T-independent and T-dependent B Lymphoblasts: Helper T Cells Prime for Interleukin 2–induced Growth and Secretion of Immunoglobulins that Utilize Downstream Heavy Chains

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Summary

Resting B cells enlarge, enter the cell cycle, and change their surface phenotype when activated via the surface immunoglobulin (Ig) receptor, but subsequent cell growth and antibody production is relatively limited. To identify stimuli that might prime B cells for enhanced function in vitro, we have compared the effects of anti-Ig with helper T (Th) cells on the formation of B lymphoblasts and the subsequent ability of the blasts to grow and secrete Ig. The B blasts first were induced by either anti-Ig, anti-Ig plus T cell–derived lymphokines, or alloreactive T blasts. Each population of B blasts showed enhanced expression of cell surface adhesion molecules, interleukin 2 receptor (IL-2R) p55, and MHC products, as well as decreased expression of IgD. The allo-activated B blasts were distinctive in expressing low levels of Thy-1 and increased reactivity with peanut agglutinin, a marker of germinal center B blasts in situ. The function of the different populations of B blasts was also different. Whereas anti-Ig or anti-Ig plus lymphokines primed for enhanced responses to lipopolysaccharide (LPS), the B blasts induced by Th cells were insensitive to LPS. B lymphoblasts that had been activated in the presence of helper factors or Th cells responded vigorously to recombinant IL-2 with growth and Ig secretion, and this response was enhanced in the presence of anti-Ig. The B blasts activated directly by Th cells, but not by anti-Ig plus lymphokines, were primed to secrete high levels of IgG1 and IgA. Therefore, the phenotype and function of a B lymphoblast depends upon the manner in which it is primed. When primed by Th cells, IL-2 proves to be the predominant mediator of clonal expansion and antibody secretion.

In vitro models of murine B cell activation have mainly relied on T cell–independent stimulation by anti-Ig (1–5) or LPS (6, 7). Anti-Ig triggers the B cell via its antigen receptor, but, even when administered as a multivalent ligand, does not induce extensive B cell growth nor antibody secretion unless another mitogen, for example LPS, is included. LPS stimulation has the disadvantage that it does not allow the discrete steps of B cell activation to be studied independently. Furthermore, antibody responses to most antigens are dependent on a MHC class II (Ia)–restricted interaction between T cells and B cells (8–11). Two experimental systems for the activation of resting B cells that require a cognate interaction between T cells and B cells have been developed. In one system, antigen-specific B cells were activated by histocompatible, antigen-specific T cells or T cell clones plus antigen (12, 13). However, large numbers of antigen-specific B cells can not easily be generated in this system to investigate their activation and differentiation at a molecular level. Alternatively, systems have been developed whereby class II–reactive T cells activate allogeneic B cells in a polyclonal fashion (14–17). Some B cell growth and Ig secretion was observed in these systems.

To compare the effect of anti-Ig with Th cells on B cell activation, we have separated the B cell response into two steps (3). In the initial step, B lymphoblasts were generated by one of three regimens: anti-Ig, anti-Ig plus T cell–derived lymphokines, or alloreactive Th cells. In the second step, each of these types of primed B blasts were studied for their response to anti-Ig, LPS, and/or lymphokines. We demonstrate that activation of B cells during direct B cell–T cell interaction leads to the generation of a distinct population of B lymphoblasts. These blasts are remarkable in that they proliferate and secrete IgM and high levels of IgG1 and IgA in response to IL-2 and are insensitive to stimulation by LPS.

Materials and Methods

Animals. 8–20-wk-old (BALB/c × DBA/2)F_1 (CD_2F_1), B6.H-2^k, and C57BL/6 mice were purchased from the Trudeau Institute (Saranac Lake, NY).

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Lymphokines and Mitogens. Purified murine rIL-1 was provided by P. Lomedico (Hoffman-La Roche, Inc., Nutley, NJ). Purified human rIL-2 was generously provided by Cetus Corp. (Emeryville, CA). Purified murine rIL-4 was generously provided by either Dr. P. C. Isakson (University of Virginia, Charlottesville, VA) or Dr. E. S. Vitetta (University of Texas Southeastern Medical Center, Dallas, TX). Murine rIL-5 was purchased from Genzyme (Boston, MA). Purified murine rIL-6 was kindly provided by Dr. J. Van Snick (Ludwig Institute for Cancer Research, Brussels, Belgium). Purified murine rIL-7 was purchased from Biosource International (Westlake Village, CA); purified human rIL-7 was also generously provided by Sterling Drug Inc. (Malvern, PA). Murine rIFN-γ was purchased from Amgen Biologicals (Thousand Oaks, CA). Granulocyte/macrophage (GM)[1]-CSF was kindly provided by S. Gillis (Immunex, Seattle, WA). Anti-lymphokine and anti-receptor antibodies used to demonstrate lymphokine specificity included S4B6.1 (IL-2) (18), 3C7 (IL-2Rp55) (19), 7D4 (IL-2Rp55) (19), 11B11 (IL-4) (20), and TRFK-5 (IL-5) (21). LPS from *Salmonella typhimurium* was purchased from Difco Laboratories (Detroit, MI).

Dendritic Cells (DC). Single cell suspensions were obtained by collagenase digestion of splenic tissue. Low density splenic cells, obtained by flotation on dense BSA gradients (density = 1.08), were adhered to tissue culture dishes (Falcon Labware, Oxnard, CA). After 1–1.5 h, the nonadherent cells were removed by vigorous pipetting. The adherent cells were cultured overnight, whereupon the dendritic cells became nonadherent. The overnight released cells were collected and readhered to plastic tissue culture dishes for 1 h to remove contaminating macrophages.

T Cells. T cells from CD_2F_1 mice were prepared from erythrocyte-depleted suspensions of mesenteric lymph nodes and spleen by passage through nylon wool columns. CD4-enriched T cells were prepared by treating the nylon wool–nonadherent leukocytes with TIB 150 (αLyt-2.2) (22) and TIB 229 (αI-$A^{b,d}$) (23) followed by complement-mediated lysis using baby rabbit serum as a source of complement (Pel Freez Biologicals, Rogers, AR).

B Cells. B cells were prepared from erythrocyte-depleted, single cell suspensions of splenic tissue by treatment with a cocktail of antibodies including TIB 99 (24), C3PO (25), and TIB 150 (22) (αThy-1.2, αLyt-1.2, and αLyt-2.2, respectively) followed by complement lysis. Adherent cells were removed by passage over a Sephadex G-10 column (26). High density B cells were obtained by density fractionation on a discontinuous Percoll gradient (3). High density B cells were those harvested from bands that formed above the 1.09- and 1.085-g/ml layers.

Allo-T Blasts. Allo-T blasts were made as described previously (27). Briefly, CD_2F_1 (H-2^d) $CD4^+$ T cells at 5×10^6/ml were cocultured with DC from B6.H2^k (H-2^k) at a concentration of $5–10 \times 10^4$/ml in a total volume of 10 ml in Hepes-buffered RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 10% heat-inactivated FCS (Hazelton Research Products, Inc., Lenexa, KA), 2 mM glutamine, 100 μg/ml streptomycin, 10 μg/ml gentamicin, 100 μg/ml penicillin, and 50 μM 2-ME in a humidified 7% CO_2 environment at 37°C. DC were treated with 1,500 rad from a ^{137}Cs source before addition to the cultures. After 48 h, DC-T cell clusters were harvested on a continuous Percoll gradient. The clusters were recultured at $4–5 \times 10^5$/ml. After an additional 48 h, the allo-T blasts released from the clusters were harvested from the top of a continuous Percoll gradient. Residual DC were removed by antibody-mediated cytolysis.

Allo-stimulated B Lymphoblasts (Allo-B Blasts). B cells at 10^6/ml (B6.H-2^k unless otherwise indicated) were cocultured with irradiated (1,500 rad), I-A^k-reactive allo-T blasts at a concentration of 2×10^5/ml in a total volume of 10 ml. At the times indicated, the cells were harvested, the T cells depleted as described above, and the dead cells and debris were removed by flotation on a dense BSA gradient.

Anti-Ig B Blasts (αIg Blasts). B cells at 2×10^6/ml were cultured with 5 μg/ml anti-Ig-coupled Sepharose in RPMI 1640 supplemented as described above. At the time indicated, the anti-Ig blasts were isolated free of the Sepharose beads by centrifugation through Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway NJ) as described previously (3).

Anti-Ig/EL-4 Blasts (αIg/EL-4 Blasts). B cells at 2×10^6/ml were cultured with 5 μg/ml anti-Ig-coupled Sepharose in RPMI 1640 supplemented as described above. During the final 24 h, phorbol dibutyrate (PdBu)-induced EL-4-conditioned media (partially purified, concentrated, and depleted of PdBu by precipitation with 50% then 85% saturating ammonium sulfate followed by extensive dialysis [EL-4 Sn]) was added at a 1% final concentration (vol/vol). This supernatant contains at least IL-2, IL-4, and IL-5. At the time indicated, the αIg/EL-4 blasts were isolated free of the Sepharose beads by centrifugation through Ficoll-Paque.

Fluorescent Flow Cytometric Analysis. The antibodies used for staining are described in Table 2. mAbs were used in the form of hybridoma culture supernatants or 50% ammonium sulfate precipitates of serum-free hybridoma supernatants supplemented with 1% Neutridoma SP (Boehringer Mannheim Biochemicals, Indianapolis, IN). Briefly, $2–10 \times 10^5$ cells/sample were incubated with either directly fluoresceinated reagent or hybridoma supernatant diluted in Dulbecco's modified PBS containing 1 mg/ml BSA and 0.02% NaN_3 (PD/BSA/N_3) at 4°C for 40 min. Samples were washed three times in PD/BSA/N_3. Samples initially incubated with hybridoma supernatant were resuspended in the appropriate fluoresceinated secondary reagent as indicated. FITC mouse anti-rat Ig (MAR-Ig), FITC goat anti-mouse Ig (GAM-Ig), FITC GAM-μ, and FITC rabbit anti-hamster Ig (RAH-Ig) were purchased from Jackson Immunoresearch (West Grove, PA). FITC-GAM-IgG2a and FITC-GAM-IgG2b were purchased from Southern Biotechnology Institute, Inc. (Birmingham, AL). GAM-δ was the generous gift of Drs. Fred Finkleman (Uniformed Health Services, Bethesda, MD) or E. S. Vitetta. Cells were fixed in 3.7% formaldehyde in PD and analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA).

Cell Cycle Analysis. Cell cycle analysis was performed as described (28). Briefly, cells were resuspended in PD/BSA/N_3 at a concentration of 5×10^6/ml. Triton X-100 solution (Sigma Chemical Corp., St. Louis, MO) was added to 1%, followed by the addition of propidium iodide to 0.1 mg/ml (Molecular Probes, Eugene, OR). The cells were analyzed on a FACScan using the program entitled "Sum of Broadened Rectangles" (29).

Assays of B Lymphoblast Proliferative Responses. B lymphoblasts or B cells were cultured with additions as indicated for 24–48 h as indicated in a 200-μl final volume. Cells were pulsed with [^{3}H]thymidine (New England Nuclear, Boston, MA) with a specific activity of 20 Ci/mmol for 12 h, harvested onto glass fiber filters, and counted on a scintillation counter (1205 Betaplate; Pharmacia-LKB). Data are represented as the mean counts per minute of triplicate cultures. Viable cell recovery was determined by counting in the presence of 0.04% Trypan blue.

Ig Isotype-specific ELISA. Immulon 1 plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with 0.5 μg/well of isotype-specific antibodies (Southern Biotechnology Associates, Birming-

[1] *Abbreviations used in this paper:* DC, dendritic cell; GAM, goat anti-mouse; GM, granulocyte/macrophage; MAR, mouse anti-rat; PdBu, phorbol dibutyrate; PNA, peanut agglutinin; RAH, rabbit anti-hamster.

ham, AL) in 0.1 M borate buffered saline (BBS), pH 8.3, overnight at 4°C. The plates were washed and blocked with BBS-1% BSA (BBS-BSA). Culture supernatant diluted in BBS-BSA was added and incubated for 4 h at room temperature or overnight at 4°C. Plates were washed and 20 ng of the appropriate alkaline phosphatase–labeled isotype-specific antibody (Southern Biotechnology Associates) in BBS-BSA was added to each well for 4 h at room temperature. Plates were washed and 100 μg of *p*-nitrophenyl phosphate (Xymed Laboratories, San Francisco, CA) in 0.1 M 2-amino-2-methyl-1,3-propanediol, pH 10.3, was added per well. The plates were read at 410 nM on a microplate reader (MR700; Dynatech Laboratories, Inc.). Standard curves were generated using the following myeloma proteins: MOPC 104E (μ), MOPC 21 (γ_1), RPC-5 (γ_{2a}), MPC-141 (γ_{2b}), J606 (γ_3), and TEPC15 (α) (Litton Bionetics, Kensington, MD). Data are represented as the mean Ig concentration of quadruplicate cultures.

Results

MHC Class II–restricted Activation of B Cells by Allo-T Blasts. Class II–reactive, CD4$^+$ T blasts were generated in the MLR (27) and used to activate allogeneic B cells. Typically, T cells from H-2^d mice were stimulated with H-2^k dendritic cells, and then the T blasts were cultured with allogeneic Ia^k, syngeneic Ia^d, or third-party Ia^b B cells. The allo-T blasts formed large, stable clusters with the allogeneic B cells but not syngeneic or third-party B cells (data not shown). Within 1 d of co-culture, the T blasts induced DNA synthesis in the allogeneic B cells but not in B cells from syngeneic mice, and <10% of the response in B cells from third-party mice (Fig. 1 *A*). The function of the alloreactive T blasts was Ia^k restricted since the stimulation was blocked by anti-Ia^k antibodies (Fig. 1 *A*).

By light scatter analysis, virtually all the B cells began to enlarge on the first day, and most were large blasts on the second day of co-culture with T blasts (Fig. 1 *B*). The recovery of viable B lymphoblasts was ~80% of the number of input cells. By cell cycle analysis, a large proportion of the B cells were in the S, G_2, and M phases of the cell cycle (Table 1). This activation of B cells as measured by forward light scatter, entry into cell cycle, and DNA synthesis was similar to that of B cells stimulated by anti-Ig (3) and αIg plus EL-4 Sn (our unpublished observation).

Table 1. *Cell Cycle Analysis of Allo-B Blasts*

	Day 0	Day 1	Day 2	Day 3
		%		
G_1	96	75	53	64
S	4	24	33	31
G_2 + M	0	1	14	5

Allo-B blasts were harvested after 24, 48, and 72 h of T cell–B cell co-culture. The numbers represent the percent of cells in G_0/G_1, S, and G_2/M at the indicated times post-stimulation.

Phenotype of B Blasts. Fluorescence-activated flow cytometry was used to determine the cell surface phenotype of the three types of B lymphoblasts: i.e., αIg, αIg/EL-4, or allo-B blasts (Fig. 2 and Table 2). Several cell surface molecules were upregulated to varying degrees on B lymphoblasts, relative to B cells, regardless of the means of activation. These included cell surface adhesion molecules (ICAM-1, Pgp-1, and LFA-1), MHC class I and II products, as well as the IL-2Rp55 subunit. These markers of cell activation were expressed on the vast majority of each of the B lymphoblast populations demonstrating the homogeneity of the activated cells. The increased expression of Ia, as well as the IL-2Rp55 subunit, was consistently greater in B lymphoblasts that received T cell help. These molecules were upregulated within 24 h after activation (Fig. 3).

A decrease in surface IgD was observed on all B lymphoblasts, but this change was also greater in those lymphoblasts generated in the presence of T cell–derived lymphokines or T cells. In contrast, surface IgM did not change significantly in any of the blast populations. The decrease in IgD was detectable on a subpopulation of blasts by day 2 but was dramatically reduced on almost all of the allo-stimulated B blasts by day 3 (Fig. 3).

In addition, there were phenotypic changes that required direct T cell–B cell interaction (Fig. 2). Allo-B blasts increased MHC class I expression to a greater extent than αIg or αIg/

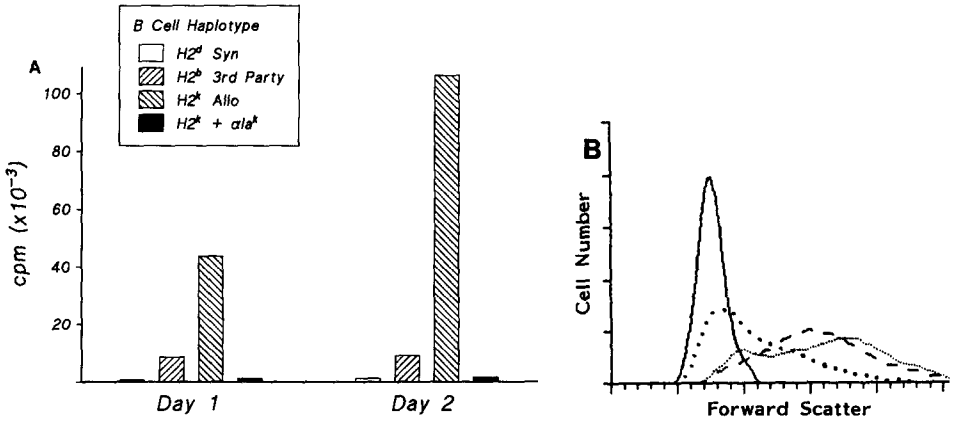

Figure 1. Generation of B lymphoblasts by allo-T blast stimulation. (*A*) Freshly isolated high density splenic B cells (MHC haplotype as indicated) at 10^6/ml were cultured with irradiated allo-T blasts at 2 × 10^5/ml. At the time indicated, 200 μl of the cells was pulsed for 6 h with 1 μCi [^{3}H]TdR, harvested, and counted. (*B*) Time course of blast transformation of B cells as assessed on a FACScan (increase in forward light scatter). Day 0 (—); day 1 (• • • •); day 2 (·····); day 3 (----).

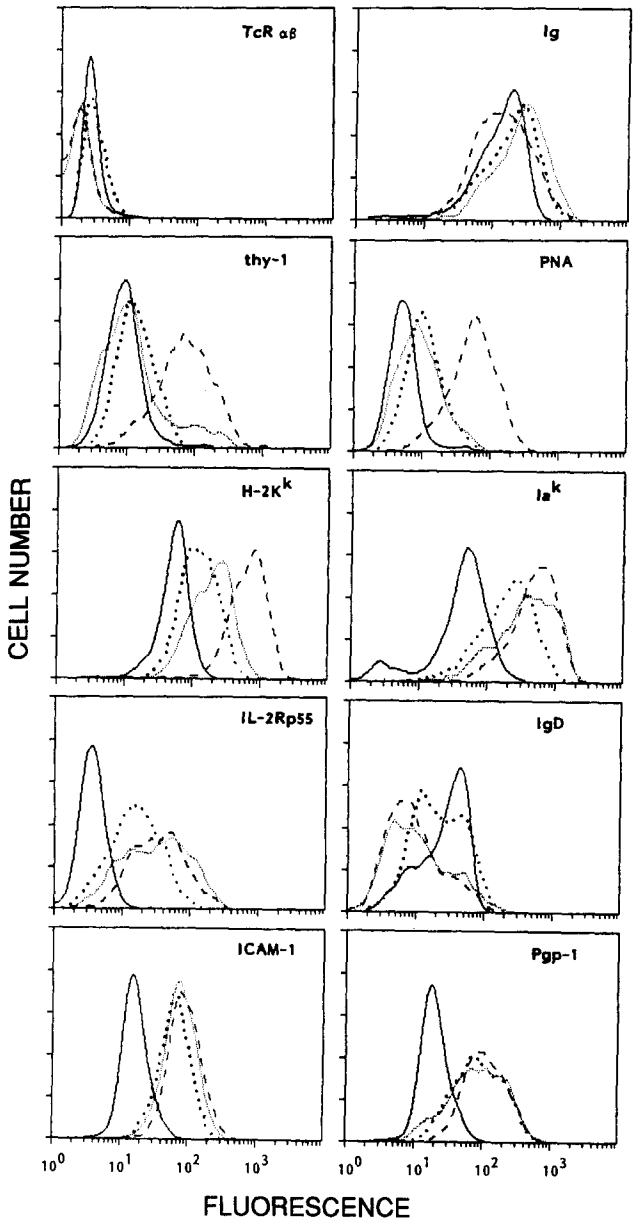

Figure 2. Phenotypic characterization of B lymphoblasts and B cells. B cells or 3-d B lymphoblasts were analyzed with antibodies to cell surface antigens or lectins. B cells (—); αIg blasts (• • • •); αIg/EL-4 blasts (·····); allo-B blasts (----).

EL-4 blasts. The majority of allo-B blasts also expressed low levels of Thy-1. αIg blasts were Thy-1$^-$ and only a small fraction (10%) of 2–3-d αIg/EL-4 blasts expressed Thy-1 at low levels (data not shown). This phenotypic change is likely mediated in part by IL-4, which has been shown to induce the expression of Thy-1 on some murine B cells (30). However, IL-4 apparently must work in concert with either LPS (30) or T cells (as shown here) to induce Thy-1 on B cells.

Finally, stimulation by allo-T blasts enhanced reactivity with peanut agglutinin (PNA). This high reactivity with PNA implies that T cell–B cell interaction may result in the loss of terminal sialic acid residues from some surface glycoproteins. Increased reactivity with PNA and Thy-1 was detectable by day 1 and was maximal by day 2 (Fig. 3). This analysis of phenotype indicates that the majority of B cells were uniformly activated, and that when B cells are activated directly by Th cells, a distinct phenotype is induced that has some similarities to germinal center B blasts in situ, i.e., IgD^{low} (31) and PNA^{high} (32).

Proliferative Responses of B Cells and B Blasts. None of the purified blast populations continued to incorporate [^{3}H]TdR nor divided if re-cultured in the absence of additional stimuli. In the case of B cells and B lymphoblasts activated by anti-Ig, viability dropped to 30–50% by 24 h (3), whereas virtually all the αIg/EL-4 blasts and allo-B blasts remained viable. This enabled us to study the requirements for maintaining cell viability and growth using anti-Ig, LPS, and lymphokines as stimuli.

B cell activation, regardless of the method used, primed the blasts for subsequent restimulation with anti-Ig-Sepharose or anti-IgD-Sepharose. Priming was consistently greater in those B blasts that received T cell help (Table 3, Exp. A, and data not shown).

All three types of B blasts examined were primed to respond to the lymphokine-rich supernatant from PdBu-stimulated EL-4 thymoma cells containing at least IL-2, -4, and -5 (Table 3, Exp. A). Responsiveness to EL-4 Sn was greatest in those B lymphoblasts primed in the presence of T cell–derived lymphokines or alloreactive T cells. Individual lymphokines were also tested for their capacity to induce B lymphoblast proliferation, including rIL-1, -2, -4, -5, -6, and 7, IFN-γ, and GM-CSF. Consistent with previously published reports (3), αIg blasts were relatively insensitive to each of the individual lymphokines tested (Table 3, Exp. B, and data not shown) and any combinations of lymphokines tested (data not shown). In contrast, B lymphoblasts generated in the presence of T cell help were primed for responsiveness to IL-2 but remained unresponsive to the other lymphokines tested (Table 3, Exp. B, and data not shown). The response to IL-2 was not augmented by addition of other lymphokines, notably rIL-4, rIL-5, or rIL-6 (data not shown). Neutralizing anti-IL-2 plus anti-IL-2R antibody inhibited the response to rIL-2 and, more importantly, the antibodies inhibited the response of allo-B and αIg/EL-4 blasts to EL-4 Sn by >90%. This result suggests that IL-2 was the predominant growth factor in EL-4 Sn responsible for the response of B blasts that had been generated in the presence of T cell help.

B lymphoblasts exhibited a synergistic response to a combination of anti-Ig or anti-IgD plus EL-4 Sn or IL-2, regardless of the means of activation. Other lymphokines including IL-4 and IL-5 had little or no effect on B lymphoblast growth, even in combination with anti-Ig (Table 3 and data not shown).

The pattern of B lymphoblast responses when viable cell recoveries were determined was similar. In the case of B lymphoblasts generated in the presence of T cell help (soluble or direct cell contact), the cell recoveries at 24 h were ~200–250% with anti-Ig or anti-IgD, ~200–300% with rIL-2 or EL-4 Sn, and ~300–350% with αIg plus rIL-2 or EL-4 Sn. The cell recoveries of αIg blasts stimulated with αIg or αIgD (45–130%), rIL-2 (75–100%), or EL-4 (150%)

Table 2. *Changes in Surface Phenotype after In Vitro Activation of B Cells*

Antigen/CD no.	mAb; ATCC* no.	Allo-B	αIg/EL-4	αIg
Class I MHC	11-4.1; TIB 95 (60)	3+	2+/3+	2+
Ia	M5/114.15.2; TIB 120 (61)	2+/3+	2+	1+/2+
IL-2Rp55/CD25	3C7; TIB 222 (19)	2+	2+	1+
ICAM-1/CD54	YN-1 (62)	2+	2+	2+
LFA-1/CD11a	FD441.8; TIB 213 (63)	2+	2+	2+
Pgp-1/CD44	18C8 (64)	2+	2+	2+
Mel-14	Mel-14.d54; HB132 (65)	NC	NC	NC
Ig	GAM-Ig	NC	NC	NC
IgM	GAM-μ	NC	NC	NC
IgD	JA12.5 (66)	1−	1−	1−/NC
Thy-1.2	B5.5 (67)	1+/2+	NC	NC
PNA	PNA	1+/2+	NC/1+	NC/1+
LCA/CD45	M1/9.3.4; TIB 122 (68)	NC	NC	NC
B220/CD45R$_A$	RA3-3A1/6.1; TIB 146 (69)	NC	NC	NC
CD45R$_B$	MB23G2; HB 220 (70)	NC	NC	NC
Heat-stable Ag	J11d; TIB 183 (71)	NC	1+	1+
Fc$_\gamma$R$_{II}$/CD 32	2.4G2; HB 197 (72)	NC	NC	NC
Fc$_\epsilon$R$_{II}$/CD 23	B3B4 (73)	NC‡	NC‡	NC

B cells or 3-d lymphoblasts were analyzed with antibodies to cell surface antigens or lectins. The change in surface phenotype refers to the change in mean fluorescence intensity relative to high density B cells: 1+ = 0–0.5 log increase; 2+ = 0.5–1 log increase; 3+ = >1 log increase; 1− = decrease; NC = no change.

B cells and lymphoblasts were: T cell marker negative (CD3 [74], TCR-α/β [75], and Lyt-1 [76]; macrophage marker negative (Mac 1 [77] and F4/80 [78]); and DC marker negative (33D1 [79]).

* American Type Culture Collection.

‡ Although the mean fluorescence intensity exhibited little or no consistent change, the staining on the allo-B blasts and the αIg/EL-4 blasts became very heterogeneous.

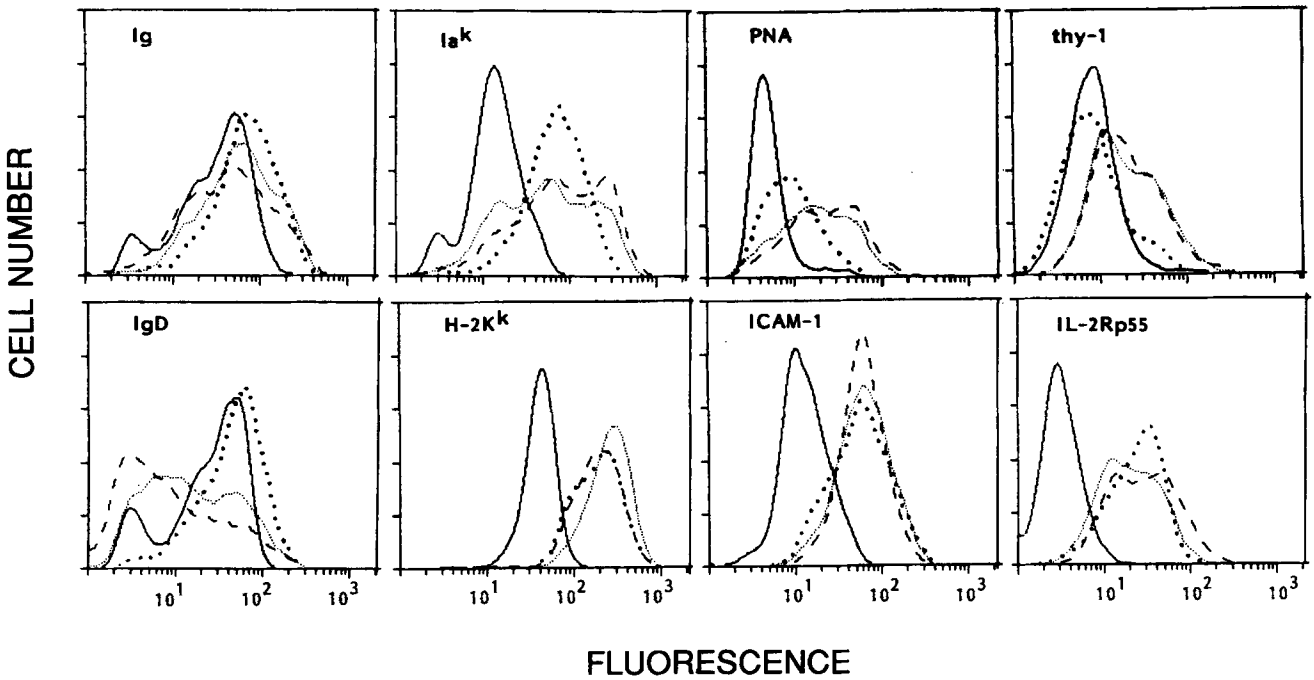

Figure 3. Time course of phenotypic changes of allo-B blasts. Allo-B blasts were harvested after 24, 48, and 72 h of T cell–B cell coculture. Allo-B blasts or high density B cells were stained with antibodies to cell surface antigens or lectins. B cell (day 0) (——); day 1 (• • • •); day 2 (·····); day 3 (----).

Table 3. *Effect of Cytokines on B Lymphoblast Proliferation*

Exp.	Additions	[^{3}H]TdR incorporation			
		B cell	αIg blast	αIg/EL-4 blast	Allo-B blast
			cpm × 10^{-3}		
A	None	0.2	1.6	2.4	1.8
	αIg	0.9	18.8	45.0	32.2
	IL-2	0.2	6.4	134.8	125.0
	EL-4 Sn	1.0	16.0	228.1	152.1
	αIg/EL-4 Sn	12.9	353.2	698.0	293.1
	LPS	2.5	322.9	309.6	7.5
B	None	0.2	2.0	3.2	2.0
	αIg	0.4	17.2	17.4	103.1
	IL-2	0.1	11.4	71.1	70.7
	αIg/IL-2	0.4	123.5	253.5	233.8
	IL-4	0.1	2.4	3.6	2.7
	αIg/IL-4	0.3	24.1	27.8	83.4
	IL-5	0.1	7.1	12.1	8.4
	IL-6	0.1	2.1	2.9	2.2
	IL-7	0.1	1.1	3.0	1.8

B cells or 3-d (Exp. A) or 2-d (Exp. B) B lymphoblasts were recultured at 2.5 × 10^5 ml in 200 μl final volume with additions as indicated. After 24 h, the cultures were pulsed with 0.5 μCi [^{3}H]TdR for 12 h and harvested. Additions were: IL-2, 10 U/ml; IL-4, 100 U/ml; IL-5, 100 U/ml; IL-6, 4 ng/ml; IL-7, 100 U/ml; αIg-Sepharose, 5 μg/ml; EL-4 Sn, 0.5% (vol/vol); and LPS, 20 μg/ml.

suggested that these lymphoblasts did not proliferate to the same extent as the T-dependent B blasts. However, the potential of αIg blasts to divide was indicated by their response to αIg plus lymphokines (200–280%). There was no evidence of cell division by the resting B cells with any stimuli since the cell recovery was always <60%.

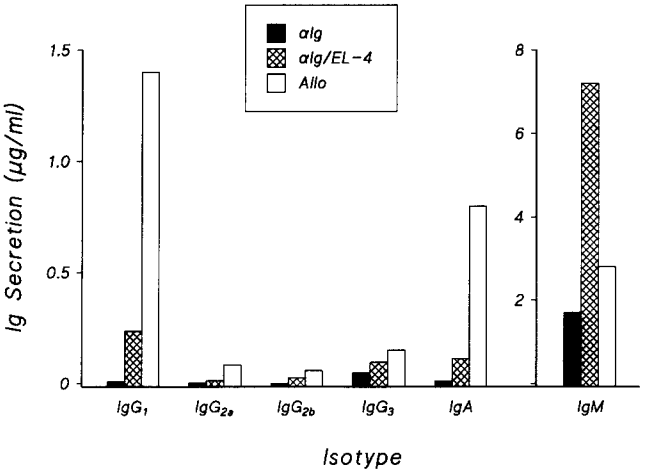

Figure 4. Effect of re-stimulation of B lymphoblasts with T cell–derived lymphokines on the secretion of Ig isotypes. 3-d B lymphoblasts were re-cultured at 2.5 × 10^5/ml in 200 μl final volume with 0.5% (vol/vol) EL-4 Sn. After 60 h, the supernatants were collected and tested in an isotype-specific Ig ELISA. In addition, IgE was tested and was uniformly below the limit of detection (4 ng/ml) of the ELISA.

Ig Secretion by B Lymphoblasts. An analysis of the quantity and isotype of the Ig secreted provides an additional means of assessing B cell activation and differentiation. T cell help, whether in the form of T blasts or activated T cell–conditioned medium, primed B cells for enhanced secretion of immunoglobulins of most isotypes (Fig. 4). However, the activation of B cells by allo-T blasts resulted in enhanced production of immunoglobulins utilizing downstream heavy chain constant regions, most notably γ_1 and α, while the amount of IgM was reduced relative to αIg/EL-4 blasts. The patterns of isotypes secreted were the same whether the cells were restimulated with rIL-2 or EL-4 Sn ± anti-IgD. (data not shown).

Time Dependence of B Cell Priming by Allo-T Blasts. We investigated the kinetics of the functional priming of allo-B blasts during the primary co-culture of T blasts and B cells. Allo-B blasts were primed for enhanced responsiveness to anti-Ig-Sepharose within 24 h (Fig. 5). In contrast, the allo-B blasts required 2 d of co-culture with allo-T blasts for maximal priming for the proliferative responses to IL-2 and EL-4 Sn. The 2-d requirement for priming of allo-B blasts to respond to IL-2 was unexpected because the increase in IL-2Rp55 subunit peaked by day 1 (Fig. 3). This phenomenon could be due to a requirement for expression of the p70 subunit of the IL-2R, which may be upregulated with different ki-

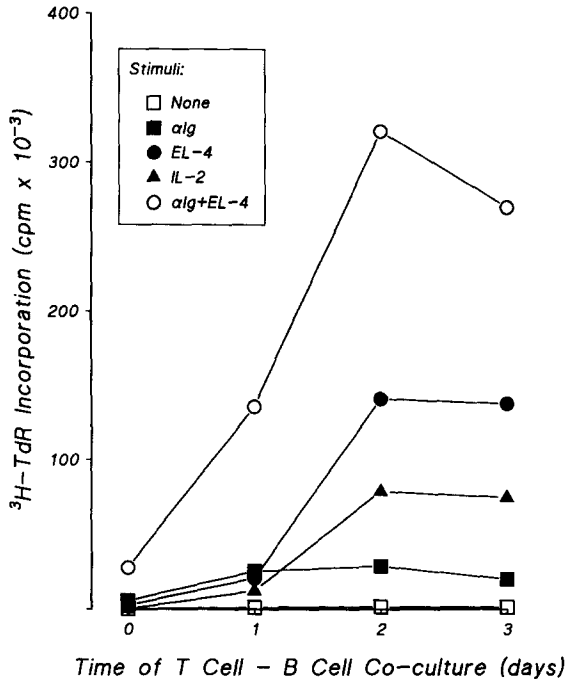

Figure 5. Time course of priming of allo-B blasts for proliferation. Allo-B blasts were harvested at 24, 48, and 72 h of T cell–B cell co-culture. Allo-B blasts or B cells were re-cultured at 2.5 × 10^5/ml in 200 μl final volume with the stimuli as indicated. After 24 h, the cultures were pulsed with [^{3}H]TdR for 12 h and harvested. Additions were: αIg-Sepharose, 5 μg/ml; EL-4 Sn, 0.5% (vol/vol); IL-2, 10 U/ml.

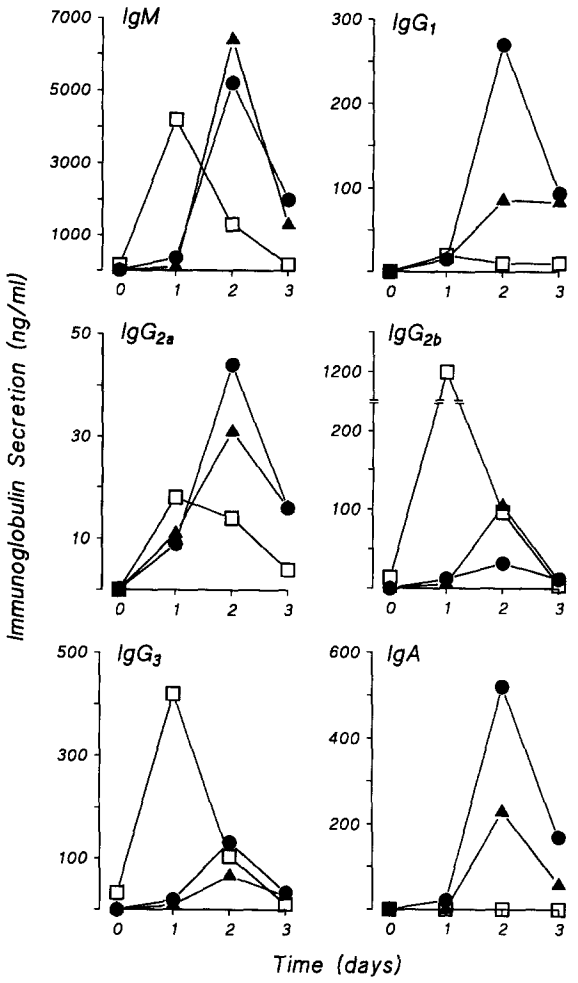

Figure 6. Time course of priming of allo-B blasts for Ig secretion. Allo-B blasts were harvested at 1, 2, and 3 d of T cell–B cell co-culture. Allo-B blasts or B cells (t = 0) were re-cultured at 2.5 × 10^5/ml in 200 μl final volume with additions as indicated. After 60 h, the supernatants were removed and tested in an isotype-specific Ig ELISA. Additions were: EL-4 Sn, 0.5% (vol/vol) (●); IL-2, 10 U/ml (△); LPS, 20 μg/ml (□).

netics. The enhanced production of Ig by allo-B blasts upon restimulation with IL-2 or EL-4 Sn also required 2 d of allo-T blast stimulation (Fig. 6).

Response of B Lymphoblasts to LPS. Bacterial LPS is a mitogen for murine B cells. B cell activation through the IgR by anti-Ig-Sepharose primed the B cells for enhanced responsiveness to LPS (Table 3) (3). In contrast, allo-B blasts were remarkably insensitive to LPS as measured by proliferation (Table 3), cell viability (data not shown), or Ig production (Fig. 6). This lack of response to LPS by allo-B blasts was dependent on the time of co-culture of B cells with allo-T blasts (Fig. 6 and data not shown) and was inversely related to the kinetics of priming for responses to anti-Ig-Sepharose and/or lymphokines. The LPS response of allo-B blasts generated by 1 d of T-B co-culture was remarkable in that high levels of IgG2b and IgG3 were detected. These findings suggest that co-culture with allo-T blasts either preferentially expands a unique population of LPS-resistant B cells or induces a specific unresponsiveness to LPS through an unidentified pathway.

Discussion

In this study, we generated murine B lymphoblasts by co-culture with primary alloreactive T blasts. We compared the phenotype and function of allo-stimulated B blasts after re-stimulation with that of B lymphoblasts stimulated via mIg alone or with lymphokines. The allo-B blasts were shown to have a distinct phenotype and distinct requirements for the subsequent growth and induction of Ig secretion compared with the anti-Ig and anti-Ig/EL-4 blasts.

Priming of B Cells to Proliferate and Secrete Ig. Activation of resting B cells with anti-Ig primes them for entry into S phase as well as immunoglobulin secretion in response to re-stimulation with T cell–derived lymphokines (3) and anti-Ig (33). We found that activating B cells in the presence of T cell help further enhanced this priming and rendered the blasts responsive to rIL-2. The magnitude of the proliferative response and the quantity and quality of the antibody response suggest that the B blasts generated in the presence of T cell help either represent a further differentiated state than those lymphoblasts generated under T-independent conditions, or the stimulation of a different subset of B cells. We think the latter is less likely in view of the large percentage of B cells that blast transform under each of these conditions.

The interaction of B cells and alloreactive T cells is required to restimulate the T cell blasts to produce B cell–stimulating factors, including IL-2 (~15 U/ml) and IL-4 (~30 U/ml) (34, 35, and unpublished observation). Thus, the allo-T blasts provide lymphokines active on the B cells but may also provide a direct signal to the B cells via cell surface Ia. A putative Ia-mediated signaling of B cells may be supported by production of IL-4 by the T blasts, since it has been shown that IL-4 induces increased expression of MHC class II molecules (36) and primes B cells to respond to anti-Ia antibodies (37). The generation of allo-B blasts presumably occurs independently of the Ig-mediated pathway of B cell activation; although we can not eliminate the possibility that some of the B cells in these cultures also receive mIg-mediated signals, for example, in the form of antigens present in the FCS. However, the large percentage of cells that were induced to blast transform makes it less likely that a specific Ig-mediated signal is required. Thus, during a normal immune response, the B cell may initially be stimulated by specific antigen. The antigen can subsequently be internalized, processed, and presented in the context of Ia to antigen-specific T cells. This may in turn further activate the B cells via the second signaling pathway, mediated by Ia.

Resistance of Allo-B Blasts to LPS. LPS has been well documented as a mitogen for murine B cells (6). In addition, αIg blasts (3) and αIg/EL-4 blasts are primed for enhanced responsiveness to LPS. In contrast, B lymphoblasts activated by allo-T blasts exhibited a time-dependent loss of sensitivity to LPS. The mechanism by which LPS stimulates B cells is largely unknown. The enhanced LPS response of B blasts primed with anti-Ig may reflect an increase in the expression of a putative "LPS receptor" or more efficient signaling. Conversely, the low response to LPS observed in allo-B blasts could be due to downregulation of the putative receptor or an uncoupling of the receptor from the signaling pathway. In addition, the insensitivity might be due to a specific selection of a population of B cells that do not respond to LPS. Alternatively, allo-stimulation of B cells may render them lymphokine dependent. If this were the case, it would be predicted that when stimulated with LPS alone they would not respond, but LPS plus lymphokines should have a synergistic effect. However, the re-stimulation of allo-B blasts with a combination of lymphokines plus LPS resulted in a response that was only additive (unpublished observation).

IL-2 Is a Predominant Stimulatory Factor for T-dependent B Lymphoblasts. The response of the two populations of T-dependent B blasts to EL-4 Sn was predominantly mediated by IL-2; i.e., the response was blocked by anti-IL-2 and similar responses were induced by rIL-2. IL-2 has been shown to induce proliferation and Ig secretion of preactivated human B cells (38–40). In the murine system, B cells activated with LPS plus anti-Ig proliferated in response to IL-2 (41). In the present study, preactivation with anti-Ig alone was not sufficient to induce IL-2 responsiveness despite upregulation of the IL-2Rp55. Since the functional high affinity IL-2R on T cells is formed by the p55/p70 heterodimeric IL-2R (42), it is possible that anti-Ig is not sufficient to induce expression of the p70 chain. The additional stimuli provided by T cell help appears to be required to induce expression of the high affinity IL-2R. Loughnan and Nossal (43) demonstrated that IL-4 and IL-5 induce expression of the p70-75 and p55 subunits of the IL-2R on B cells. Our data further suggest that T cells committed to the T_H1 phenotype, i.e., that produce IL-2 but not IL-4 (18), would be sufficient to support the latter stages of B cell responses to T cell–dependent antigens.

Regulation of Ig Isotypes. Relative to other types of B lymphoblasts, the allo-B blasts secreted increased levels of Ig that are encoded for by downstream heavy chain constant region genes. This phenomenon reflects either a selective expansion of B cells that were already committed to isotype switching or a specific induction of isotype switching dependent on cell-cell interaction. In prior work, the induction of enhanced production of these isotypes by specific lymphokines usually involved co-culture with LPS (5, 44–47) or crude activated T cell supernatants containing a complex mixture of lymphokines (4). If the enhanced secretion of these isotypes is dependent on a combination of soluble factors and cell-cell interaction, then LPS may be bypassing the requirement for T cell contact. One possibility is that isotype switching occurs more efficiently during rapid cell division that is induced by LPS or T cell help (48). IgG and IgE production was demonstrated in these systems. In the case of IgG, IFN-γ and IL-4 have been shown to regulate the production of the different subclasses (45, 46, 49, 50) and IL-4 induces IgE production (51). The regulation of IgA production is less well characterized, but transforming growth factor β and IL-5 have been reported to increase IgA production under certain conditions (44, 52, 53). The data in this study suggest that T cells, possibly by direct contact or additional lymphokines, may provide a signal that potentiates switching to IgA.

Allostimulated B Blasts Resemble Germinal Center B Cells. Several features of allo-stimulated B lymphoblasts resemble those of germinal center B cells. First, generation of germinal centers is T cell dependent (54, 55). In addition, germinal center B cells are IgD^{low} (31) and PNA^{high} (32). T cell help was required for maximal downregulation of mIgD and this decrease in mIgD on allo-stimulated B cells occurred in a time-dependent fashion, evidently without a requirement for crosslinking of mIgD. In addition, the downmodulation of mIgD was specific in that there was no change in the expression of mIgM. Most likely, mIgD is specifically modulated off the cell surface; alternatively, the activation of B cells may block synthesis of IgD. Allo-B blasts also exhibit enhanced reactivity with PNA. PNA reacts with the penultimate *N*-acetyl glucosamine residues of glycoproteins exposed by removal of the N-terminal sialic acid residues. Thus, allo-T blast–induced activation of B cells may induce neuraminidase activity on the surface of T cells or B cells (56, 57) or, alternatively, newly synthesized proteins not bearing terminal sialic acid residues may be expressed.

In addition, the allo-B blasts proliferate rapidly in response to αIg. These data are consistent with the observation that surface Ig can provide a signal that maintains the viability

of human tonsillar germinal center B cells (58). The allo-B blasts may undergo isotype switching at an enhanced rate or frequency, which is also a property of germinal center B cells (59). In conclusion, these data suggest that the allo-B blasts may reflect a stage in differentiation more closely related to germinal center B cells than αIg or αIg/EL-4 blasts.

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