INDEPENDENT REGULATION OF IgM, IgD, AND Ia ANTIGEN EXPRESSION IN CULTURED IMMATURE B LYMPHOCYTES

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Newly-formed B cells found in the peripheral lymphoid organs are thought to be derived primarily from bone marrow pre-B cells (reviewed in 1). The pre-B cells express cytoplasmic μ chains, but are negative for the mature B-cell markers IgM, IgD, and Ia (2–4). We have been interested in the mechanisms that regulate Ia antigen expression. Expression of Ia by macrophages is under the control of a lymphokine derived from activated T cells, IFN-γ (5, 6), and our goal has been to determine what signals may control Ia expression in developing B cells. Early studies indicated that Ia could be induced on a fraction of bone marrow cells by cAMP, LPS or a number of other agents (7, 8). However, these analyses relied on cytotoxicity assays on impure cell populations, and it was not possible to assess precisely which cells were expressing Ia. While another study has implicated factors elaborated by adherent cells in the maturation of pre-B cells into more differentiated Ia-bearing cells (9), it is not certain whether the induction of B cell surface markers is a direct or indirect effect due to factors elaborated by auxiliary cells.

Also still unsettled is the relative timing of appearance of surface IgM, IgD, and Ia antigens. Lala et al. (10) have shown that nascent bone marrow B cells acquire Ia and IgM concomitantly. On the other hand, Kearney et al. (11) have shown that a percentage of IgM^+ B cells in neonatal animals lacks Ia and IgD. Others have been less successful in demonstrating such a population of cells (12). Thus it has been unclear whether the expression of Ia and Ig by pre-B cells is coordinately regulated.

Recently (13) it has become possible to maintain in vitro cultures of bone marrow that contain both pre-B and B cells. We have examined such long-term bone marrow cultures for a number of cell surface antigens using the FACS. We find that the cells present in such cultures are pre-B cells and B cell–like, based on the expression of the markers IgM, B-220, Lyb-2.1 and ThB, suggesting that the pre-B cells in this culture system might provide a good source of cells with which to study the appearance of IgM, IgD, and Ia expression. By the use of two-color FACS, we have been able to place bone marrow pre-B cells directly in culture. The cells rapidly expand in vitro, providing enriched populations of pre-B cells that can be examined without the delay associated with the typical

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Whitlock culture (13) starting with total bone marrow. The results of this study show clearly that the signals leading to Ig and Ia expression are separable. Further, the long-term cultures of bone marrow generate an IgM^+, Ia^-, IgD^- cell whose phenotype closely matches that found on a population of immature B cells in the spleens of young mice. Finally, we find that PMA provides a stimulus for IgD expression on these immature B cells.

Materials and Methods

Animals. BALB/c (Simonsen Laboratories, Gilroy, CA), AKR and DBA/2 (The Jackson Laboratory, Bar Harbor, ME) mice were maintained in our animal facility.

Reagents. Reagents were obtained from the following suppliers: LPS (*E. coli* 055:B5; Difco Laboratories, Detroit, MI), Dextran sulfate (Gibco, Grand Island, NY), Con A, PMA, 5′-azacytidine and A23187 (Sigma Chemical Co., St. Louis, MO), FITC (Molecular Probes, Roseville, MN), Texas Red (Jackson Immunoresearch, Avondale, PA), propidium iodide (Calbiochem Behring, San Diego, CA), and biotin succinimide ester (Biosearch, San Rafael, CA). IFN-γ (recombinant) was generously provided by Genentech, Inc. (South San Francisco, CA). NZB serum was obtained by bleeding 4-wk-old NZB mice purchased from The Jackson Laboratory.

Monoclonal Antibodies. The following mAb were used in this study: MK-D6 (anti-Ia^d) (14), 10-3.6 (anti-Ia^k) (15), 10-2.16 (anti-Ia^k) (15), 14-4-4 (anti-I-E) (16), 34-1-2 (anti-H-2^d) (17), 331.12 (anti-μ) (18), 10-4.22 (anti-δ) (15), 187.1 (anti-κ) (19), H49-4.3 (anti-ThB) (20), 14.8 (anti-B220) (3), M1/70.15.11.5 (anti-MAC 1) (21), and 10-1.D.2 (anti-Lyb-2.1) (22). The mAb AF3-12.1 was kindly provided by Dr. A. Stall (Dept. of Genetics, Stanford Univ., Stanford, CA). This antibody reacts with murine H-2^k (H-2K.23) but is nonreactive with *d, f, j, p, q, r, s, u,* and *v* haplotypes (A. Stall, unpublished observations). All protein A–binding antibodies were purified from culture supernatants using protein A affinity chromatography (23). Other antibodies were purified by ammonium sulfate fractionation followed by DEAE-Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden) chromatography (24). Antibodies were fluorescein-conjugated using FITC (25). Two types of indirect staining were employed for Texas Red immunofluorescence. The first consisted of biotinylation of antibodies followed by incubation with Texas Red–labeled avidin. The second consisted of haptenation of antibodies with Pan-f (Pan-Ab Laboratories, San Jose, CA) followed by staining with Texas Red–coupled anti-Pan-f antibody. Texas Red coupling to avidin and to anti-Pan-f antibody was performed according to the method of Titus and coworkers (26). Biotinylation of the mAb was carried out according to the supplier's instructions. Antibodies were haptenated with Pan-f by incubating 1–2 mg antibody in 1.0 ml PBS with 0.1 ml of 1.0 M $NaHCO_3$ (pH 9.5) and 0.2 ml of Pan-f (10 mg/ml in water) for 3 h at 37 C. After incubation, the antibodies were dialyzed overnight against PBS.

Cell Lines. The following cell lines were used to obtain culture supernatants: WEHI-3 (27), P338D1 (28), RAW 264 (29), and FS6-14.13 (30). FS6-14.13 was kindly provided by Dr. A. Zlotnick (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA). BMM-1 is an adherent MAC-1^+ cell line obtained from bone marrow (J. R. Dasch and P. P. Jones, unpublished observations). The supernatants were obtained by removing cells by centrifugation after 24 h in culture.

Bone Marrow Isolation. Bone marrow cultures were initiated by the method of Whitlock and Witte (13). Femurs were removed from mice asceptically. Bone marrow cells were obtained by flushing femurs with RPMI 1640 (Gibco) containing 5×10^{-5} M 2-ME and 5% newborn calf serum (NCS) with a 25-gauge needle attached to a 5-ml syringe. Cells were initially plated in 5 ml at 10^6 cells/ml or 3.5×10^5 cells/ml in RPMI 1640 Plus 2-ME and 5% FCS in 60-cm culture plates. Lots 310625 and 503259 FCS from Irvine Scientific (Santa Ana, CA) were used in this study.

Sorted Pre-B Cells. Bone marrow cells were collected, washed, and stained with optimal amounts of FITC–anti-μ and biotin–anti-ThB for 20 min at 4°C. The cells were washed, resuspended with Texas Red–labeled avidin, and incubated an additional 20 min at 4°C.

Propidium iodide (1 μg/ml) was included for the last 5 min of incubation to stain dead cells (31). The cells were then washed and sorted. Cells stained with Texas Red–labeled ThB but negative for FITC–anti-μ were collected (see below). Generally, 10,000–15,000 ThB^+, IgM^- sorted cells in RPMI with 2-ME and 15% FCS were added to a feeder layer previously established from bone marrow adherent cells. The feeder layers were prepared according to the method of Whitlock and Witte (13) by plating bone marrow at 3.5×10^5 cells/ml at least 3 wk before adding sorted cells. At this concentration of cells, there is rarely outgrowth of nonadherent lymphoid cells.

Two-color Immunofluorescence. These analyses were carried out using a modified dual laser FACS II (Becton Dickinson Immunocytometry Systems, Sunnyvale, CA) in the FACS Facility directed by Dr. L. Herzenberg, Department of Genetics, Stanford University. An argon ion laser was used to excite fluorescein (488 nm), while a rhodamine 6G dye laser was used to excite Texas Red (605 nm). This instrument is equipped with logarithmic amplifiers for the fluorescence channels to measure light scatter and antibody-fluorochromes bound to cells. In addition, propidium iodide (measured in a third detector) was used to eliminate dead cells that are not eliminated by light scatter. A VAX II/780 computer was used to collect and store individual measurements on 10,000 cells as list mode data for later analysis. Data on both forward angle and 90° (obtuse) light scatter were collected on the computer. The use of this FACS system is described in detail elsewhere (32).

FACS Data Analysis. The computer programs for these analyses were developed by Mr. Wayne Moore in the Dept. of Genetics, Stanford Medical School. Two-color staining data are presented as contour-type plots that are representations of three-dimensional surfaces. Cells with varying levels of red and green fluorescence are located on a 64×64 grid. The frequency of cells at any location on the grid defines an elevation at that location. Like contour maps, contour lines were drawn to represent equal step changes in the elevation (frequency) (33). The analyses in this study were gated to remove dead (propidium iodide–stained) cells, and scatter-gated to remove large cells.

Results

Long-term Cultures Express Pre-B and B Cell Antigens. We have established a number of long-term Whitlock bone marrow cultures to characterize their cell surface phenotype more completely. Fig. 1 summarizes the immunofluorescence staining profiles of seven of these cultures examined 4 wk after the cultures were initiated. Actual staining profiles are presented below in Fig. 3. These cultures were stained with FITC-labeled mAb against μ H chains, Ia, B-220, and ThB. The cultures clearly contain both ThB^+ and $B\text{-}220^+$ cells. In addition, up to 15% of the cells were stained by anti–μ chain antibodies. However, the cultures were uniformly negative for expression of Ia antigens. In a total of 20 independent cultures derived from mice of three strains and examined at a variety of time points, we have never detected appreciable numbers of Ia^+ cells in culture. The cultured cells strongly express MHC class I antigens (data not presented). Thus, in agreement with others (13), we can describe these cultures as containing both pre-B ($B\text{-}220^+$, ThB^+, IgM^-) and B cells (IgM^+). The expression of IgM has been confirmed by staining with both anti-κ and heterologous anti-μ antibody reagents (data not presented).

Sorted Pre-B cells Can Be Placed Directly into Culture. In an effort to increase the frequency of B cells within the culture system, we have established long-term cultures by directly sorting pre-B cells from bone marrow and placing them in tissue culture. Others (34) have found that purified pre-B cells survive poorly in culture. We found, however, that when placed in culture on previously established adherent cell layers, the pre-B cells undergo considerable expansion

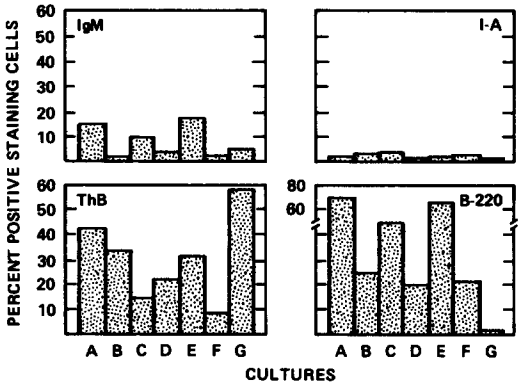

FIGURE 1. Long-term cultures express pre-B and B cell markers. BALB/c-derived long-term cultures were initiated by the method of Whitlock and Witte (13). Nonadherent cells were removed from the plates for analysis 4 wk after the initiation of the cultures. Each culture represents an independently-arising population of cells. The cells were labeled with either fluorescein- or hapten (Pan-f)-coupled antibodies. Haptenated antibodies were visualized with Texas Red–coupled antihapten antibody. Antibodies used were 331.12 (anti-μ), MK-D6 (anti-Ia^d), 14.8 (anti-B220), and H49-4.3 (anti-ThB). Negative control antibodies were 10-2.16 (anti-Ia^k) and M1/70 (anti-MAC 1).

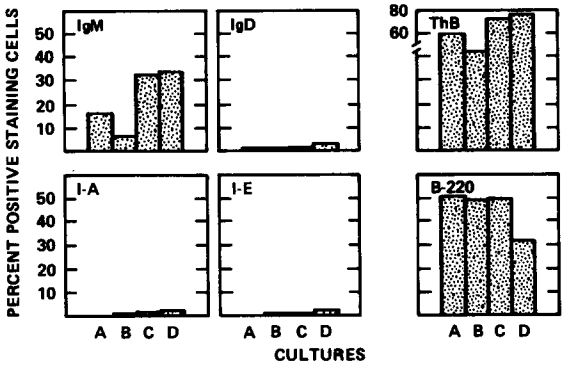

FIGURE 2. Long-term cultures derived from sorted pre-B cells show enhanced expression of B cell markers. Cultures were initiated with cells isolated by two-color FACS sorting of a population of bone marrow cells bearing the ThB antigen but lacking sIg. Culture *A* had been in culture for ~3 mo before this analysis while cultures *B–D* were analyzed 2 wk after sorting. Staining was carried out with the mAb described in Fig. 1. In addition two other antibodies were used: 14-4-4 (anti-I-E) and 10-4.22 (anti-δ).

without the lag associated with Whitlock cultures. Typically, cultures were established in 1 wk, vs. 3 wk for Whitlock cultures. The difference in rate reflects the need to establish an adherent layer before the initiation of lymphoid cell growth and the presence of increased numbers of cells capable of in vitro proliferation in the sorted population.

Fig. 2 shows the phenotypic characterization of four cultures of cells maintained in vitro for at least 2 wk after two-color immunofluorescent sorting. Cells were sorted on the basis of expression of ThB but absence of sIgM. These ThB^+, sIg^- cells were >90% small lymphocytes by light scatter criteria. The cultured cells arising from the sorted cells appear as small lymphoid cells both morphologically and by light scatter. When examined 2 wk after sorting, several cultures

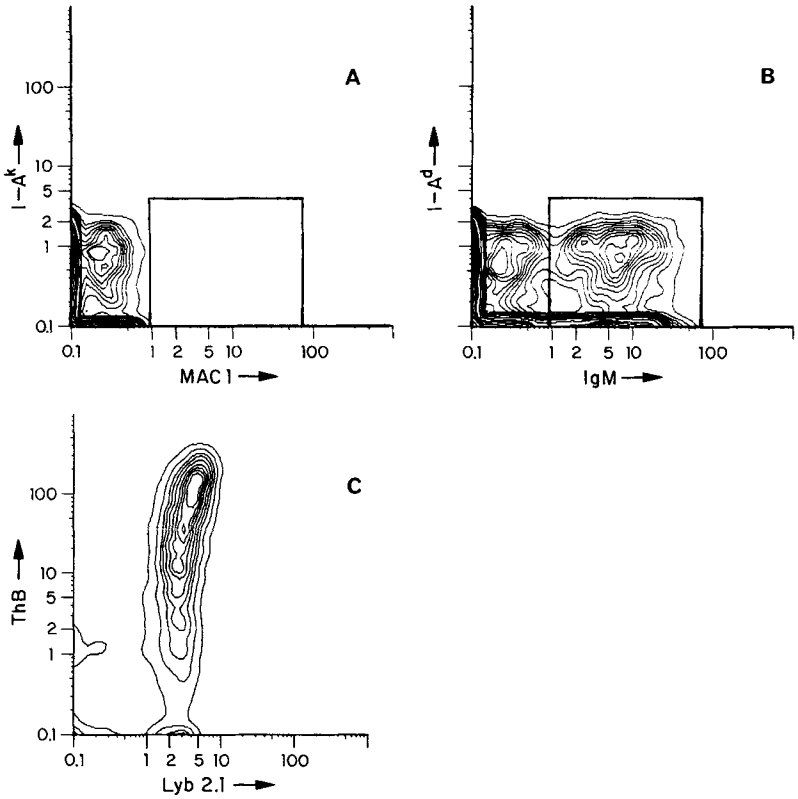

FIGURE 3. Two-color analysis of a typical long-term culture. Long-term bone marrow cultures of pre-B cells derived by sorting were stained with three combinations of antibodies. The cells in *A* and *B* were from BALB/c cultures harvested 2 wk after sorting. The cells in *C* were taken from DBA/2 cultures 3 wk after sorting. Antibodies used were same as in Figs. 1 and 2. Lyb-2.1 was detected using FITC-labeled 10-1.D.2 antibody. Increasing Texas Red and fluorescein staining are shown on the *y* and *x* axes, respectively.

contained an average of 56% (42–79%) cytoplasmic μ chain–positive cells (data not presented) confirming their classification as pre-B cells. When they were placed in culture, the sorted cells were initially surface Ig (sIg)[1]-negative. However, as can be seen in Fig. 2, each culture contained substantial numbers of IgM^+ cells, which appear by day 7 of culture. Thus, these cultures contain requisite elements allowing the acquisition of sIgM by pre-B cells. However, these cultures remained uniformly negative for both I-A and I-E Ia antigens, and IgD (Fig. 2).

The FACS analysis profiles of a typical culture obtained by two-color sorting are presented in Fig. 3, *A* and *B*. Cells were stained with combinations of haptenated and fluoresceinated antibodies. The binding of haptenated antibodies to the cells was visualized by addition of Texas Red–coupled antihapten antibody. Clearly a population of cells stained for IgM (Fig. 3*B*, boxed population) but there is no staining over background using anti-Ia^d.

The results presented in Figs. 2 and 3 are from cultures in which sorted cells

[1] *Abbreviation used in this paper:* sIg, surface Ig.

were plated on syngeneic feeder layers. Although we believed the lymphoid cells that grew out were derived from the sorted population, this was not proven. Additional sorts have been performed in which the sorted cells have been plated on feeder layers derived from allogeneic mice or mice that differ in Ig H chain allotypes or Lyb-2. The resulting long-term cultures contain lymphoid cells that express class I MHC products or the alloantigen appropriate to the sorted cells. These cultures were similar to the syngeneic cultures in that they express IgM but not Ia or IgD. In the course of these analyses, we found that, in addition to the other B cell markers, the long-term cultures derived from DBA/2 mice express the alloantigen Lyb-2.1 (Fig. 3*C*). In three cultures derived by two-color sorting, an average of 93% of the cells were found to stain for Lyb-2.1. The contour plots show that, although most of the cells stain for both ThB and Lyb-2.1, the staining patterns for each antigen are quite distinct.

A Population of Cells in Young Spleen Expresses IgM but Not Ia or IgD. Some question remains about the relative timing of Ia and Ig expression in vivo (10–12). Therefore, we reinvestigated this question using two-color FACS analysis. Fig. 4 shows two-color FACS analysis in which splenic cells from either 2-wk-old mice or adult mice were simultaneously stained with antibodies against Ia and IgM or Ia and IgD. As reported previously (35, 36), adult sIg^+ cells are found to also express Ia. In contrast, in the young spleen, a sizable population of cells that express IgM appear to lack expression of Ia (Fig. 4, boxed population). The percentage of B cells in 2-wk spleen is 36%. ~31% of the B cells appear to lack Ia in 2-wk spleen. Virtually all cells that express IgD do express Ia. Thus it can be shown that normal 2-wk-old animals have a population of IgM^+, Ia^-, IgD^- spleen cells, which is lost in adult animals. It seems plausible to suggest that the long-term cultures may contain cells arrested at a similar stage of differentiation.

Long-term Cultures Derived from Young and Adult Bone Marrow Express the IgM^+, Ia^- Phenotype. We thought it possible that the pre-B and B cells with in vitro growth potential might be restricted to the early weeks of life. This was particularly of concern inasmuch as Whitlock cultures were always initiated with bone marrow derived from 3–5-wk-old mice (13, 37). Therefore, we investigated whether long-term cultures of pre-B and immature B cells could be initiated using cells derived from adult bone marrow. This was accomplished both by setting up Whitlock cultures with bone marrow taken from 12-wk animals, and by directly sorting adult pre-B cells onto feeder layers derived from 4-wk mice. Both of these strategies led to the establishment of long-term cultures of cells which are phenotypically indistinguishable from the cultures derived from cells of young mice (data not presented). The fact that the long-term cultures of adult bone marrow yield phenotypically similar cultures suggests that pre-B cells are able to undergo in vitro expansion and differentiation into IgM^+, Ia^-, IgD^- immature B cells regardless of age.

Induction of Ia and IgD Expression with Exogenous Agents. To investigate the regulation of Ia and IgD expression on immature B cells we have treated our long-term cultures with a number of agents that affect lymphocyte and macrophage differentiation (Table I). None of the agents induced Ia expression after 48 h of culture. Two of the inducers, IFN-γ and PMA, did have affects on other surface antigens on the cultured cells. Although it did not induce Ia expression

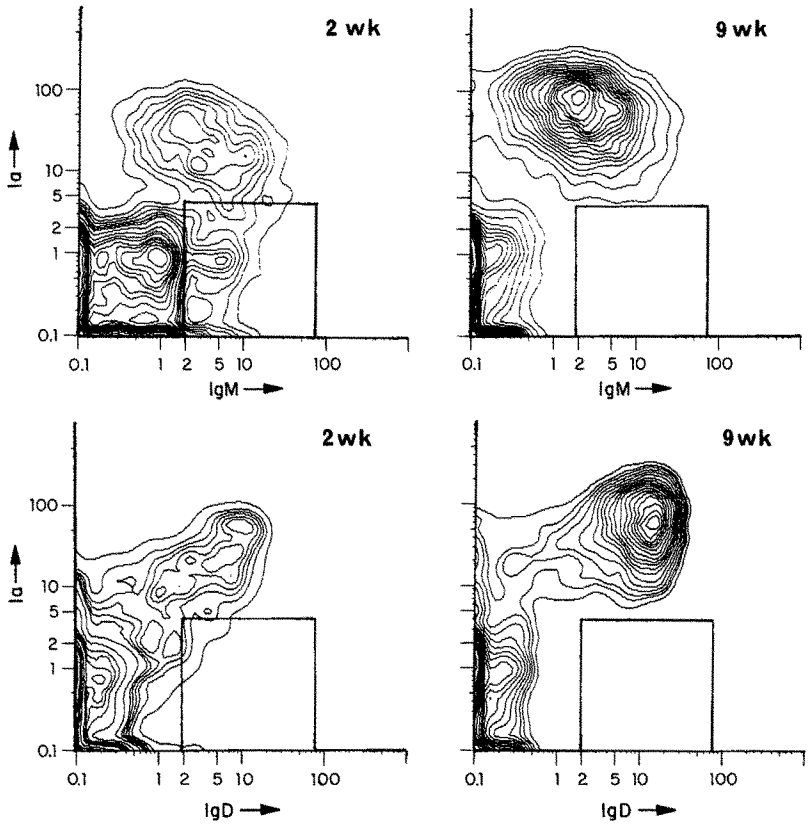

FIGURE 4. A population of splenic lymphocytes in young BALB/c mice expresses IgM but not Ia. Cells were simultaneously labeled for examination of Ia and IgM expression or Ia and IgD expression. In this experiment, cells were labeled with FITC–anti-μ or –anti-δ along with Pan-f haptenated anti-Ia^d (MK-D6). The hapten-coupled antibody was visualized with Texas Red–labeled antihapten antibody.

as it does on macrophages, IFN-γ caused a 30-channel increase in mean fluorescence of H-2 expression (data not presented). This represents a two-fold increase in H-2 expression. PMA appears to be a potent inducer of IgD expression (Fig. 5). While there is virtually no detectable IgD expression in the absence of PMA, addition of 10 ng/ml PMA caused a substantial number of the cells to express IgD. Based on the number of IgM expressing cells in the culture, 55–82% were found to express IgD following PMA treatment. Staining with anti-κ showed that the total number of Ig$^+$ cells was approximately equal to the number of IgM$^+$ cells (data not presented). This suggests that IgD expression is occurring on cells also expressing IgM. Expression of another surface marker, B-220, was not affected by PMA treatment (data not presented). Thus, the effect of PMA is specific for IgD expression, and is not a generalized phenomenon increasing expression of all surface antigens. The results of these experiments suggest that not only are the long-term cultured cells responsive to external stimuli, but that expression of Ia and IgD are separable events, responsive to different stimuli.

TABLE I
Summary of Agents Examined for Induction of Ia Expression on Long-term Cultured Cells

Agent	Concentration
Mitogens and activators	
LPS	0.01–100 μg/ml
Dextran sulfate	20 μg/ml
PMA*	1–500 ng/ml
A23187	0.5 μM
PMA and A23187	10 ng/ml and 0.5 μM
Anti-μ antibody	0.1–10 μg/ml
Anti-Lyb-2.1 antibody*	10–50 μg/ml
4-wk NZB serum	1:50, 1:200
Lymphokines	
IFN-γ*	20, 100 U/ml
BSF1 containing supernatant*‡	25%
T cells/supernatants	
Supernatant from Con A–activated spleen cells	20%
Con A (plus irradiated spleen cells)	10 μg/ml
Con A (plus irradiated thymocytes)	10 μg/ml
Macrophage supernatants	
WEHI-3	10%, 50%
P388D1	50%
RAW264	50%
BMM1	10–50%

* These reagents were also tested for inducing activity in combination with 5′-azacytidine (2.4 μM).
‡ Supernatant was obtained from overnight culture of the T cell hybridoma, FS6-14.13, in the presence of Con A.

Discussion

The molecular events underlying the differentiation of pre-B cells into functional B cells are largely unknown. The current studies are aimed at better defining the phenotypic and functional characteristics of bone marrow pre-B cells maintained in long-term culture. In agreement with earlier studies (13), we find that Whitlock cultures clearly contain B-lineage cells. In addition, we have not found T cell or macrophage markers on the nonadherent long-term cells (cells were negative for Thy-1, Ly-1, Lyt-2, MAC 2 and MAC 3; data not presented). Inasmuch as the cells lack Ly-1, they appear to be distinct from the recently described Ly-1$^+$ B cell subpopulation (38, 39). These long-term cells show characteristic staining patterns for various antigens, i.e., ThB expression is always quite heterogeneous while Lyb-2 and H-2 staining are quite homogeneous (Fig. 3*C*). It seems possible that the null cells present in the cultures represent earlier phenotypes in the B cell differentiation pathway.

In an effort to restrict the types of cells contained within the cultures, we have adopted a strategy whereby FACS-sorted pre-B cells are placed directly in culture. This yields cultures that contain increased numbers of IgM$^+$ cells that are >90% positive for the B-lineage marker Lyb-2.1 (22). Based on the ability of these

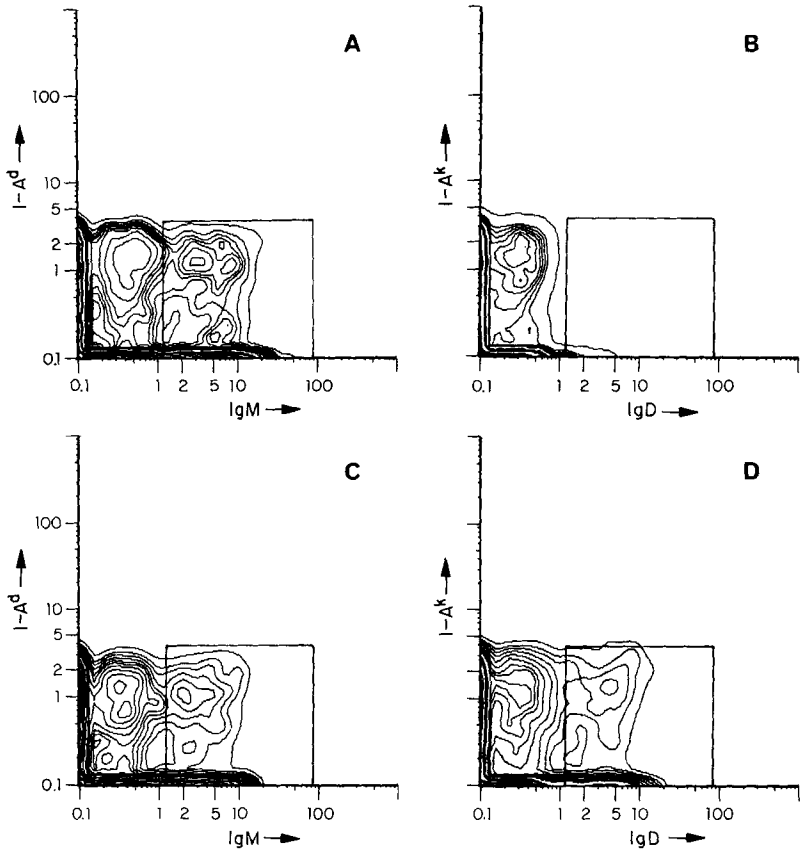

FIGURE 5. PMA induces IgD expression in cultured long-term bone marrow cultures. Cells were incubated for 2 d in the presence of 10 ng/ml PMA. They were then stained for IgM or IgD along with anti-Ia. The cells in this experiment were of BALB/c origin, so the anti-I-A^k antibody is a negative control. *A* and *B* depict a culture incubated without PMA, while *C* and *D* depict a culture incubated with PMA. In this experiment, 82% of the IgM-expressing cells became IgD^+ after PMA treatment.

sorted cells to undergo rapid expansion without the 3–4-wk lag associated with the Whitlock cultures, it seems likely that we are seeing direct expansion of pre-B cells.

Virtually all of the Ig^+ cells in the cultures have the IgM^+, Ia^-, and IgD^- phenotype. Although cells with a similar phenotype are not found in the spleen of adult mice, they can be found in immature mice. Inasmuch as cells similar to this population, which we call immature B cells, are demonstrable in vivo, we feel that these cultured cells represent a cell type that is trapped at a particular stage of their differentiation pathway. Our hope is that the cultured cells will be capable of further differentiation under the proper stimulus.

The functional capabilities of the long-term cultured cells are largely unexplored. It is known that a population of B cells found in immature mice, which expresses IgM but not IgD (40–42), can apparently, respond to both thymus-dependent (43, 44) and -independent antigens (41, 45). Whether IgD or Ia

antigens are expressed during the course of these antibody responses has not been ascertained. However, work by Kincade (46), suggests that neonatal δ^- cells do not acquire IgD in short-term culture. Further study should clarify whether Ia or IgD expression is a requisite part of the acquisition of responsiveness to thymus-dependent or -independent antigens.

Recently, Kurland et al. (47) and Nagasawa et al. (48) have been able to return long-term cultured cells to an in vivo environment. These transferred cells became LPS-responsive, and were capable of making a PFC response. Although the cells that reconstitute these responses are not completely characterized, these studies suggest that the long-term pre-B or immature B cells are capable of further maturation in vivo. Our results also show that the cells maintained in vitro are responsive to some external stimuli, PMA and IFN-γ.

The transcription of IgM and IgD in resting lymphocytes is the result of complex splicing of a long primary RNA transcript that encodes both μ and δ exons (49). In the case of cells expressing only IgM, there appears to be cleavage of the transcript at a specific site by an endonuclease (endase) (50). Several apparent sites where such an endase could cut have been identified (51) within the constant region genes for μ and δ. Blattner and Tucker (51) have hypothesized that by altering levels of the endase at different stages of differentiation, alternate mRNA species would be formed. In support of this hypothesis, they report that δ-specific RNA can be found in the nuclei of neonatal B cells despite the fact that these cells do not express IgD. This RNA could represent pieces of message clipped from a primary transcript by endase. Our observation that PMA dramatically increases IgD expression is of considerable interest with respect to this hypothesis. One might expect that the induction of IgD expression would be preceeded by a decrease in the activity of the putative endase allowing alternative transcripts to be generated.

PMA has been found to cause alterations in the phenotypic expression and differentiative state of a number of cell types (reviewed in 52). Among other effects on cells, PMA has been shown to activate the Ca^{++} and phospholipid-dependent protein kinase (kinase C) (53–55). The activated kinase C is thought to alter the phosphorylation state of regulatory molecules within the cell, thereby affecting the activation state of the cell. PMA has been reported to have several effects on mature B lymphocytes, including membrane depolarization and increasing the levels of expression of the Ia antigens (56). Our findings suggest that PMA, and hence kinase C, may also alter the processing of Ig H chain RNA transcripts in immature B cells. The lack of effect of PMA on Ia antigens in this culture system suggests that, although PMA can have effects on the level of Ia expression on mature cells, it does not trigger expression on pre-B cells.

Summary

Long-term cultured bone marrow cells were characterized with respect to a number of B and pre-B cell markers. Cells expressing ThB, B-220, and IgM were found within cultures set up according to the procedure of Whitlock and Witte (13). This culture system was modified by placing sorted pre-B cells (ThB^+, IgM^-) from bone marrow in culture with previously-established bone marrow adherent layers. These cultures commenced growth without the lag associated

with the Whitlock cultures. These cultured nonadherent cells show a high frequency of IgM^+ cells, but do not express either IgD or Ia, and we refer to them as immature B cells. Cells with a similar phenotype (IgM^+, Ia^-, IgD^-) are found within the spleens of young but not adult mice. The phorbol ester PMA induces expression of IgD on the cultured immature B cells, but has no effect on Ia expression. This suggests that the processing of H chain RNA transcripts may be affected by protein kinase C. These results demonstrate that the appearance of IgM, IgD, and Ia are independently controlled in long-term cultured B-lineage cells.

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References

1. Kincade, P. W. 1981. Formation of B lymphocytes in fetal and adult life. *J. Exp. Med.* 31:177.
2. Owen, J. J. T., M. D. Cooper, and M. C. Raff. 1974. In vitro generation of B lymphocytes in mouse foetal liver, a mammalian 'bursa equivalent'. *Nature (Lond.).* 249:361.
3. Kincade, P. W., G. Lee, T. Watanabe, L. Sun, and M. P. Scheid. 1981. Antigens displayed on murine B lymphocyte precursors. *J. Immunol.* 127:2262.
4. Abney, E. R., and R. M. E. Parkhouse. 1974. Candidate for immunoglobulin D present on murine B lymphocytes. *Nature (Lond.).* 252:600.
5. Steinman, R. M., N. Nogueira, M. P. Witmer, J. D. Tydings, and I. S. Mellman. 1980. Lymphokine enhances the expression and synthesis of Ia antigens on cultured mouse peritoneal macrophages. *J. Exp. Med.* 152:1248.
6. McNicholas, J. M., D. P. King, and P. P. Jones. 1983. Biosynthesis and expression of Ia and H-2 antigens on a macrophage cell line are stimulated by products of activated spleen cells. *J. Immunol.* 130:449.
7. Hammerling, U., A. R. Chin, J. Abbott, and M. P. Scheid. 1975. The ontogeny of murine B lymphocytes. I. Induction of phenotypic conversion of Ia^- to Ia^+ lymphocytes. *J. Immunol.* 115:1425.
8. Hammerling, U., A. R. Chin, and J. Abbott. 1976. Ontogeny of murine B lymphocytes: Sequence of B-cell differentiation from surface-immunoglobulin–negative precursors to plasma cells. *Nature (Lond.).* 73:2008.
9. Kincade, P. W., G. Lee, C. J. Paige, and M. P. Scheid. 1981. Cellular interactions affecting the maturation of murine B lymphocyte precursors in vitro. *J. Immunol.* 127:255.
10. Lala, P. K., G. T. Johnson, F. C. Battye, and G. T. V. Nossal. 1979. Maturation of B lymphocytes. I. Concurrent appearance of increasing Ig, Ia and mitogen responsiveness. *J. Immunol.* 122:334.
11. Kearney, J. F., M. D. Cooper, J. Klein, E. R. Abney, R. P. E. Parkhouse, and A. R. Lawton. 1977. Ontogeny of Ia and IgD on IgM-bearing B lymphocytes in mice. *J. Exp. Med.* 146:297.
12. Mond, J. J., S. Kessler, F. D. Finkelman, W. E. Paul, and I. Scher. 1980. Heterogeneity of Ia expression on normal B cells, neonatal B cells, and on cells from B cell–defective CBA/N mice. *J. Immunol.* 124:1675.

13. Whitlock, C. A., and O. N. Witte. 1982. Long term culture of B lymphocytes and their precursors from murine bone marrow. *Proc. Natl. Acad. Sci. USA.* 79:3608.
14. Kappler, J. W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen inducible, H-2 restricted, interleukin producing T cell hybridomas. *J. Exp. Med.* 153:1198.
15. Oi, V. T., P. P. Jones, J. W. Goding, L. A. Herzenberg, and L. A. Herzenberg. 1978. Properties of monoclonal antibodies to mouse Ig allotypes, H-2 and Ia antigens. *Curr. Top. Microbiol. Immunol.* 81:115.
16. Ozato, K., N. M. Mayer, and D. H. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* 124:533.
17. Ozato, K., N. M. Mayer, and D. H. Sachs. 1982. Monoclonal antibodies to mouse major histocompatibility complex antigens. *Transplantation (Baltimore).* 34:113.
18. Kincade, P. W., G. Lee, L. Sun, and T. Watanabe. 1981. Monoclonal rat antibodies to murine IgM determinants. *J. Immunol. Methods.* 42:17.
19. Yelton, D. E., C. Desaymard, and M. E. Scharff. 1981. Use of monoclonal anti-mouse immunoglobulin to detect mouse antibodies. *Hybridoma.* 1:5.
20. Eckhardt, L. A., and L. A. Herzenberg. 1980. Monoclonal antibodies to ThB detect close linkage of Ly-6 and a gene regulating ThB expression. *Immunogenetics.* 11:275.
21. Springer, T., G. Galfre, D. S. Secher, and C. Milstein. 1978. Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigens. *Eur. J. Immunol.* 8:539.
22. Subbarao, B., and D. E. Mosier. 1983. Induction of B lymphocyte proliferation by monoclonal anti-Lyb 2.1 antibody. *J. Immunol.* 130:2033.
23. Goding, J. W. 1978. Use of staphlococcal protein A as an immunological reagent. *J. Immunol. Methods.* 42:17.
24. Melgyesi, G. A., G. Fust, J. Gergely, and H. Bazin. 1978. Classes and subclasses of rat immunoglobulins: interaction with the complement system and staphlococcal protein A. *Immunochemistry.* 15:125.
25. Goding, J. W. 1976. Conjugation of antibodies with fluorochromes. Modification to standard methods. *J. Immunol. Methods.* 13:215.
26. Titus, J. F., R. Haugland, S. O. Sharrow, and D. M. Segal. 1982. Texas Red, a hydrophilic, red-emitting fluorophore for use with fluorescein in dual parameter flow microfluorometer and fluorescence microscopic studies. *J. Immunol. Methods.* 50:193.
27. Warner, N. C., M. A. S. Moore, and D. J. Metcalf. 1969. A transplantable myelomonocytic leukemia in BALB/c mice: cytology, karyotype, and muramidase content. *J. Natl. Cancer Inst.* 43:963.
28. Karen, H., B. Handwerger, and J. Wunderlich. 1975. Identification of macrophage-like characteristics in a cultured murine tumor line. *J. Immunol.* 113:894.
29. Ralph, P., and I. Nakoinz. 1977. Antibody-dependent killing of erythrocyte and tumor targets by macrophage-related cell lines: enhancement by PPD and LPS. *J. Immunol.* 119:950.
30. Leibson, H. J., P. Marrack, and J. W. Kappler. 1981. B cell helper factors. I. Requirement for both interleukin 2 and another 40,000 mol wt factor. *J. Exp. Med.* 154:1681.
31. Yeh, C.-J. G., B.-L. Hsi, and W. P. Faulk. 1981. Propidium iodide as a nuclear marker in immunofluorescence. II. Use with cellular identification and viability studies. *J. Immunol. Methods.* 43:269.
32. Parks, D. R., and L. A. Herzenberg. 1984. Fluorescence activated cell sorting: theory, experimental optimization and applications in lymphoid cell biology. *Methods Enzymol.* 108:197.
33. Hardy, R. R., K. Hayakawa, D. R. Parks, L. A. Herzenberg, and L. A. Herzenberg. 1984. Murine B cell differentiation antigens. *J. Exp. Med.* 159:1169.

34. Coffman, R. J. 1982. Surface antigen expression and immunoglobulin gene rearrangement during mouse pre-B cell development. *Immunol. Rev.* 69:5.
35. Goding, J. W., G. J. V. Nossal, D. C. Shreffler, and J. J. Marchalonis. 1975. Ia antigens on murine lymphoid cells: distribution, surface movement and partial characterization. *J. Immunogenet.* 2:9.
36. Hammerling, G. 1976. Tissue distribution of Ia antigens and their expression on lymphocyte subpopulations. *Transplant. Rev.* 30:64.
37. Whitlock, C. A., D. Robertson, and O. N. Witte. 1984. Murine B cell lymphopoiesis in long term culture. *J. Immunol. Methods.* 67:353.
38. Manohar, V., E. Brown, W. M. Leiserson, and T. M. Chused. 1982. Expression of Lyt-1 by a subset of B lymphocytes. *J. Immunol.* 129:532.
39. Hayakawa, K., R. R. Hardy, D. R. Parks, L. A. Herzenberg. 1983. The "Ly-1 B" subpopulation in normal, immunodefective, and autoimmune mice. *J. Exp. Med.* 157:202.
40. Goodman, S. A., E. S. Vitetta, U. Melcher, and J. W. Uhr. 1975. Cell surface immunoglobulin. XIII. Distribution of IgM- and IgD-like molecules on small and large cells of mouse spleen. *J. Immunol.* 114:1646.
41. Layton, J. E., B. L. Pike, F. L. Pattye, and G. J. V. Nossal. 1979. Cloning of B cells positive or negative for surface IgD. I. Triggering and tolerance in T-independent systems. *J. Immunol.* 123:709.
42. Marshall-Clarke, S., K. D. Keeler, and M. E. Parkhouse. 1983. The expression of surface IgD on B cells responsive to thymus-independent and thymus-dependent antigens and its requirement for B cell triggering. *Immunology.* 48:393.
43. Zan-Bar, I., E. S. Vitetta, F. Assisi, and S. Strober. 1978. The relationship between surface immunoglobulin isotype and immune function of murine B lymphocytes. III. Expression of a single predominant isotype on primed and unprimed B cells. *J. Immunol.* 147:1374.
44. Mosier, D. E., J. J. Mond, and E. A. Goldings. 1977. The ontogeny of thymic-independent antibody responses in vitro in normal mice and mice with an X-linked B lymphocyte defect. *J. Immunol.* 119:1874.
45. Waldschmidt, T. J., J. E. Layton, S. F. McFadden, J. W. Uhr, and E. S. Vitetta. 1985. $sIgD^-$ and $sIgD^+$ B cells responding to thymus-independent antigens have a different lymphokine requirement. *J. Immunol.* 135:865.
46. Kincade, P. W., K. S. Landreth, and G. Lee. 1982. Apparent differences in B-lineage differentiation occurring in fetal and adult life. *Ann. N.Y. Acad. Sci.* 399:296.
47. Kurland, J. I., S. F. Ziegler, and O. N. Witte. 1984. Long-term cultured B lymphocytes and their precursors reconstitute the B-lymphocyte lineage in vivo. *Proc. Natl. Acad. Sci. USA.* 81:7554.
48. Nagasawa, R., O. Kanagawa, T. V. Tittle, and J. M. Chiller. 1985. In vivo maturation of pre-B cells derived from long-term cultured bone marrow. *J. Immunol.* 135:965.
49. Liu, C. P., P. W. Tucker, J. F. Mushinski, and F. P. Blattner. 1980. Mapping of heavy chain genes for mouse Immunoglobulins M and D. *Science (Wash. DC).* 209:1348.
50. Nelson, K. J., J. Haimovich, and R. P. Perry. 1983. Characterization of productive and sterile transcripts from the immunoglobulin heavy-chain locus: processing of μm and μs mRNA. *Mol. Cell. Biol.* 3:1317.
51. Blattner, F. R., and P. W. Tucker. 1984. The molecular biology of immunoglobulin D. *Nature (Lond.).* 307:417.
52. Nishizuka, A. 1984. The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature (Lond.).* 308:693.
53. Castigna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. 1982.

Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* 257:7847.

54. Kraft, A. J., and W. B. Anderson. 1983. Phorbol esters increase the amount of Ca^{++}, phospholipid-dependent protein kinase associated with plasma membrane. *Nature (Lond.).* 301:621.
55. Niedel, J. E., L. J. Kuhn, and G. R. Vandenbark. 1983. Phorbol diester receptor copurifies with protein kinase C. *Proc. Natl. Acad. Sci. USA.* 80:36.
56. Monroe, J. G., J. E. Niedel, and J. C. Cambier. 1984. B cell activation. IV. Induction of cell membrane depolarization and hyper-I-A expression by phorbol diesters suggests a role for protein kinase C in murine lymphocyte activation. *J. Immunol.* 132:1472.