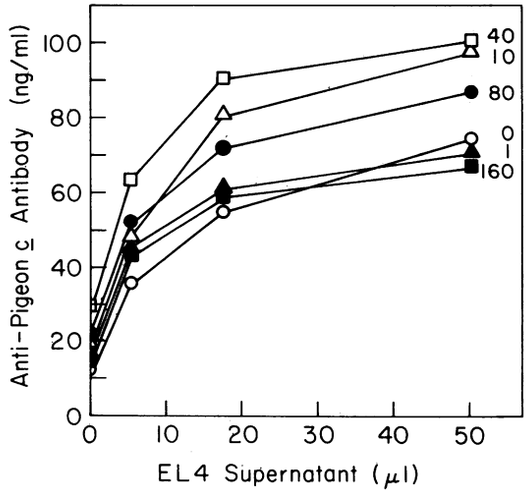

FIG. 4. Pigeon *c*-specific antibody response of B cells from CBA/J mice as a function of the volume of EL4 supernatant at various concentrations of pigeon *c*. The concentrations of pigeon *c* is shown in μg/ml. Each point in the figure represents the average of the results of two separate experiments, in which the SEM was less than 10%.

Nevertheless, lymphokine activation of B cells is not a generally observed phenomenon. A possible explanation for this discrepancy is that, in our experiments, the B-cell response to the EL4 supernatant alone was not the result of the stimulation of naive resting B cells, but rather of B cells that had already encountered antigen *in vivo*. This is an ambiguity inherent in the interpretation of any experiment that uses primary B cells, which may have encountered either antigen or activating factors *in vivo*, so as to influence their subsequent stimulation by one or both *in vitro*. However, in the present case, even though one might argue that a portion of B cells may have been exposed *in vivo* to antigens that cross-react with the chemically simple DNP determinant, this is particularly unlikely for the highly specific, conformationally dependent, antigenic determinant on pigeon *c* (35). A second consideration is the length of time a B cell might be expected to remain in a growth factor receptive state after antigenic stimulation *in vivo*. Published results suggest that this period may be no longer than 18 hr (4). The probability that the B-cell population encountered both of these determinants in the 18 hr preceding the experiment is even lower.

An alternative explanation for the generally observed inability of lymphokines alone to activate B cells might be found in the common use of anti-Ig to purify B cells. Anti-Ig is known to trigger early activation events that result in an increase in the cell size (4). This activation presumably

occurs through cross-linking of receptors, leading to their internalization, so that after an anti-Ig exposure, as during purification, the B cell will replace its surface Ig receptors. However, nothing is known about the fate of postulated activating factor receptors on the cell surface during these events. If the density or number of such receptors is decreased in the enlarged anti-Ig-treated cell, it may no longer be able to respond to signals from growth factors alone and require the synergistic effect of a combination of signals delivered by both anti-Ig or antigen and growth factors. Our own experience has been that B cells, obtained by anti-Ig panning, were less responsive and at times refractory to activating factors alone (unpublished observations). If some such explanation proves to be correct, the differences between resting and anti-Ig-panned B cells are not trivial and may lend valuable insights into the B-cell activation mechanism.

A second important observation was that the presence of a soluble antigen could augment the response of the B-cell population over that produced by the activating factors. It remains to be determined if the cells that responded to the activating factor(s) and antigen are a subset of those that responded to activating factors alone. If resting B cells have both activating factor and immunoglobulin receptors on their surfaces, it is possible that a signal through either receptor, of the appropriate magnitude, could induce the expected response. This would account for the slight but significant effect of the antigen alone, attributable to those B cells that have a high affinity for the antigen. Conversely, the response to activating factors alone may be due to those B cells that have a large number of T-cell factor receptors. In the presence of both antigen and activating factors a greater number of cells would meet the minimal threshold binding requirements for stimulation, accounting for the increased magnitude of the response.

Since B cells can be activated through exposure to antigen and T-cell-derived factors, is there a role for MHC-restricted interactions in the B-cell activation pathway? Recent studies (36, 37) indicate that B cells may serve as antigen-presenting cells in the activation of class II MHC-restricted T cells. This suggests that intimate interactions may be possible between T and B cells, leading to the triggering of both, and possibly to higher-order phenomena, such as isotype switching (29, 38, 39), the generation of memory cells (40, 41), and the modulation of the induction of tolerance (42, 43). However, these are all suppositions, the only clearly demonstrated function of T cells in B-cell activation being the production of soluble growth and differentiation factors. It remains to be determined over what distances such factors can travel under physiological conditions and remain effective. If such distances are short, direct B-cell–T-cell interactions could be significant.

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