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# DEXTRAN-CONJUGATED ANTI-Ig ANTIBODIES AS A MODEL FOR T CELL-INDEPENDENT TYPE 2 ANTIGEN-MEDIATED STIMULATION OF Ig SECRETION IN VITRO

## I. Lymphokine Dependence[1]

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**We have previously demonstrated that dextran-conjugated anti-IgD antibodies (anti-δ-dex) stimulate high levels of B cell proliferation at concentrations that are 1000-fold lower than that required by unconjugated anti-Ig. We now show that anti-δ-dex may provide a suitable model to study Ig secretion stimulated by soluble T cell-independent type 2 Ag exemplified by TNP-Ficoll. Thus, both TNP-Ficoll and anti-δ-dex stimulate low to undetectable levels of Ig secretion when cultured with resting B cells. Addition of IL-5 or IL-2 stimulated enhanced anti-TNP responses in the presence of TNP-Ficoll, or induced polyclonal Ig secretion in the presence of anti-δ-dex. Both TNP-Ficoll and anti-δ-dex conjugates stimulated Ig production by Percoll-separated low density (partially activated) B cells in the absence of added lymphokines. These findings point to the similarities in the activation requirements of TNP-Ficoll and anti-δ-dex and suggest that dextran-anti-Ig conjugates, which can induce B cell activation irrespective of Ag specificity, may provide a useful model for studying various parameters that characterize the responses to soluble TI type 2 Ag.**

Cross-linking of sIg[3]M or sIgD by anti-Ig antibodies induces a series of events in vitro that culminate in B cell proliferation (1–7). Such stimulated B cells do not, however, secrete Ig unless T cells or T cell-derived lymphokines are present (2–6). Even responses to Ag that were previously classified as TI have been shown to require lymphokine-mediated help in vitro to induce resting B cells to secrete Ig (8). The lymphokine dependency of TI Ag can be bypassed when preactivated rather than resting B cells are used. Thus, the haptenated polysaccharide TNP-Ficoll stimulates anti-TNP responses when cultured with low-density, preactivated B cells but can only stimulate resting B cells to secrete antibody when cultured in the presence of a mixture of cytokines (9). To more closely examine the activation requirements for this group of antigens, some investigators have studied the responses of purified Ag-specific B cells (10–12). This approach, however, presents significant limitations in that it is a very time consuming process that yields only a small number of cells that can be studied. Furthermore, the purification process entails interaction of Ag and sIgR that in itself may influence subsequent responsiveness of the cell. To avoid these difficulties, other investigators have used anti-Ig antibody as a model for Ag-mediated sIg cross-linking leading to B cell activation (1–7). Although this approach has the advantage of stimulating large numbers of B cells, it does not provide an accurate model for studying responses to TI type 2 Ag. Thus, an antibody response to the prototype TI type 2 Ag TNP-Ficoll is stimulated at low Ag concentrations in the absence of lymphokines when preactivated B cells are used, although in all reported studies to date, anti-Ig, even at high Ag concentrations, stimulated antibody responses only in the presence of combinations of lymphokines (2–4). When insolubilized anti-Ig is used as the sIg cross-linking stimulus, higher levels of Ig secretion are stimulated even in the presence of limited combinations of lymphokines (5). These findings suggested that insolubilized anti-Ig antibody provides a model for studying TI responses whereas soluble anti-Ig antibody-stimulated responses may provide a model that more closely resembles TD responses because unlike insolubilized anti-Ig they require combinations of many lymphokines to induce even lower levels of Ig secretion. The limitation of using Sepharose bound anti-Ig is that it is difficult to quantitate the concentrations of anti-Ig antibody to which B cells are exposed, and therefore difficult to closely compare the concentration dependency of an Ag-specific response such as TNP-Ficoll to that of a polyclonally B cell activated response stimulated by anti-Ig Sepharose. Furthermore, because Sepharose-bound anti-Ig is

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[3] Abbreviations used in this paper: sIg, surface Ig anti-δ-dex, dextran coupled anti-IgD antibody; D10, D10G4.1 T cell clone; sup, supernatant; TI, T cell independent.

particulate it may not provide a suitable model for studying activation mediated by soluble Ag. We believed therefore that designing a chemically well defined agonist whose characteristics would mimic those of soluble type 2 TI Ag that could be used at equivalent concentrations and that would stimulate large numbers of B cells would provide a useful model that could be used to study B cell activation stimulated via a TI pathway. In view of our recent finding that dextran-conjugated anti-IgD antibody (anti-δ-dex) induces high levels of B cell proliferation at concentrations that are 1000-fold lower than that required by unconjugated anti-Ig (13), we wished to examine whether it would also stimulate Ig secretion in vitro. The results demonstrate that similar to haptenated polysaccharide type 2 Ag, (1) anti-δ-dex stimulates partially activated B cells to secrete Ig in the absence of added cytokines, and (2) stimulates resting B cells to secrete Ig in the presence of lymphokines. These findings suggest that dextran-conjugated anti-Ig antibodies may provide valuable tools for studying the events that influence Ig secretion by soluble type 2 Ag.

## MATERIALS AND METHODS

*Mice.* Female DBA/2 and CBA/J mice were obtained from The Jackson Laboratories, Bar Harbor, ME, and were used at 6 to 8 wk of age.

*Antibodies and other reagents.* The monoclonal anti-T cell reagents, anti-Thy-1.2 (clone 30-H12) (14), anti-CD4 (clone GK1.5) (15), and anti-CD8 (clone 53-6.7) (15) were grown as ascitic fluid in nude mice. The mouse mAb against rat IgG κ-chain, MAR 18.5 (16), was produced by cells grown in tissue culture. The anti-FcR antibody 2.4G2 (17) was grown as ascitic fluid in nude mice. The mAb Hδ$^a$/1 (18) with specificity for the heavy chain of IgD (δ) was grown in nude mice. Antibodies were purified as described previously (19). Conjugation of the Hδ$^a$/1 antibody to dextran was done as described (13), and the Hδ$^a$/1-dex conjugate that was used in these experiments had on average six molecules of anti-δ antibody per dextran molecule. Concentrations of dextran-conjugated antibodies that are noted in the text reflect only the anti-Ig antibody concentration and not that of the entire dextran conjugates. Thus, in all experiments equivalent protein concentrations of anti-δ and anti-δ-dex were used.

Purified XMG-6 (rat IgG1 anti-murine IFN-γ) (20), S4B6 (rat IgG2a anti-murine IL-2) (21), 11B11 (rat IgG1 anti-murine IL-4) (22), and TRFK-5 (rat IgG1 anti-murine IL-5) (23) were purified from ascites as described (19). J4-1 (a rat IgG1 anti-NP) (24) and a rat IgG2a anti-β galactosidase (cell line was the generous gift of Dr. John Abrams, DNAX, Palo Alto, CA), were used as control antibodies in experiments with the anti-lymphokine antibodies. rIL-5 was produced using the recombinant baculovirus AeNPV-IL-5 in an SF9 cell system and was a kind gift of Dr. G. Harriman (National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD). One U of IL-5 activity was contained in 125 pg of purified IL-5. Murine IL-4 purified by affinity chromatography using anti-IL-4 mAb was a kind gift of Dr. W. E. Paul (NIAID, NIH), and murine rIL-2 was purchased from Cetus Corp., Emeryville, CA. Murine rIL-6 was a kind gift of Dr. Richard Nordan (National Cancer Institute NIH).

*B cell purification.* Suspensions of single spleen cells were washed three times with RPMI 1640 (M. A. Bioproducts, Walkersville, MD) plus 10% FCS (GIBCO, Grant Island, NY) and treated with the following antibodies: monoclonal anti-Thy-1.2 (1 μg/ml), anti-CD4 (2.5 μg/ml), and anti-CD8 (1/600 dilution of ascitic fluid), respectively, per $10^7$ spleen cells for 30 min on ice. This was followed by treatment with newborn rabbit complement (10%) (Pel-Freez, Rogers, AR), in the presence of a 1/10 dilution of tissue culture fluid containing the anti-rat κ-chain mAb MAR 18.5 at 37°C for 45 min.

Percoll gradients (Pharmacia, Uppsala, Sweden) were used to separate large (low density) B cells from small (high density) B cells. Gradients consisting of 70, 65, 60, and 50% Percoll (with densities of 1.086, 1.0815, 1.074, and 1.062 g/ml, respectively) were prepared. Splenic B cells obtained as described above were layered onto cold Percoll gradients and spun at 3000 rpm for 15 min. The high density (small) cells were collected from the 70 to 65% interface, the low density (large) cells were collected from the 65 to 60 and 60 to 50% Percoll interface. The average percentage of Ig$^+$ cells in the various fractions was 90 to 95% in 60 to 65% and 65 to 70% fractions and 88 to 94% in the 50 to 60% fraction. The B cells yield was 56% in the 65 to 70% Percoll fraction, 29% in the 60 to 65% fraction and 15% in the 50 to 60% fraction.

*Measurement of [$^3$H]-Tdr incorporation.* B cells were cultured for 48 h in a final volume of 0.2 ml in modified Mishel-Dutton medium in flat bottom 96-well trays (Costar, Cambridge, MA). [$^3$H]-Tdr (1.0 μCi) (Amersham Corp., Arlington Heights, IL) with a sp. act. of 20 Ci/mmol was added to the cultures for the final 18 h and cultures were harvested with a PHD cell harvester (Cambridge Technology, Watertown, MA) onto glass fiber filters. Specific incorporation of thymidine was analyzed by liquid scintillation spectroscopy and results are expressed as the arithmetic mean of triplicate cultures.

*Measurement of antibody-producing cells.* Cultures prepared as described above were assayed for IgM + IgG-secreting cells by a protein A-SRBC plaque assay (25). Rabbit anti-mouse IgG + IgM (Jackson Immunoresearch, Inc., West Grove, PA) was used for developing the assay. Measurement of B cells secreting anti-TNP antibodies was enumerated with a hemolytic plaque assay using TNP-conjugated SRBC as described (26).

*Preparation of CD4$^+$ T cell supernatant.* Cells from the conalbumin specific CD4$^+$ T cell clone D10.G4.1 (27) were cultivated at $10^5$/ml for 24 h with conalbumin (100 μg/ml) and $5 \times 10^5$ T-depleted, 3000rad irradiated spleen cells from C3H mice. The cell-free supernatant was kept frozen at −20°C until use. D10.G4.1 is defined as a Th2 clone based on its secretion of IL-4, IL-5, and IL-6 and the absence of IL-2 and IFN-γ production (20). The batches of D10 supernatant used in this report had on average 95 U/ml of IL-5 and 365 U/ml of IL-4. Both lymphokine levels were determined by ELISA (23) with reagents kindly provided by Dr. G. Harriman (NIAID, NIH).

## RESULTS

*High levels of in vitro Ig production are induced by stimulation with anti-δ-dex conjugates.* We have previously shown that stimulation with dextran conjugated anti-IgD mAb (anti-δ-dex) induces B cell proliferation that is approximately 10 times more than the peak proliferative response induced by unconjugated anti-δ antibodies and does so at concentrations that are 100 to 1000-fold lower than that required by unconjugated anti-δ (13). This finding suggested that increasing the valency of the anti-Ig antibody from a bivalent to a multivalent conjugate enhanced its mitogenic potential and imparted to it the characteristics of TI type 2 Ag that are also multivalent. In order for these conjugates to be used as accurate models for TI type 2 Ag, it was essential to demonstrate that they stimulate B cells not only to proliferate but also to secrete Ig. To test for this we cultured resting B cells at $2.5 \times 10^4$/ml with anti-δ or anti-δ-dex over a wide range of concentrations and measured numbers of Ig-secreting cells 5 days later (Fig. 1). By themselves, neither of the agonists stimulated increases in numbers of Ig-secreting

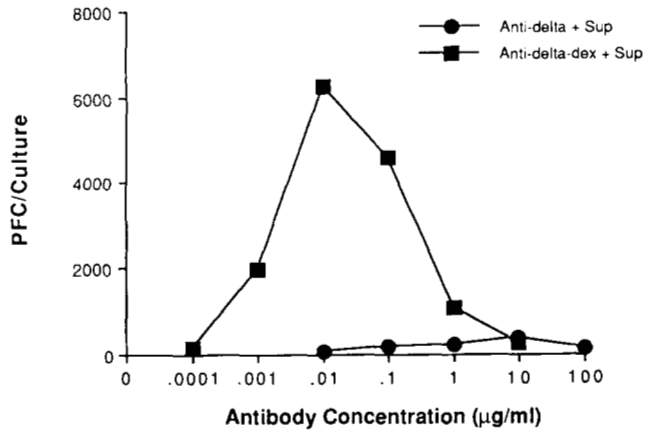


*Figure 1.* Dose-response profile of the induction of Ig-producing cells (PFC) by anti-δ-dex. The $2.5 \times 10^4$ resting B cells from DBA/2 mice were stimulated for 5 days in the presence of the indicated antibody concentrations and 10% D10 supernatant. Stimulation of the cells with antibodies alone induced 0 to 8 PFC per culture and stimulation with the supernatant gave 84 PFC per culture.

cells over that seen in medium only (data not shown). However, in the presence of an optimal concentration of supernatant obtained from the Th2 $CD4^+$ T cell clone, D10.G4.1 (D10 supernatant), anti-δ-dex stimulated more than 6000 Ig-secreting cells/culture as compared to 200 stimulated by unconjugated anti-δ. It should be noted that the concentration of anti-δ-dex that stimulated the highest level of Ig production (0.01 μg/ml) was the same as that required for induction of optimal levels of proliferation (13). However, even at concentrations of anti-δ-dex that stimulated lower levels of proliferation equivalent to that stimulated by unconjugated anti-Ig, Ig secretion was stimulated only by the former and not by the latter agonist.

To exclude the possibility that different kinetics or cell dose response may have accounted for the relatively poor to undetectable Ig production by cells stimulated with unconjugated anti-δ antibody, kinetics and cell dose response profiles were studied. No convincing increase in Ig production was stimulated by anti-δ antibody irrespective of the day on which the response was measured (Fig. 2). Similar findings were also observed when anti-μ mAb were used. The absence of Ig production was also not a consequence of a suppressive effect mediated by the binding of the uncoupled antibody to B cell FcγRII because addition of anti-FcγRII mAb (2.4G2) to the cultures did not lead to an increase in the number of Ig-secreting cells stimulated by any dose of anti-δ antibody tested (Table I).

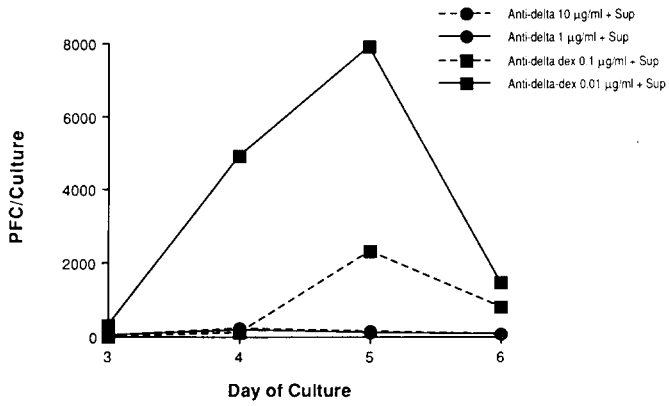


*Figure 2.* Kinetics of the response to anti-δ-dex and anti-δ. CBA/J mice resting B cells ($2.5 \times 10^4$) were stimulated for the indicated periods either with dextran-coupled Hδ$^a$/1 antibody or Hδ$^a$/1 at the indicated concentrations in the presence of 10% D10 supernatant. The data shown are the differences between the response induced by the antibodies in the presence of the supernatant and that induced by the supernatant alone. Supernatant only stimulated from 54 to 138 Ig-producing cells per culture depending on the day the response was measured.

TABLE I

*Effect of anti-γFcRII receptor antibody on induction of Ig production by unconjugated anti-δ and by dextran-conjugated anti-δ antibodies*[a]

| Stimuli | Reagent Added to Culture | | | |
|---|---|---|---|---|
| | Medium | D10 Sup | 2.4G2 | D10 Sup + 2.4G2 |
| | (Ig-producing cells/culture) | | | |
| Medium | 30 | 198 | 36 | 214 |
| Hδ$^a$/1-dex | 68 | 7340 | 18 | 6656 |
| Hδ$^a$/1 100 μg/ml | 38 | 322 | 10 | 234 |
| 10 μg/ml | 44 | 386 | 16 | 300 |
| 1 μg/ml | 12 | 350 | 28 | 346 |
| 0.1 μg/ml | 32 | 296 | 28 | 190 |

[a] Resting B cells obtained from CBA/J mice ($2.5 \times 10^4$) were stimulated for 5 days either in the presence of D10 supernatant or medium. To some cultures 10 μg/ml of the antibody 2.4G2 was added. The numbers shown represent the number of PFC induced in each culture. Hδ$^a$/1-dex was used at the concentration of 0.01 μg/ml.

Optimal Ig secretory responses to anti-δ-dex plus D10 supernatant were observed with cell numbers ranging from $10^4$ to $2.5 \times 10^4$/well ($5 \times 10^4$ to $1.25 \times 10^5$/ml), but the largest response, nearly three IgM-secreting cells per input cell, was seen at the lowest cell density tested: $0.6 \times 10^4$ cells/well (Fig. 3).

*Anti-δ-dex stimulates Ig secretion in partially activated B cells in absence of added lymphokines.* We have previously demonstrated that TI type 2 Ag are unable to stimulate antibody responses in resting B cells in the absence of added lymphokines. However, when cultured with size fractionated B cells these Ag stimulated in vitro antibody responses in the larger sized B cell fraction in the absence of added lymphokines (28). We wished to study whether similar differences could be observed in the activation requirements of resting vs partially activated B cells when stimulated with anti-δ-dex. B cell fractions that were separated on a discontinuous Percoll gradient were cultured for 5 days with anti-δ or anti-δ-dex in the absence of added cytokines (Fig. 4). Although anti-δ-dex was unable to stimulate an increase in Ig production by resting B cells (70% Percoll fraction

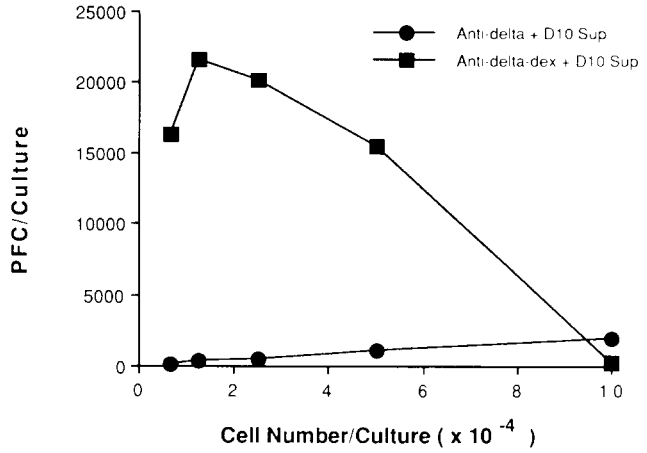


*Figure 3.* Measurement of Ig-producing cells induced by anti-δ-dex and anti-δ mediated stimulation of different B cell numbers. The indicated numbers of resting B cells from DBA/2 mice were cultured for 5 days with dextran-coupled Hδ$^a$/1 antibody (0.01 μg/ml) or Hδ$^a$/1 (10 μg/ml) either alone or in the presence of 10% D10 supernatant. The PFC obtained by stimulation with medium alone and D10 supernatant, respectively, was 4 and 48 at $0.65 \times 10^4$ cells/well, 1 and 124 at $1.25 \times 10^4$ cells/well, 12 and 260 at $2 \times 10^4$ cells/well, 24 and 848 at $5 \times 10^4$ cells/well, and 92 and 1808 at $10^5$ cells/well.

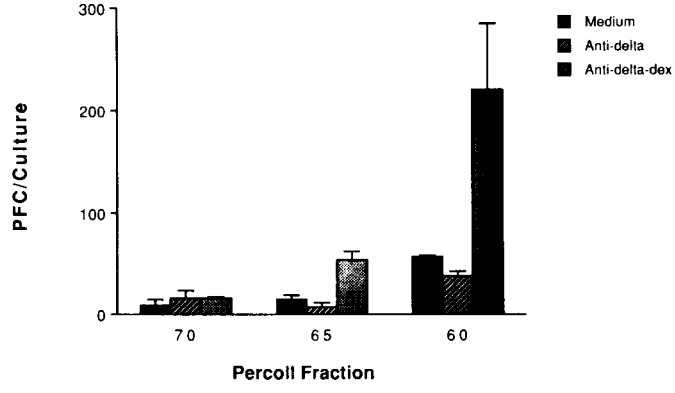


*Figure 4.* Induction of antibody producing cells by stimulation of in vivo pre-activated and resting B cells. $2.5 \times 10^4$ cells obtained from DBA/2 mice and separated in percoll gradient were stimulated for 5 days with medium, Hδ$^a$/1 (10 μg/ml) and dextran-coupled Hδ$^a$/1 antibody (0.01 μg/ml) in the absence of supernatant. Results are expressed as mean ± SD of triplicate cultures.

cells) in the absence of added lymphokines, it stimulated Ig secretion in the partially activated B cells from the 60% Percoll fraction (Fig. 4). On average approximately 1% of the input number of B cells was stimulated to secrete Ig under these conditions. Although we think it is unlikely, the possibility exists that the anti-δ-dex-stimulated responses of low density B cells reflect the presence of low density accessory cells. Unconjugated anti-Ig that stimulated little, if any, increase in Ig-secreting cells even in the presence of lymphokines, was able to stimulate low density partially activated B cells to secrete Ig in the presence of lymphokines (data not shown). Although partially activated B cells responded better than resting B cells to anti-δ-dex in the absence of lymphokines, similar levels of antibody-producing cells were observed in all three cell fractions in the presence of anti-δ-dex plus D10 supernatant (data not shown).

*Characterization of lymphokines present in D10 supernatant that are necessary for induction of Ig secretion by anti-δ-dex.* The supernatant of the D10.G4.1 T cell line has high concentrations of both IL-4 and IL-5 (20). To test whether either or both of these lymphokines were essential for supporting PFC responses in the presence of anti-δ-dex, we attempted to inhibit responses by the addition of anti-IL-4 and anti-IL-5 mAb. Each antibody markedly inhibited the antibody response induced by anti-δ-dex in the presence of the D10 supernatant, whereas an isotype-matched control antibody had no demonstrable effect (Fig. 5*A*). Antibodies specific for IL-2 and IFN-γ lymphokines were also added to cultures stimulated with anti-δ-dex and D10 cell supernatant and did not affect the numbers of Ig-secreting cells induced in these cultures. To further verify whether the inhibition mediated by anti-IL-4 and anti-IL-5 mAb was a specific consequence of neutralization of the lymphokines present in the supernatant, we attempted to reverse the inhibition by addition of IL-4 or IL-5 to cultures containing anti-IL-4 or anti-IL-5, respectively (Fig. 5*B*). Antibody-mediated inhibition of PFC responses was completely reversed by the addition of the appropriate lymphokine. To address the possibility that these antibodies were inhibiting an early step in B cell activation, we studied their effect on anti-Ig-mediated B cell proliferation. Both anti-IL-4 and anti-IL-5 had no effect on the proliferative response as reflected by thymidine incorporation or total cell count measured at 48 h of culture, but a high degree of inhibition of cell proliferation was mediated by both mAb at later times (Table II; Fig. 6).

*rIL-5 or IL-2 synergize with anti-δ-dex to stimulate Ig secretion.* Although anti-IL-4 and anti-IL-5 inhibited the ability of D10 sup to support the stimulation of Ig-secreting cells, it was possible that these lymphokines were necessary but not sufficient cofactors for Ig secretion. To test this, we added graded doses of recombinant-derived IL-5 and/or IL-4 to anti-δ-dex-stimulated-resting B cells and measured numbers of Ig-secreting cells. Although IL-5 cosynergized with anti-δ-dex to induce Ig secretion (Table III), IL-4 did not, nor did it appreciably enhance the ability of IL-5 to induce Ig secretion (Table III). rIL-2 also stimulated Ig production by anti-δ-dex-activated B cells (Fig. 7). This IL-2-mediated response was not dependent on endogenous production of IL-5 because Ig production was unaffected by the presence of anti-IL-5 antibody (Fig. 7). IL-6 by itself also did not co-synergize with anti-δ-dex to stimulate Ig secretion but moderately enhanced responses in the presence of IL-5 (Table III). Even though in some experiments the combination of IL-5 plus IL-6 and anti-δ-dexstimulated responses as large as those induced by IL-2 plus anti-δ-dex, neither of these combinations stimulated resting B cells to make antibody responses as large as those induced by anti-δ-dex plus D10 supernatant.

*Effect of recombinant lymphokine on in vitro response to the type 2 Ag, TNP-Ficoll.* Inasmuch as we were trying to use anti-δ-dex conjugates as a model for

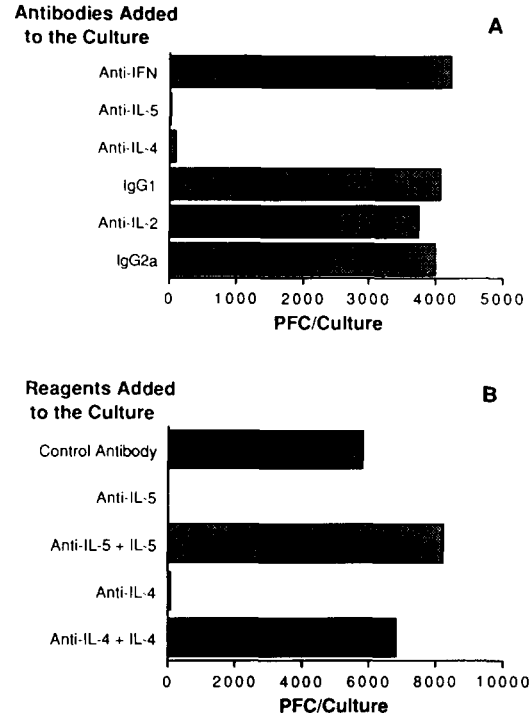


*Figure 5.* Effect of the neutralization of the lymphokines present in the D10 supernatant in the anti-δ-dex induction of PFC. *A*, Resting B cells from DBA/2 mice ($2.5 \times 10^4$) were stimulated with 0.01 μg/ml of dextran-coupled Hδ$^a$/1 antibody in the presence of 5% D10 supernatant for 5 days. The antibodies XMG-6 (anti-IFN-γ), TRFK-5 (anti-IL-5), 11B11 (anti-IL-4), J4.1 (control IgG1), S4B6 (anti-IL-2), and a control rat IgG2a were added to some cultures at 20 μg/ml. Cultures stimulated with dextran-coupled Hδ$^a$/1 antibody or D10 alone presented 8 and 16 PFC, respectively. *B*, Resting B cells from DBA/2 mice ($2.5 \times 10^4$) were stimulated for 5 days with dextran-coupled Hδ$^a$/1 antibody 0.01 μg/ml and 6% D10 sup. The antibodies indicated and described in A were added at 0.025 μg/ml to the cultures. Cultures stimulated in the presence of the control antibody J4.1 showed similar responses even at the concentration of 2.5 μg/ml. A total of 198 Ig-producing cells per culture were stimulated by D10 supernatant alone.

TABLE II

*Effect of anti-IL-4 and anti-IL-5 mAb in the proliferative response induced by anti-δ-dex*[a]

| Stimuli | Antibody Added to Culture | | |
|---|---|---|---|
| | Anti-IL-4 | Anti-IL-5 | Control Antibody |
| | ($^3$H-thymidine incorporation (cpm)) | | |
| Medium | 1,510 (±99) | 1,527 (±170) | 1,062 (±282) |
| Hδ$^a$/1 dex | 71,138 (±9,795) | 108,697 (±3,944) | 83,152 (±4,732) |
| Hδ$^a$/1 dex + sup | 131,042 (±3,501) | 123,800 (±16,339) | 137,905 (±6,402) |

[a] Resting B cells obtained from DBA/2 mice ($10^5$) were stimulated in the presence of the indicated reagents. The antibodies 11B11 (anti-IL-4), TRFK-5 (anti-IL-5), and J4-1 (control antibody) were added to some cultures at 20 μg/ml. Hδ$^a$/1 dex was used at 0.01 μg/ml and the D10 sup at 5%. The numbers indicate the incorporation of tritiated thymidine after 48 h of culture. Results are expressed as mean ± SD of triplicate culture.

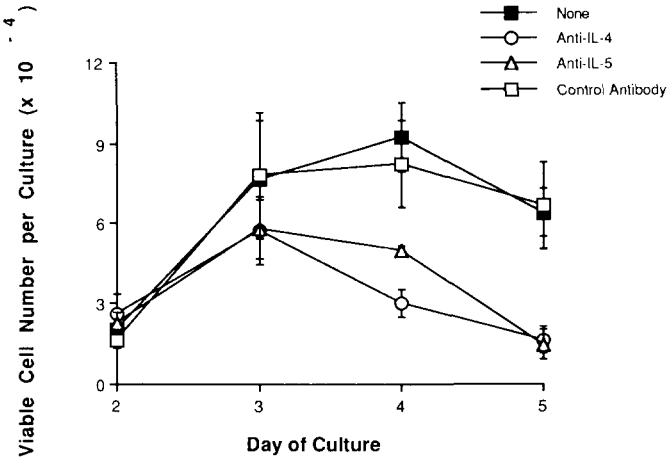


*Figure 6.* Effect of anti-IL-4 and anti-IL-5 mAb on the proliferative response induced by anti-δ-dex. The 2.5 × $10^4$ resting B cells from DBA/2 mice were stimulated with anti-δ-dex 0.01 μg/ml in the presence of 20% D10 supernatant and either with or without the indicated antibodies at 5.0 μg/ml. Cell number was counted on the indicated days with a hemocytometer. The antibodies showed no effect on proliferation induced by anti-δ-dex alone that was less than that induced in the presence of the antibody plus the supernatant. Results are expressed as mean ± SD of triplicate cultures.

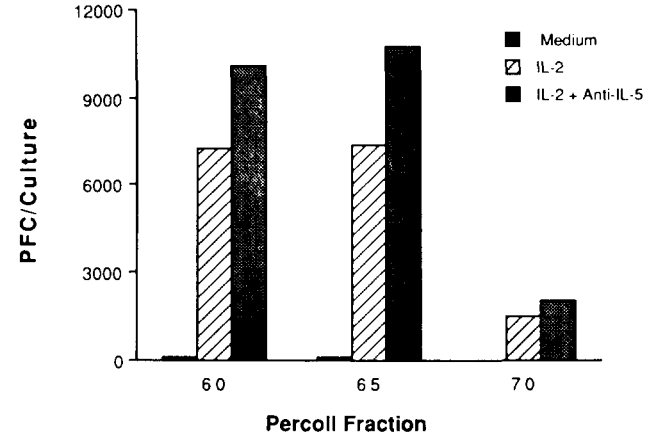


*Figure 7.* Induction of antibody-producing cells by stimulation of partially activated low density and resting high density B cells with anti-δ-dex and IL-2. B cells from DBA/2 mice (2.5 × $10^4$) obtained by separation of total splenic B cells in Percoll gradient were stimulated for 5 days with dextran-coupled Hδ[a]/1 antibody 0.01 μg/ml and IL-2 100 U/ml. Some cultures were stimulated in the presence of the anti-IL-5 antibody TRFK-5 at 2.5 μg/ml.

TABLE III

*Response to anti-δ-dex in presence of different lymphokine combinations*[a]

| Cytokines | | PFC/Culture | |
|---|---|---|---|
| IL-5 (U/ml) | IL-4 (U/ml) | Without IL-6 | With IL-6 (100 U/ml) |
| 50 | 50 | 198 | 348 |
| 5 | 50 | 22 | 16 |
| NA[b] | 50 | 4 | 34 |
| 50 | 5 | 262 | 456 |
| 5 | 5 | 24 | 26 |
| NA | 5 | 2 | 8 |
| 50 | NA | 196 | 394 |
| 5 | NA | 12 | 30 |
| NA | | 2 | 6 |
| 200 | NA | 1776 | ND |

[a] DBA/2 resting B cells (2 × $10^4$/culture) were stimulated for 5 days with Hδ[a]/1-dex 0.01 μg/ml in the presence of the indicated lymphokine concentrations. Unstimulated cultures and cultures stimulated with lymphokines alone gave between two and six PFC.

[b] NA, No addition.

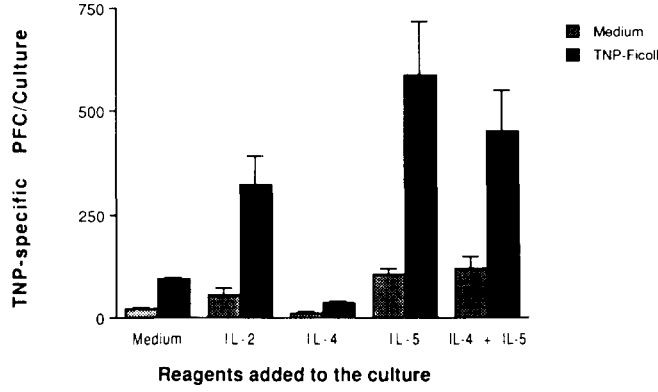


*Figure 8.* Response of resting B cells to TNP-Ficoll in the presence of recombinant lymphokines. Resting B cells ($10^6$) from DBA/2 mice were stimulated with TNP-Ficoll 0.01 μg/ml. rIL-2 100 U/ml, rIL-4 50 U/ml, or rIL-5 10 U/ml were added to some cultures. Anti-TNP antibody producing cells were measured after 3 days of culture. Results are expressed as mean ± SD of triplicate cultures.

studying responses to type 2 Ag, we needed to compare directly the lymphokine requirements for anti-δ-dex-stimulated Ig secretion with those necessary to obtain Ig production with the prototype type 2 Ag TNP-Ficoll. We have previously demonstrated that this Ag stimulates low to undetectable anti-TNP responses in resting B cells but induces substantial Ig production in the presence of T cell-derived cytokines (28). The results of the experiment shown in Figure 8 extend these observations and demonstrate that both rIL-2 and IL-5 independently enhance Ig production by TNP-Ficoll-stimulated resting B cells. It should be noted that when studying Ag-specific responses a 50-fold greater concentration of a polyclonal population of cells (5 × $10^6$/ml) is required compared to the lower concentrations of B cells that were used when studying anti-δ-dex stimulated polyclonal responses. One cannot therefore directly compare the absolute magnitude of responses stimulated by anti-δ-dex and that stimulated by TNP-Ficoll. Similar to that seen with anti-δ-dex, IL-4 at all doses tested was without effect on the TNP-Ficoll-stimulated response (data not shown). These data suggest that anti-δ-dex and TNP-Ficoll-stimulated Ig secretion have similar lymphokine requirements.

## DISCUSSION

We recently presented evidence demonstrating that anti-Ig antibody conjugated to dextran carrier may provide a suitable model for studying T cell-independent B cell activation (13, 29). Inasmuch as our previous studies examined only events that occurred within 48 h after stimulation, it was important to extend these findings to show that these conjugates could also stimulate Ig secretion under conditions in which Ig production is obtained by the prototype type 2 Ag TNP-Ficoll. The data demonstrate that as with TNP-Ficoll, anti-δ-dextran induced little, if any, Ig secretion in resting B cells. However, anti-δ-dex but not unconjugated anti-δ stimulated large numbers of Ig-secreting cells when cultured with preactivated, low density B cells in the absence of lymphokines (Fig. 4). Under these conditions approximately 1 of 100 input B cells was stimulated to secrete Ig. This is not dissimilar from the frequency of TNP-specific B cells that are stimulated by TNP-Ficoll when large activated B cells are

cultured in the presence of TNP-Ficoll only. However, high numbers of Ig-producing cells were induced when a Th2-derived, lymphokine-rich supernatant was added to anti-δ-dex-stimulated resting B cells. When low numbers of input cells were stimulated, the number of Ig-secreting cells in some experiments considerably exceeded input cell number. In contrast to the findings of others (2–4) we observed that unconjugated anti-Ig antibody over a wide range of concentrations had little if any ability to stimulate B cells to secrete Ig even in the presence of an antibody that blocks potential FcγR-mediated inhibition. This difference may reflect the fact that we used resting B cells, as well as anti-δ mAb whereas others used populations containing activated B cells and used heterologous anti-μ antibodies that are more efficient cross-linkers. This possibility is supported by our data that show that unconjugated anti-Ig induces low levels of Ig secretion in the presence of lymphokines when activated B cells were used (data not shown).

Our findings support the hypothesis that dextran conjugated anti-Ig, which as with other type 2 Ag stimulates high levels of in vitro Ig secretion at low Ag concentrations in the presence of lymphokines, may provide a more suitable model than unconjugated anti-Ig for studying TI responses. Although the reasons for the differences in stimulatory capacity of conjugated and unconjugated anti-Ig are not known, evidence from our laboratory suggests that it may be related to the extent and duration of sIg cross-linking that is stimulated by these agonists (29). The finding that the lower concentrations of anti-δ-dex (10 ng/ml) that do not completely modulate sIgD (29) were the most stimulatory for proliferation (13) and for Ig secretion suggests that these dextran conjugates may mediate repetitive and prolonged sIg-directed B cell activation. This mode of stimulation cannot be induced by higher concentrations of unconjugated anti-Ig antibodies that rapidly modulate membrane IgD. A recent report by Dintzis et al. (30), demonstrating that antibody responses against haptenated polymers are determined by hapten density and molecular mass of the carrier Ag, also emphasizes the requirement for a threshold amount of sIg cross-linking to induce TI-dependent Ig secretion. This repetitive mode of stimulation may not be required to activate B cells to become efficient APC. Thus, unconjugated anti-Ig antibody, such as T dependent Ag, can efficiently enhance the expression of MHC class II Ag (31). However, in contrast to multiepitope Ag, induction of Ig secretion by relatively inefficient sIg cross-linkers that are unable to mediate prolonged or repetitive sIg-mediated signaling, requires the added stimulation conferred by cognate T cell help. Although it is not clear how long this sIg-mediated signal is required, recent evidence from Isakson (6) suggests that 24 h of stimulation with a particulate multivalent anti-Ig conjugate might suffice to sensitize the B cell to subsequent encounters with lymphokines.

To study the identity of the lymphokines in the D10 supernatant that were responsible for inducing anti-δ-dex-mediated Ig secretion, as well as to determine whether Th1-derived lymphokines may also play a role in TI-stimulated responses, we examined the effects of recombinant IL-2, IL-4, IL-5, and IL-6 on in vitro Ig secretion. The results shown here are in accordance with many previous reports that demonstrated a role for IL-2, IL-5, and IL-6 either singly or in combination for induction of Ig secretion (32–39). Thus, we have shown that IL-2 and IL-5 by themselves fully supported anti-δ-dex-stimulated Ig secretion, whereas IL-6 mediated solely an enhancing effect. Thus, although we cannot definitely exclude a role for small numbers of IL-2-responsive non-B cells in these in vitro responses, we can conclude that Ig-stimulated responses can proceed in the absence of IL-5, because anti-IL-5 did not block IL-2-mediated effects.

A role for IL-4 in these responses was more difficult to interpret. Previous studies have shown that IL-4 has an enhancing effect on the early stages of B cell responses to TI type 2 Ag (40, 41), but anti-IL-4 cannot suppress the in vitro antibody response to TNP-Ficoll in the presence of IL-4 containing supernatant produced by EL-4 cells (28). Others have shown that IL-4 synergizes with IL-5 to induce IgA secretion by B cells as well as influence the in vitro Ig secretory response of B cells to TI Ag (42, 43). IL-4 did not by itself nor together with IL-5 over a wide range of concentrations enhance Ig secretion by anti-δ-dex-stimulated B cells. However, monoclonal anti-IL-4 (11B11) always inhibited anti-δ-dex + D10 supernatant induction of Ig secretion by more than 75%. The specificity of this inhibition was evident from the finding that high concentrations of IL-4 could overcome this inhibition. It is not clear to us why the IL-5 in the D10 supernatant did not act in the presence of anti-IL-4 antibody, because we knew from other experiments that the activity of D10 to support Ig secretion reflected the presence of adequate concentrations of IL-5 in the supernatant. Thus, the magnitude of the anti-δ-dex-stimulated response was comparable whether we used the D10 supernatant at 20 or 10% concentration, suggesting that the active lymphokines were present in more than limiting concentrations. Furthermore, IL-5 even at concentrations as low as 50 U/ml, in the absence of other added lymphokines, was in four of four experiments able to support Ig secretion in vitro. Inasmuch as anti-δ-dex plus D10 sup stimulated a much larger Ig response than anti-δ-dex plus IL-5, it is possible that D10 sup contains an as yet unidentified cytokine that acts synergistically with IL-4 to stimulate Ig secretion.

We have previously demonstrated that there are no apparent differences in the early biochemical events that are stimulated by high concentrations of anti-Ig or anti-Ig dextran. Both stimulate phosphatidyl inositol bisphosphate hydrolysis with generation of comparable but not identical concentrations of the various inositol phosphate isomers (44) and both stimulate tyrosine phosphorylation of identical substrates.[4] Nonetheless, dextran conjugated but not unconjugated anti-Ig stimulates Ig secretion. Moreover, at lower concentrations of anti-δ-dex that are still capable of stimulating Ig production, we observe barely detectable increases in inositol phosphate and no observable increase in tyrosine kinase activity (Brunswick, M., and J. J. Mond. Manuscript in preparation). This raises the possibility that additional signal transduction pathways may be recruited by dextran-conjugated anti-Ig that culminate in Ig secretion.

Taken together, our data demonstrate that responses stimulated by dextran-conjugated anti-Ig and by TNP-Ficoll show many similarities including: 1) similar lymphokine responsiveness; 2) the ability to stimulate partially activated B cells in the absence of lymphokines and resting B cells in the presence of lymphokines; and 3) similar antigen concentration requirement for induction

of the response by both. This suggests that anti-δ-dextran conjugates may provide a suitable model for studying the biochemical characteristics of responses to soluble TI type 2 Ag and for delineating the "physical-chemical" parameters that characterize this group of Ag.

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