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T-INDEPENDENT AND T-DEPENDENT STEPS IN THE MURINE B CELL RESPONSE TO ANTIIMMUNOGLOBULIN

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The response of B cells to mitogen includes three phases: Entry into the cell cycle, G_0 to G_1 transition; DNA synthesis and cell division; and Ig secretion (1, 2). There is evidence that T cells can influence each of these phases. T cell effects have been most easily studied with anti-Ig as the polyclonal stimulus (3, 4). When small B cells are incubated with anti-Ig, a large fraction enlarge and show increased levels of RNA (5, 6). The T cell product, B cell stimulatory factor 1 (BSF-1)[1] or IL-4, may be an important costimulant that prepares the anti-Ig-stimulated B cell to enter S phase (7–10). IL-4 also induces the B lymphoblast to secrete IgG1 (10, 11). Other lymphokines, distinct from IL-4, will induce anti-Ig and dextran sulfate–primed B cells to show increased proliferative and secretory activity (12–17).

To further understand the contributions of T cells, we have divided the polyclonal anti-Ig response into two experimental phases: The formation of B lymphoblasts and their subsequent growth and Ig secretion. We report on a pathway of lymphoblast development that originates from homogeneous populations of small, resting, mouse spleen B cells and does not require T cells or the T cell product IL-4. The subsequent capacity of these blasts to proliferate and secrete Ig is, however, critically dependent on lymphokine(s) distinct from IL-1, -2, -3 and -4.

Materials and Methods

Animals. 8–20-wk-old CD2F1 (Charles River Breeding Laboratories, Inc., Wilmington, MA) or BALB/c (Cumberland Farms, Clinton, TN) female mice were used routinely. C3H/HeJ and C3HeB/FeJ female mice were from The Jackson Laboratory, Bar Harbor, ME.

Antibodies. Anti-Thy-1.2 mAb (IgMκ) was partially purified by ammonium-sulfate precipitation of culture supernatant from HO-13.4 hybridoma cells obtained from the American Type Culture Collection (ATCC, TIB 99, Rockville, MD). Culture supernatants from C3PO and HO-2.2 (ATCC TIB 150) were used as sources of cytotoxic anti-Lyt-1.2

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[1] *Abbreviations used in this paper:* BCGF-II, B cell growth factor II; BSF-1/IL-4, B cell stimulatory factor (or IL-4); Sn, cell-free conditioned medium or supernatant.

and anti-Lyt-2.2, respectively. Affinity-purified goat anti–mouse IgD (anti-δ) and goat anti–mouse IgM (anti-μ) were the generous gift of Dr. E. S. Vitetta (University of Texas Health Science Center, Dallas, TX). Affinity-purified goat anti–mouse IgM (anti-μ, μ chain–specific) and goat anti–mouse Ig (GAM Ig, heavy and light chain–specific) were purchased from Jackson Immunoresearch (Avondale PA), and anti-IgG1 was from Southern Biotechnology Associates, Inc. (Birmingham, AL). Coupling of these antibodies to CNBr–Sepharose CL4B (Pharmacia Fine Chemicals, Piscataway, New Jersey) was accomplished at a ratio of 1 mg protein per 1 ml packed, swollen resin according to the method recommended by the manufacturer. The mAb 11B11 (18) was generously provided by Dr. W. E. Paul (National Institutes of Health, Bethesda, MD) and affinity purified on anti–rat Ig immunoadsorbent. Culture supernatant from the B21.2 hybridoma was used as a source of anti-I-A (19).

B Cell Preparations. T-depleted spleen cells (hereafter referred to as B cells) were routinely prepared by treating total splenic white cells with a cocktail of antibodies directed against Thy-1.2, Lyt-1.2, and Lyt-2.2 followed by complement (C) lysis (baby rabbit complement; Pel-Freeze Biologicals, Rogers, AR) or anti-Thy-1.2 and C. The T-depleted cell population was not responsive to 5 μg/ml Con A as measured by [^{3}H]TdR incorporation at 72 h of culture (data not shown). Adherent cells were depleted by one or two Sephadex G-10 passages as indicated (20).

Preparation of Anti-Ig Blasts. Anti-Ig blasts were prepared by culture of B cells at 1–2 $\times$ 10^6/ml with 5 μg/ml anti-Ig-coupled Sepharose for 48–72 h in Hepes-buffered RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2 mM glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamycin, 50 μM 2-ME, 5 or 10% vol/vol heat-inactivated FCS (KC Biological Inc., Lenexa, KA or Gibco Laboratories) in a humidified 7% CO_2 environment at 37°C. Anti-Ig blasts were isolated free of Sepharose beads by centrifugation through Ficoll-Paque (Pharmacia Fine Chemicals). Cells and beads in a volume of 3–5 ml were layered on a cushion of 3–4 ml Ficoll-Paque, and centrifuged at 400 *g* for 10–15 min. Viable cells (free of Sepharose beads and dead cells) were collected from the interface and washed extensively in a balanced salt solution before reculture in 200 μl microcultures with bacterial LPS (*Salmonella typhimurium*; Difco Laboratories Inc., Detroit MI) or lymphokines from activated T cells.

Density Fractionation of B Cells and Blasts. Percoll (Pharmacia Fine Chemicals) was made isotonic with 10X concentrated PBS. Fractions of density 1.09 g/ml, 1.085 g/ml, 1.08 g/ml, 1.075 g/ml, 1.065 g/ml, and 1.055 g/ml were prepared with isotonic Percoll and PBS. Unstimulated or stimulated (Sepharose–anti-Ig) cells were resuspended in the 1.075 g/ml layer. 2-ml fractions were layered in a 15-ml conical tube (Sarstedt, Inc., Princeton, NJ, or Falcon Labware, Oxnard, CA) and centrifuged for 30 min at 800 *g*. High-density B cells were those harvested from bands that formed above the 1.09 g/ml and 1.085 g/ml layers. >90% of the Sepharose–anti-Ig–stimulated cells (blasts) were collected from bands at 1.065 g/ml and 1.075 g/ml. Total cells loaded on each gradient varied from 0.8–2 $\times$ 10^8 viable B cells or blasts.

Preparation of Lymphokine-enriched Supernatants. Supernatants from activated EL-4 thymoma cells were collected 24–48 h after treating the cells with 20 ng/ml PMA (Sigma Chemical Co., St. Louis, MO). Supernatants were concentrated (5–10-fold) and partially purified by ammonium-sulfate precipitation as previously described (21). Alternatively phorbol dibutyrate (PDBU, Sigma Chemical Co.)-induced EL-4 supernatant was prepared under serum-free conditions as previously described (11, 22).

Recombinant Lymphokines. Recombinant lymphokines were generously provided as follows: Murine IL-1 by Dr. P. Lomedico (Hoffman-La Roche, Nutley, NJ); human IL-2 by Dr. S. Rudnick (Biogen, Cambridge, MA); murine IL-3 by Dr. Yokata (DNAX, CA); and murine IL-4 (BSF-1) Dr. T. Mossman (DNAX).

Assays of Anti-Ig Lymphoblast Function. Proliferation in response to recombinant lymphokines, EL-4 cell-free conditioned medium (EL-4 Sn) or LPS was assayed by reculturing 48–72-h blasts prepared as described above with lymphokine-containing supernatants or LPS. The cell density, length of incubation, and concentrations of stimuli were varied as indicated in the Results.

TABLE I

EL-4 Sn Does Not Induce Growth or IgM Secretion of High-density B Cells

Cell density	[^{3}H]TdR uptake			IgM secretion		
	Nothing added	EL-4 Sn	LPS	Nothing added	EL-4 Sn	LPS
gm/ml		*cpm* × 10^{-3}			*ng/ml*	
>1.08	1.0 ± 0.6	0.5 ± 0.2	1.1 ± 0.2	0	49 ± 20	194 ± 27
≤1.075	3.9 ± 0.4	24.6 ± 6.8	64.4 ± 8.8	40 ± 8	836 ± 171	1914 ± 203

Freshly isolated splenic B cells were fractionated on dense Percoll gradients and cultured at 2 × 10^5/ml alone or with 5 μl EL-4 Sn or 20 μg/ml LPS for 48 h then pulsed overnight with 0.5 μCi [^{3}H]thymidine or culture supernatants harvested at 78 h and assayed for IgM.

Detection of Ig Secretion. IgM and IgG1 in culture medium were measured by RIA as previously described (11).

FACS Analysis. Cells were incubated with 2 μg/ml FITC-conjugated goat-anti–mouse Ig (Scandic, Vienna, Austria), anti-I-A (clone B21-2), or mouse anti–rat Ig (Boehringer Mannheim Biochemicals, Indianapolis, IN) as a control for 60 min at 4°C then washed in PBS and fixed in formalin. Forward light scatter and fluorescence were analyzed on a Becton Dickinson & Co. (Mountain View, CA) FACS 440 at the New York Blood Center, New York.

Cytospin Preparations. Cells were resuspended at 2–5 × 10^5/ml in complete medium and 200 μl were spun onto slides in a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, PA). Samples were air dried, treated with acetone for 10 min at room temperature, and air dried again. Samples were stained with FITC-GAM Ig to detect mouse Ig or FITC-MAR Ig as a control. Samples were preserved in glycerol/PBS and photographed on a Labophot (Nikon Inc., Garden City, NY) equipped with epifluorescence.

Results

High-density B Cells Do not Proliferate or Secrete IgM when Cultured with Activated T Cell–conditioned Medium. Bulk populations of splenic B cells contain stimulated and unstimulated populations that can be separated on the basis of different buoyant densities (5). Low-density B cells (≤1.075 g/ml in Percoll gradients) from freshly isolated spleen respond vigorously to LPS or EL-4 Sn and have presumably been preactivated in vivo (12–14; Table I). However, small, high-density, resting B cells (>1.080 g/ml) exhibit little proliferation or IgM secretion when cultured in the presence of T cell–conditioned medium (12–14; Table I). Therefore, we used such small resting B cells, isolated by Percoll density gradient centrifugation, as a more homogeneous starting population to generate B lymphoblasts in vitro.

Phenotype and Physical Properties of High-density B Cells and Anti-Ig Blasts. Stimulation of purified high-density B cells with Sepharose–anti-Ig followed by flotation on Ficoll-Paque and Percoll, provided highly enriched populations of B lymphoblasts. The density and size of the anti-Ig blasts generated in vitro (as described in Materials and Methods) were compared with freshly isolated high-density spleen B cells. >90% of recovered cells were found in low-density fractions of 1.075 and 1.065 g/ml. This fraction contained all of the EL-4 Sn–responsive cells (data not shown). Forward light scatter profiles, which provided an approximation of cell size, clearly distinguished most high-density B cells from

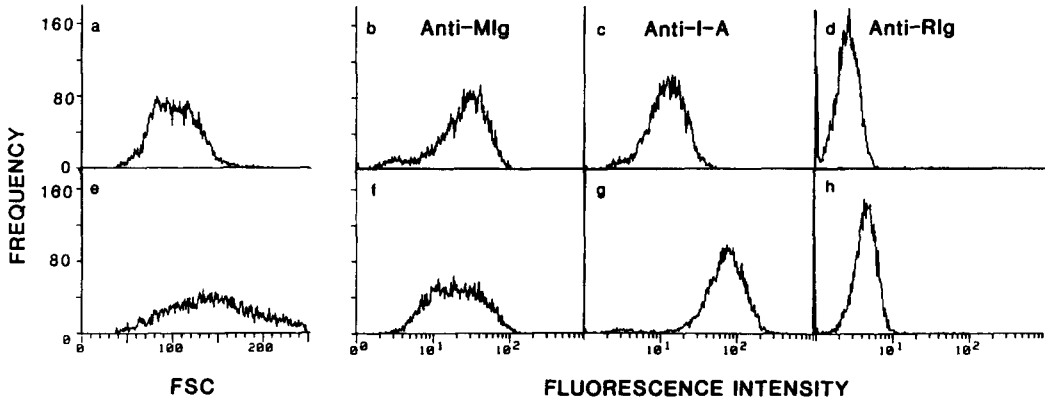

FIGURE 1. FACS analysis of small resting B cells and anti-Ig blasts. Freshly isolated splenic B cells ($p > 1.08$ g/ml [*a–d*]) or 3-d anti-Ig blasts ($p < 1.065$ g/ml [*e–h*]) were fixed in 3.7% formalin and examined on the FACS 440. Forward light scatter profiles (*a* and *e*) and fluorescent distribution are shown. The cells were stained before fixation for 1 h at 4°C with FITC-conjugated goat anti-mouse Ig (*b* and *f*), anti-I-A (*c* and *g*), or mouse anti-rat Ig as a negative control (*d* and *h*).

most anti-Ig blasts (Fig. 1, *a* and *e*). The homogeneity of the population was further evidenced by the fact that all cells were sIg^+ and I-A rich (Fig. 1).

Experimental Parameters Influencing the Generation of Anti-Ig–induced Lymphoblasts. A number of variables were tested for their effect on the yield of B blasts generated in response to anti-Ig–Sepharose and the responsiveness of these blasts to EL-4 Sn or LPS. The yield and responsiveness of blasts was not significantly affected by any of the following variables pertaining to the input population of B cells (Table II): (*a*) The buoyant density of the B cells (Exp. 1); (*b*) adherent-cell depletion (Exp. 3). The use of Sephadex G10 depletes most splenic dendritic cells and macrophages, so that these accessory cells do not seem essential for the anti-Ig response; or (*c*) surface IgM- (sIgM) versus sIgD-mediated activation (Exp. 2). Anti-μ and anti-δ were equivalent in stimulating entry into cell cycle and responsiveness to T cell factors, although somewhat less effective than anti-Ig (Table II, Exp. 2).

Role of BSF-1 in the Generation of Anti-Ig Blasts. The results described above were obtained with a starting population of T-depleted spleen cells. As indicated in Fig. 1, the B cells and anti-Ig blasts were >95% Ig^+. In addition, neither the starting population nor the resulting anti-Ig blasts contained Thy-1^+ or Lyt-1^+ T cells detectable by immunofluorescence (data not shown). Previous results (7, 9) have, however, suggested an important role for the T cell–derived lymphokine BSF-1 for B cell responses to anti-Ig. To address whether the production of BSF-1/IL-4 by contaminating T cells (or other cell types) during the primary culture contributed to the response observed, we prepared anti-μ blasts in the presence of an mAb (11B11) that neutralizes BSF-1 activity (18). A concentration of 11B11 mAb was added that completely inhibited the induction of IgG1 secretion (see below), another BSF-1-dependent response. In several experiments, similar yields of blasts were obtained from control anti-μ-activated cultures and cultures stimulated in the presence of anti-BSF-1 mAb (Table III). Blasts were then cultured with LPS or EL-4 Sn and assayed for proliferation and IgM secretion.

TABLE II

Yield and Response of Anti-Ig Blasts Made under Various Conditions

Exp.	Cells used to prepare blasts*	Cells recovered	Response of blasts to secondary stimuli ([^{3}H]TdR incorporation)		
			None	LPS‡	EL-4 Sn§
		$\times 10^{-5}$		$cpm \times 10^{-3}$	
1*,‖	Unfractionated	2.4	6.9 ± 0.1	149 ± 9.0	18.6 ± 1.8
	1.85 g/ml	1.7	2.9 ± 0.2	81.8 ± 1.9	10.6 ± 0.5
	1.080 g/ml	3.2	3.1 ± 0.4	100.7 ± 12.1	10.3 ± 0.7
	1.075 g/ml	3.6	3.6 ± 2.1	100.9 ± 18.1	14.9 ± 1.1
2*,¶	anti-Ig	13.0	1.1 ± 0.5	93.7 ± 6.4	28.3 ± 0.7
	anti-μ	4.9	1.5 ± 0.3	82.4 ± 8.5	22.4 ± 1.8
	anti-δ	4.4	2.0 ± 0.4	66.5 ± 4.5	30.2 ± 1.8
3**	T-depleted	8.8	2.9 ± 1.1	168.5 ± 17.2	25.5 ± 0.1
	T and G10-depleted	9.4	1.5 ± 0.6	152.1 ± 24.4	19.0 ± 1.9

In all experiments, 2×10^6 viable B cells/ml were cultured for 48–72 h with Sepharose anti-Ig. Blasts were recultured at 2×10^5/ml in a total volume of 200 μl. Viable cell recovery per 10^6 input cells was determined by counting in the presence of trypan blue.

* Number of cells represented is the number recovered before centrifugation through Ficoll-Paque, except for experiments marked by asterisk, where the numbers shown are final yield of cells.

‡ LPS was added at 10 μg/ml.

§ EL-4 Sn was added at 1% vol/vol.

‖ T-depleted and G-10-passed B cells were fractionated on discontinuous Percoll gradients as described in Materials and Methods. Cells from each fraction were cultured with Sepharose–anti-Ig for 72 h. Densities are of the cells seeded in the culture.

¶ B cells were cultured for 72 h with Sepharose-anti-Ig, -anti-μ, or -anti-δ.

** B cells depleted of T cells or T and adherent cell depleted by two Sephadex G-10 passages, were cultured with anti-Ig Sepharose for 72 h.

As shown in Table III, blasts prepared in the presence of anti-BSF-1 were functionally indistinguishable from control blasts.

In three experiments, we found that the preparation of anti-Ig blasts from T- and adherent cell–depleted splenic B cells in the presence of 100 U/ml of rIL-4 (BSF-1) resulted in a twofold greater yield of blasts on day 3 but had no effect on their subsequent growth or IgM secretion when stimulated with EL-4 or LPS (Table III).

We conclude that BSF-1 is not essential for the generation of B lymphoblasts from Sepharose–anti-Ig–stimulated mouse spleen B cells.

The Proliferation and Ig Secretion of Anti-Ig Blasts. The majority of blasts did not survive in secondary cultures in the absence of exogenous stimuli. However, in the presence of lymphokines the number of viable cells recovered at 20–24 h of culture was 150–200% of the number of cells seeded in the secondary culture. Since B cells would not have divided more than once in this time period, a 1.5–2-fold recovery signifies the division of at least 50% of the blasts. The rapid nature of this response was also apparent when [^{3}H]TdR incorporation was measured at different times (Table IV). When the kinetics of the response of small B cells and anti-Ig blasts to LPS were compared, anti-Ig blasts responded much more rapidly (Fig. 2). Detectable responses were observed by 12 h of culture and approached a maximal value by 24–48 h. In contrast, small B cell

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TABLE III
Response of Anti-Ig Blasts Made in the Presence of Monoclonal Anti-BSF-1

Exp.	Addition to primary culture	Addition to secondary culture	[^{3}H]TdR incorporation	IgM
			cpm × 10^{-3}	*μg/ml*
1	Control	None	1.6	0.02
		LPS	68.6	0.40
		EL-4 Sn (1 μl)	49.4	0.45
		EL-4 Sn (5 μl)	90.5	1.19
	Anti-BSF-1	None	0.8	0.03
		LPS	83.3	0.46
		EL-4 Sn (1 μl)	35.1	0.32
		EL-4 Sn (5 μl)	129.7	0.90
2	Control	None	2.6	
		LPS	88.6	
		EL-4 Sn (1 μl)	27.3	
		EL-4 Sn (5 μl)	42.8	
	Anti-BSF-1	None	2.7	
		LPS	133.4	
		EL-4 Sn (1 μl)	34.6	
		EL-4 Sn (5 μl)	51.0	
3	Control	None	1.6	
		LPS	116.2	
		EL-4 Sn (2 μl)	22.1	
	Anti-BSF-1	None	1.2	
		LPS	127.5	
		EL-4 (2 μl)	12.5	
	rBSF-1	None	2.7	
		LPS	113.8	
		EL-4 Sn (2 μl)	30.2	

Splenic B cells (2×10^6/ml) were cultured for 2 (Exp. 1) or 3 (Exps. 2 and 3) d with GAM-Ig Sepharose, and in the presence of 1 μg/ml of a control monoclonal rat antibody (Exp. 1), or medium (Exps. 2 and 3), or 11B11 anti-BSF-1, or 100 U/ml rBSF-1. Blasts were isolated as outlined in Materials and Methods and recultured in microtiter plates at 2×10^5/ml for 72 h (Exp. 1) or 48 h (Exp. 2). The yield of blasts in Exp. 1 was 13×10^5 and 12×10^5 per 10^6 input cells for control blasts and blasts made in the presence of anti-BSF-1, respectively. [^{3}H]TdR was added during the last 16 h (Exp. 1) or 8 h (Exps. 2 and 3) of culture and the concentration of IgM in the 3-d culture medium was determined by RIA.

responses were not detectable until 24 h and were not maximal until 48 h (Fig. 2). These findings indicate that anti-Ig blasts are poised to enter S phase when challenged with a mitogenic stimulus.

The proportion of anti-Ig blasts that were induced by lymphokines to synthesize IgM was also remarkable (Fig. 3). The starting population of small B cells had low levels of Ig, as detected by FITC–goat anti–mouse Ig staining of cytospin preparations. At 3 d, anti-Ig blasts were larger and had patches of perinuclear anti-Ig stain. The vast majority of blasts restimulated with EL-4 Sn stained brightly for cytoplasmic Ig.

TABLE IV

Effect of EL-4 on Anti-Ig Blast Proliferation and IgM Secretion

Cells per well	EL-4 Sn	[^{3}H]TdR incorporation		IgM at 72 h
		34 h	58 h	
$\times 10^{-4}$	μl	$cpm \times 10^{-3}$		$\mu g/ml$
1	0	0.29 ± 0.08	0.29 ± 0.05	
	1	1.03 ± 0.05	0.56 ± 0.04	
	5	2.08 ± 0.15	1.13 ± 0.39	
2	0	0.73 ± 0.17	0.58 ± 0.23	0.03 ± 0.01
	1	1.92 ± 0.18	1.61 ± 0.44	0.18 ± 0.04
	5	6.92 ± 0.43	4.32 ± 1.24	0.14 ± 0.03
4	0	1.57 ± 0.14	0.77 ± 0.24	0.09 ± 0.02
	1	5.59 ± 0.24	4.01 ± 0.42	0.46 ± 0.08
	5	14.87 ± 1.45	10.03 ± 1.86	0.43 ± 0.09
10	0	6.05 ± 0.83	2.10 ± 0.91	0.31 ± 0.05
	1	18.56 ± 2.52	14.92 ± 1.85	1.38 ± 0.11
	5	12.75 ± 5.87	22.43 ± 2.11	1.45 ± 0.14

72-h anti-Ig blasts were prepared from splenic B cells and isolated as described in Materials and Methods. Cells were recultured with EL-4 Sn in a final volume of 200 μl. [^{3}H]TdR was added during the last 8 h of culture.

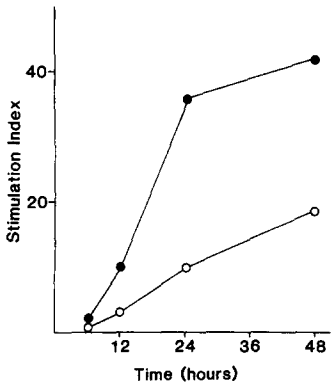

FIGURE 2. Kinetics of the LPS response of small B cells and anti-Ig blasts. 72-h anti-Ig blasts (●) or T-depleted splenic white cells (○) were prepared as described and cultured in a final volume of 200 μl with 20 μg/ml LPS at a density of 10^5 cells/ml and 5×10^5 cells/ml, respectively. Cultures were pulsed with 0.5 μCi [^{3}H]TdR per well for 4 h at the indicated times.

Several variables in the response of anti-Ig blasts were assessed. The stimulation indices of [^{3}H]TdR incorporation and IgM secretion for each dose of EL-4 Sn remained approximately the same for each cell concentration we tested (Table IV). This suggests that a constant proportion of the blasts respond over a range of cell densities. In view of the sensitivity of anti-Ig blasts to LPS we tested the possibility that the EL-4 Sn–induced response was due to contaminating LPS by comparing the response of B cells from LPS low-responder mice, C3H/HeJ, and normal responder controls, C3HeB/FeJ. The EL-4 Sn–induced proliferation and IgM secretion of B cell blasts were comparable to that of C3HeB/FeJ mice (data not shown).

Lymphokine Requirements of Anti-Ig Blasts. Addition of anti-IL-4 mAb, 11B11, did not reduce growth or IgM secretion by blasts stimulated with LPS or EL-4

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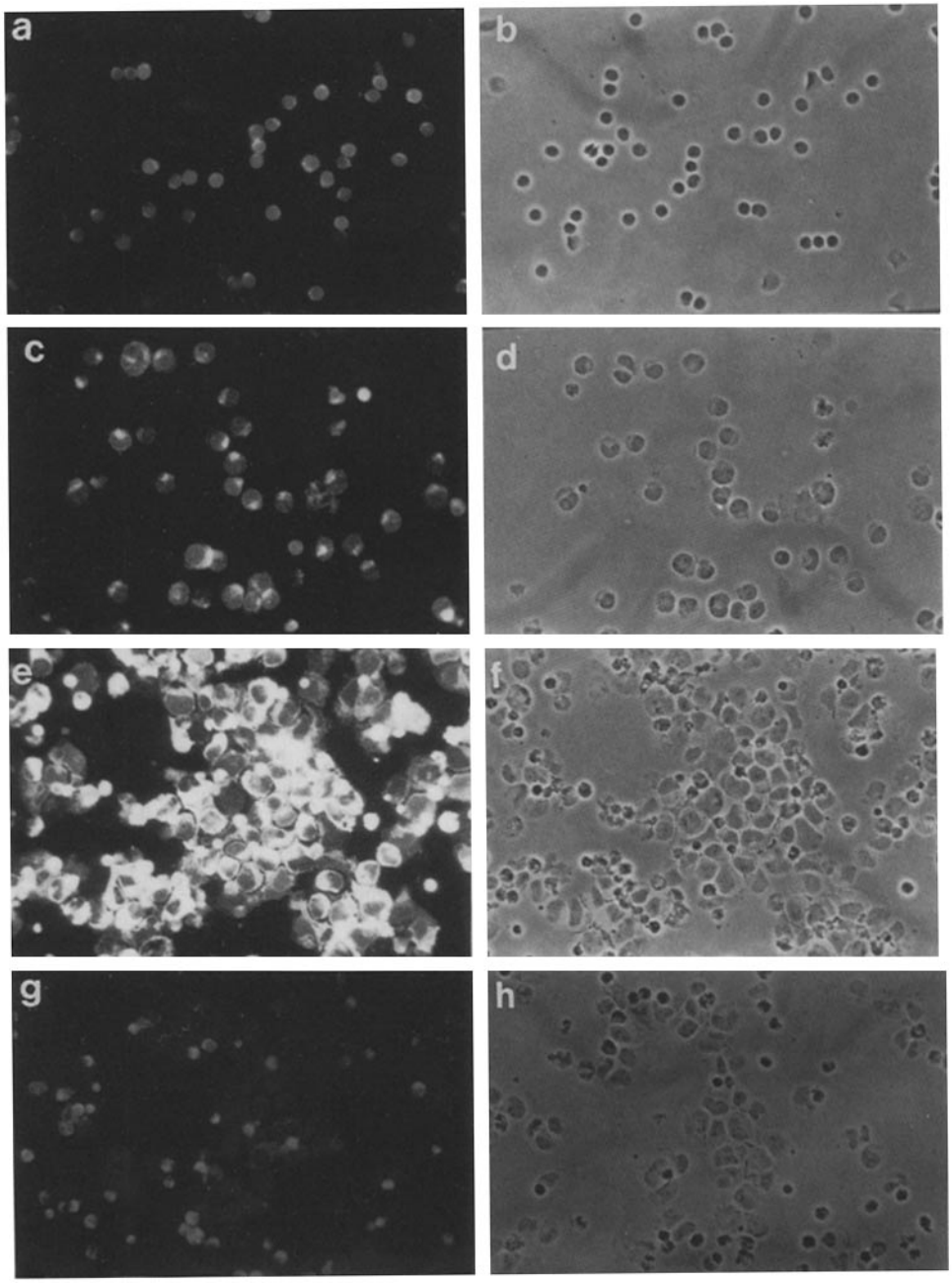

FIGURE 3. Phase contrast and cytoplasmic anti-Ig stains of B cells. Cytospins of (*a* and *b*) high-density B cells, (*c* and *d*) 3-d anti-Ig blasts, and (*e–h*) anti-Ig blasts treated for 3 d with El-4 Sn were stained with FITC–goat anti–mouse Ig (*a–f*) or FITC–mouse anti–rat Ig (*g* and *h*) as a control.

TABLE V

Effect of Anti-BSF-1 on [^{3}H]thymidine Incorporation by Anti-Ig Blasts

Exp.	11B11	[^{3}H]TdR incorporation		
		Nothing added	EL-4	LPS
		cpm × 10^{-3}		
1	−	1.6	90.5	46.4
	+	0.8	120.6	60.4
2	−	1.3	53.7	88.3
	+	0.7	67.0	103.5

Anti-Ig blasts were prepared as described in Materials and Methods in the presence of 1 μg/ml 11B11 anti-BSF-1. Blast were recultured at 2 × 10^5/ml for 48 h in the presence of 1 μg/ml isotype-matched control rat mAb or 11B11 anti-BSF-1, then pulsed with 0.5 μCi/well of [^{3}H]thymidine.

TABLE VI

Effect of Anti-BSF-1 on IgM and IgG1 Secretion of Anti-Ig Blasts

11B11		IgM secretion			IgG secretion		
Primary	Second-ary	Nothing added	EL-4	LPS	Nothing added	EL-4	LPS
			μg/ml			*μg/ml*	
−	−	<0.1	15.7	16.1	<0.1	2.1	<0.1
−	+	<0.1	23.1	13.4	<0.1	<0.1	<0.1
+	−	<0.1	16.4	11.6	<0.1	1.3	0.1
+	+	<0.1	13.1	12.4	<0.1	<0.1	<0.1

Anti-μ blasts were prepared as described in Materials and Methods. Blasts were recultured at 2 × 10^5/ml for 5 d, and IgM and IgG1 in the culture supernatant were measured by RIA. 11B11 anti-BSF-1 or a control rat mAb was added at a final concentration of 1 μg/ml during the anti-Ig stimulation in the primary culture and/or the secondary culture of blasts in the absence of any additional stimuli (nothing added), 1% vol/vol of EL-4 Sn or 20 μg/ml LPS.

Sn (Table V). In some experiments (Table VI), blasts were prepared in the presence of anti-BSF-1, then washed and recultured with or without anti-BSF-1 in the secondary cultures. Parallel cultures were carried out to 5 d so that secretion of IgG1, an IL-4-dependent response, could be assayed. This provided a positive control for the effectiveness of the anti-BSF-1 added. Again, 11B11 did not diminish the growth or IgM secretion in response to EL-4 Sn, but completely abolished the IgG1 response.

Additional experiments compared the EL-4 Sn with recombinant IL-1, IL-2, or IL-3 as stimuli for the growth of anti-Ig blasts. Although a small boost in [^{3}H]-TdR incorporation was detected when all three recombinant lymphokines were added, the lymphokines clearly did not mimic the growth-promoting activity of EL-4 culture Sn (Table VII).

Discussion

These studies characterize a pathway of polyclonal B cell development that begins with a small resting B cell and yields large numbers of proliferating

TABLE VII
Recombinant IL-1, -2, and -3 Do Not Mimic the Activity Found in EL-4 Sn

Stimuli	[^{3}H]TdR incorporation
	cpm × 10^{-3}
None	2.61 ± 0.19
LPS	88.57 ± 12.98
EL-4 Sn	50.13 ± 4.02
rIL-1 (100 U/ml)	4.73 ± 0.25
rIL-2 (100 U/ml)	6.01 ± 1.37
rIL-3 (100 U/ml)	7.57 ± 1.03
rIL-1 + 2 + 3	10.26 ± 0.56

72-h anti-Ig blasts were prepared as described in Materials and Methods and recultured at 10^5 cells/well for 15 h. [^{3}H]Thymidine was added at 7–15 h.

lymphoblasts that secrete IgM. The response to Sepharose–anti-Ig proceeds in two phases. The formation of large, Ia-rich blasts, which appears to be T independent, followed by proliferation and differentiation that is dependent on factors released by PMA-activated EL-4 thymoma cells. The lymphoblasts described in this study provide an excellent model for characterizing the factors that mediate T-dependent, B cell development.

The Formation of Lymphoblasts in Response to Anti-Ig. One purpose of these studies was to define the conditions necessary to generate lymphokine-responsive blasts from small resting B cells. Sepharose–anti-Ig, –anti-μ, or –anti-δ can all be used, the cell suspension can be depleted of most macrophages and dendritic cells by passage over Sephadex G10, and high-density resting B cells are as responsive as bulk, T-depleted spleen cells (Table II). It is possible that the signal delivered by Sepharose–anti-Ig differs from that provided by most soluble, T-dependent antigens. The two stimulation pathways do seem to differ since Sepharose–anti-Ig induces lymphoblasts that are responsive to lymphokines, while a period of direct B–T cell contact is required for hapten-carrier conjugates to induce responsiveness to lymphokines (23).

We have been unable to identify a major role for the T cell product BSF-1 or IL-4 in the Sepharose–anti-Ig response. IL-4 was discovered on the basis of two biological activities: as a costimulant for enhanced B cell proliferative responses to anti-Ig (7) and as a factor that increased switching of LPS-stimulated B cells to IgG1 (24). Our data demonstrate that lymphokine-responsive blasts can be produced and will continue to proliferate in the apparent absence of IL-4. There are some differences between our experiments and those suggesting a necessary role of IL-4 in anti-Ig signaling. Other studies (7, 25) have relied on soluble anti-Ig, which probably differs from Sepharose–anti-Ig in its activation of B cells. For example, Mond et al. (26) found that stimulation of B cells with soluble anti-Ig was inhibited by IFN-γ, whereas that induced by insoluble anti-Ig was not. The advantage to using Sepharose–anti-Ig is that it can be effectively removed from the blasts before reculturing. We also performed our initial cultures at higher cell concentrations (1–2 × 10^6 cells/ml), whereas lower cell concentrations have been used to assess BSF-1 effects (25). However, we obtain marked responses to

Sepharose–anti-Ig in low-density microcultures in the presence of monoclonal anti-BSF-1 (data not shown). Finally, although the phenotype of our initial B cells provides no evidence for previous activation in situ (high-buoyant density, small size, low levels of surface Ia), it is possible that the lymphocytes we studied have had prior contact with BSF-1.

Characteristics of Lymphoblast Response to LPS and to Lymphokines. The B blast that is induced by Sepharose–anti-Ig is poised to synthesize DNA. Within 6–12 h of applying LPS or El-4 Sn, [^{3}H]TdR uptake and cell recoveries increase. Comparable responses only begin between 24 and 48 h with small resting B cells.

In contrast to the proliferative response to LPS and EL-4 Sn, IgM secretion by anti-Ig blasts begins at about the same time (48 h) as it does with small B cells. This suggests that the entry of the lymphoblast into the G_1 phase is not sufficient to enable prompt IgM secretion. One or more cell divisions seem to be required. We have previously noted that IgM secretion by normal and tumor B cells is reduced when the cells are inhibited from passage through S phase (22).

More detailed cell cycle analyses, as with RNA and DNA binding dyes and flow cytometry, will be required to accurately determine the proportion of anti-Ig blasts that continue through the cell cycle. Nevertheless, it is clear that a substantially greater fraction of anti-Ig blasts respond to LPS than do bulk spleen B cells. At least 50% of the B blasts respond to LPS as opposed to a maximum of 10–30% of fresh spleen B cells under optimal conditions (27, 28). Specifically, we observed ≥150% recovery of anti-Ig blasts cultured overnight with either LPS or EL-4 Sn. Since 24 h is only enough time to permit cells to pass through one cell cycle, the majority of the blasts (≥50) must have responded. In preliminary autoradiographic studies, we have noted that all nuclei from anti-Ig blast cultures become labeled with [^{3}H]TdR after 24 h (data not shown). In addition, the vast majority of the blasts are producing high levels of IgM after a 3-d stimulation with EL-4 Sn (Fig. 3).

Identity of the Lymphokine(s) that Influence the Anti-Ig Blast. Recombinant IL-1, -2, -3, and -4 (alone or in various combinations) induce little or no proliferative activity or IgM release from anti-Ig blasts. In contrast, EL-4 Sn is a potent stimulus. The active material(s) in the EL-4 Sn migrates as a broad peak around 50 kD on gel filtration (data not shown). Similar findings have been reported by Muller et al. (16), who suggested that the factor was similar to the lymphokine that costimulates normal B cells with dextran sulfate and was called B cell growth factor II (BCGF-II) by Swain and coworkers (29, 30). Given the magnitude and apparent homogeneity of the anti-Ig blast response, it should be feasible to further characterize the EL-4 Sn and to test new factors derived by molecular cloning, including the BCGF-II–like gene (31).

Summary

Sepharose–anti-Ig and purified populations of small, high-density B cells have been used to study the formation and function of B lymphoblasts. Sepharose–anti-Ig converts small, Ia-poor B cells with a high-buoyant density to large, Ia-rich, B blasts with a low-buoyant density. We find that this response proceeds efficiently in the absence of IL-4 (BSF-1) as well as most T cells, macrophages, and dendritic cells. Further development of the blasts requires an additional

stimulus, such as LPS or the conditioned medium of stimulated EL-4 thymoma cells. Within 6 h, blasts begin to enter S phase and within 24 h most divide. At later times (48–72 h) most of the blasts are actively secreting IgM. Recombinant IL-1, -2, -3, and -4 have little or no effect on the B blasts, and a neutralizing mAb to IL-4 does not block the response to EL-4 Sn. We conclude that Sepharose–anti-Ig induces B cell blastogenesis in a T-independent fashion and that these blasts represent a highly enriched population of cells that respond to distinct, T cell–derived lymphokines.

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