

MECHANISMS REGULATING IgA CLASS-SPECIFIC
IMMUNOGLOBULIN PRODUCTION IN MURINE GUT-
ASSOCIATED LYMPHOID TISSUES

II. Terminal Differentiation of Postswitch sIgA-Bearing Peyer's Patch B
Cells

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It is now well established that Peyer's patches (PP)¹ are lymphoid follicles that give rise to cells that are committed to IgA development and that ultimately localize in mucosal areas (1–5). However, the mechanisms governing such commitment are not completely understood. On the one hand, PP B cells and their clonal progeny could represent a class of B cells that secrete IgA, because they are exposed to a particular kind and pattern of specific (antigenic) and/or nonspecific (T cell and/or B cell) stimuli (6, 7); on the other hand, IgA B cells may develop in PP because PP contain Ig class-specific PP T cells that direct PP B cells along certain lines of differentiation (8, 9). Recently, we have provided additional evidence favoring the latter possibility, in that we have obtained T cell clones from PP which are capable of influencing PP B cell differentiation so that it follows a pathway that leads directly from IgM expression to IgA expression (10, 11). However, these IgA-specific T cells (switch T cells) cannot in themselves provide help for terminal differentiation of PP B cells since the effect of the switch T cells is to induce cells to become surface IgA-bearing B cells rather than plasma cells secreting IgA.

Newer information regarding immunologic mechanisms governing B cell differentiation of all Ig classes are of relevance to IgA B cell differentiation. In this regard, there are extensive data which indicate that T cells or T cell-derived factors, and/or macrophages or macrophage-derived factors induced by specific and nonspecific stimuli can profoundly affect the proliferation and differentiation of T and/or B cells (12–26). These cells and the factors derived from these cells appear to act on the differentiation of certain or all B cells (3, 4, 13–15, 18, 20, 21, 23, 25, 27); the factor in this case is called T cell replacing factor (TRF) (19, 20, 23) or B cell differentiation factor (BCDF) (27). However, the factor involved in the terminal differentiation of B cells to Ig-producing cells is different from

¹ *Abbreviations used in this paper:* BCDF, B cell differentiation factor; BCPF, B cell proliferation factor; cIg, cytoplasmic Ig; Con A, concanavalin A; GALT, gut-associated lymphoid tissue(s); Ig, immunoglobulin; IL-1, interleukin 1; IL-2, interleukin 2; LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; MF, macrophage factor; MLN, mesenteric lymph nodes; PP, Peyer's patches; sIg, surface Ig; TRF, T cell replacing factor.

that involved in the proliferation of B cells; the latter factor has been named B cell growth factor (BCGF) (19) or B cell proliferating factor (BCPF) (27).

In the studies reported here we have focused on the terminal maturation of sIgA-bearing PP B cells that develop following exposure to switch T cells. More particularly, we have asked whether or not this differentiation is governed by other types of regulatory cells, such as helper T cells and/or macrophages, or factors produced by such cells. Our results indicate that the predominant line of development followed by sIgA-bearing PP B cells as they differentiate into IgA-secreting plasma cells involves a two-step process. The first step of this process involves at least two signals, one a signal leading to activation and proliferation of B cells provided by mitogen, and the other, a class-specific switch signal provided by a switch T cell (10, 11); the second step of this process involves postswitch B cells and is mediated by helper T cells and/or macrophages, as well as factors produced by these cells.

Materials and Methods

Animals. 8–16-wk-old female BALB/c mice and 8–10-mo-old Sprague-Dawley female rats were purchased from The Jackson Laboratory, Bar Harbor, ME.

Preparation and Fractionation of Single Cell Suspensions. Unseparated T and B cell suspensions were prepared from PP, mesenteric lymph nodes (MLN), spleen, and thymus as previously described (10).

Anti-Lyt-2 and Complement Treatment. In some experiments, T cells derived from MLN and spleen were enriched for a Lyt-1⁺ subset by pretreatment with anti-Lyt-2.2 allosera (1:20 dilution) (Cedarlane Laboratories, Ltd., Ontario, Canada) and "low-tox" rabbit complement (1:8 dilution) (Accurate Chemical and Scientific Co., Hicksville, NY), as previously reported (4, 10). Dead Lyt-2⁺ T cells were removed on a Ficoll-Hypaque gradient; the Lyt-1⁺ T cell-enriched fraction thus obtained contained >90% Lyt-1-bearing T cells as determined by indirect immunofluorescence.

Cloning and Surface Phenotype of Con A-Activated PP and Spleen T Cells. Both Con A-activated PP and spleen T cells were cloned using limited dilution techniques after long-term culture (2 mo) with Con A (concanavalin A, Miles Laboratories, Inc., Miles Research Products, Elkhart, IN) and IL-2, as reported previously (10). Each clone from PP (K-14) and spleen (K-17) was used in the present studies. The cloned T cells had a Thy-1.2, Lyt-1⁺, and Lyt-2⁻ surface phenotype, and bore Ia (I-A and I-E) as well as H-2 (K/D) antigens corresponding to the histocompatibility antigen phenotype of the parent cells (10).

Culture Medium. RPMI 1640 (Gibco Laboratories, Grand Island, NY) was supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 25 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol (2-ME), 25 mM HEPES buffer, and antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin, and 50 μ g/ml gentamycin). The concentration of heat-inactivated fetal calf serum (FCS) (Gibco Laboratories) and further additions of supplements were dependent on the type of culture or assay used and are indicated below.

Preparation of Interleukin-1 (IL-1)-Containing Macrophage Factors (MF)

(a) MF-1: This macrophage factor was prepared from purified macrophages obtained from mouse spleen and mesenteric lymph node cell suspensions by double plastic adherence. >99% of cells obtained were macrophages, as judged by staining for nonspecific esterase and uptake of latex beads (10). The macrophages were then cultured at 2×10^6 /ml in flat-bottomed 6-well tissue culture plates (#3506, Costar, Division of Data Packing Corporation, Cambridge, MA) and after 24 h the seeded macrophages were pulsed with 50 μ g/ml lipopolysaccharide (LPS, *S. typhimurium*, Difco Laboratories, Detroit, MI) for 4 h at 37°C, and were washed once with warm RPMI 1640. The LPS-pulsed macrophages (activated MF-1) were then incubated in 5% FCS-containing medium for 24 h at 37°C in

a humidified atmosphere of 95% air and 5% CO₂. The macrophage culture supernatants then were aspirated, centrifuged free of cells, and stored at -20°C until use. Supernatants were also obtained from cultures containing macrophages not subjected to LPS treatment (unpulsed MF-1).

(b) MF-2: This macrophage factor was prepared from a continuous macrophage-like cell line, P388D₁ (DBA/2; H-2^d). The cells were pulsed at a concentration of 2×10^6 cells/ml with LPS as above. The macrophages were then cultured and supernatants were obtained as described above. Supernatants obtained from cultures of macrophage not subjected to LPS treatment (unpulsed MF-2) were also prepared.

Preparation of Interleukin 2 (IL-2)

(a) Con A-containing IL-2: Rat spleen cell suspensions in the culture medium supplemented with 2% FCS (5×10^6 /ml) were incubated for 24 h in the presence of Con A (5 µg/ml). The supernatants were harvested, filtered through 0.45-µm Nalgene filters, and stored at -20°C until use. This IL-2 was used for cloning of T cells as well as maintenance of long-term cultured T cell and cloned T cell lines.

(b) Con A-pulsed IL-2: Rat spleen cell suspensions were first pulsed with Con A for 2 h in petri dishes. The nonadherent cells were then extensively washed at least four times in RPMI 1640, and then briefly recultured in petri dishes to remove residual adherent cells. The macrophage-depleted Con A-pulsed cells thus obtained were then cultured for 24 h at 37°C. Analogous spleen cell cultures performed in the absence of Con A were also set up. At the end of the culture period cell supernatants were harvested, filtered through 0.45-µm Nalgene filters, and stored at -20°C until use. In some experiments these supernatants were supplemented with α-MM (α-methyl-D-mannoside, Sigma Chemical Co., St. Louis, MO) (50 mM/ml).

Preparation of B Cell Differentiating Factor(s) Derived from T Cells (BCDF)

Two kinds of BCDF-enriched supernatant fluids were obtained by depleting Con-A pulsed cell supernatants (prepared as described above) of IL-2:

(a) BCDF-1: To prepare this factor IL-2-containing supernatant was absorbed with IL-2 receptor-bearing cells, an IL-2-dependent alloreactive CT-6 cell line (C57BL/6-anti-H-2^d). Absorption was performed by incubating Con A-pulsed supernatant for 18 h at 37°C with 5×10^6 /ml IL-2-dependent allo-reactive CT-6 cloned T cells. The cell-free supernatant was then filtered and stored at -20°C. In addition, similar absorptions were performed with various non-IL-2-dependent cells, including normal resting spleen cells (10^7 /ml) and BCL₁ cells (H-2^d, B cell leukemia cell line) to establish the specificity of the IL-2 absorptions. None of these negative control absorptions removed IL-2 from the Con A-pulsed supernatant.

(b) BCDF-2: This factor was prepared in the same manner as BCDF-1 except in this case the Con A-pulsed supernatant was obtained from 96-h rather than 24-h cultures.

These BCDF preparations were tested at multiple concentrations for IL-1 and IL-2 activity remaining after absorption (see below). It was found that neither BCDF-1 nor BCDF-2 contained significant amounts of IL-1 or IL-2.

Assay for IL-1 Activity (Co-stimulator Assay). The IL-1 activities of supernatants were assessed by a thymocyte proliferation assay as described by Shaw et al. (28). Briefly, thymocytes in culture medium containing 5% FCS and Con A (5 µg/ml) were placed in wells (at 10^5 cells/0.2 ml/well) of a MicroTest II culture plate (Falcon Plastics Co., Oxnard, CA) in a flat-bottomed Linbro 96-well microtiter tray. To these wells serial dilutions (1:2 to 1:1,024) of test macrophage factor(s)-containing supernatant were added in triplicate. After 72 h of incubation in a humidified 5% CO₂-95% air atmosphere the cells were pulsed with [³H]thymidine (1.5 µCi/well) for 6 h; the cells were then harvested and the radioactivity counted with a Beckman LS 8100 liquid scintillation spectrometer (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). For controls, media alone, Con A alone, or IL-2 alone were added to identical cultures. The activity of IL-1 present in each supernatant was expressed as half-maximal units, which indicates a value of reciprocal of sample dilution that yields 50% maximal cpm of [³H]thymidine incorporation into

thymocytes. Thymocyte proliferative activity was expressed in units/ml. A batch of macrophage culture supernatant with 256 U of half-maximal activity was chosen as a preparation of IL-1. The preparation did not contain any demonstrable activity of IL-2, which was determined by the following T cell growth factor microassay.

Assay for IL-2 Activity. The IL-2 activities of supernatants were assessed by a T cell growth microassay according to a modification of the method of Gillis et al. (29). Briefly, CT-6 cells were cultured in flat-bottomed microtiter plates (#3040, Falcon Plastics Co.) at $5 \times 10^3/0.2$ ml/well. IL-2-containing test samples that had been serially diluted from 1:4 to 1:1,024 in culture medium supplemented with 2% heat-inactivated FCS were then added to the wells. In some experiments α -MM (50 mM) was also added. The cells were then cultured for 18 h at 37°C in a humidified atmosphere containing 5% CO₂ and, finally, cell proliferative responses were determined by adding 1.5 μ Ci [³H]thymidine (New England Nuclear, Boston, MA) during the last 6 h. As described above, the concentration of IL-2 present in each supernatant was expressed in terms of half-maximal units. Con A-containing IL-2 supernatants routinely had between 128 and 512 half-maximal units of IL-2 activity. A batch of Con A- and IL-1 free IL-2 supernatant, which yielded 256 U of half-maximal activity, was selected for a preparation of BCDF (IL-1- and IL-2- depleted lectin-free supernatant).

In Vitro Cultures. Terminal differentiation and induction of Ig synthesis by postswitch sIgA-expressing PP B cells induced by PP cloned T cells were determined using one of several experimental designs: In the first experimental design highly purified PP B cells (2.5×10^5 /culture) were co-cultured with PP or splenic cloned T cells at a 1:4 B/T cell ratio in the presence or absence of LPS (20 μ g/ml) and/or BCDF or MF (10% vol/vol) in culture medium supplemented with 10% FCS. The cultures were incubated at 37°C for 7 d, at which time cell-free supernatants were harvested for determination of Ig secreted. Each BCDF or MF was tested preliminarily for optimal concentration of Ig production by PP B cells; these factor-containing supernatants did not themselves contain significant amounts of Ig.

In the second experimental design PP B cells (2.5×10^5 /cultured) were precultured with PP or spleen cloned T cells at a 1:4 B/T ratio and/or LPS (20 μ g/ml) at 37°C for 4 d (first culture); the cultured cells were then harvested and depleted of co-cultured cloned T cells by treatment with monoclonal anti-Thy-1.2 (New England Nuclear) and complement (Accurate Chemical and Scientific Co.) and subsequently by Hypaque-Ficoll gradient centrifugation (10). The viable PP B cells thus obtained were further cultured with MLN or spleen T or Lyt-1-enriched T cell subset at a 1:1 B/T ratio in the presence or absence of protein A (250 g/ml) at 37°C for 4 additional days (second culture). The concentration of protein A inducing optimal secretion of Ig by unseparated or B cells derived from PP was determined in preliminary experiments. Cloned T cells as well as MLN and spleen T cells did not produce significant amounts of Ig when cultured alone with either protein A or LPS for 7 d. In the third experimental design viable PP B cells (2.5×10^5 /culture), which were precultured with PP or spleen cloned T cells in the presence or absence of LPS (first culture) as in the second experimental protocol indicated above, and were then cultured in the presence of varying concentrations of BCDF-2 (optimal, 10% vol/vol) at 37°C for 4 additional days (second culture).

Radioimmunoassays for Secreted Class-specific Immunoglobulins (Ig) in Cell Cultures. Each class-specific IgM, IgG and IgA produced was determined by the ¹²⁵I-coupled double-antibody solid phase radioimmunoassay as previously reported (3, 10).

Enumeration of Class-specific Cytoplasmic Ig-Bearing Cells by Immunofluorescence. In some experiments distributions of each class-specific Ig-containing plasma cell was examined by an immunofluorescent microscope as previously described (11).

Results

In previous studies (10, 11) we have shown that cloned T cells derived from PP (cloned PP T cells), when cultured with LPS-driven B cells cause B cells to move along a pathway of differentiation that favors IgA expression. More

specifically, in cultures containing cloned PP T cells (as compared to cultures not containing such cells), LPS-induced IgM and IgG synthesis and secretion are reduced, the number of cells bearing surface(s) IgM and IgG is reduced and the number of cells bearing sIgA is greatly increased; however, IgA secretion does not occur to any significant extent. Parallel studies using cloned T cells derived from spleen (cloned spleen T cells) do not result in the appearance of sIgA-bearing cells although, in this case, the number of sIgG-bearing cells increased. These data suggest that PP cloned T cells act as "switch T cells" capable of causing a B cell class-specific switch from IgM to IgA. However, since cells expressing sIgA rather than cells producing IgA result from exposure to the cloned PP T cells, it was apparent that additional cells or cell-derived factors are necessary for terminal IgA B cell differentiation. Accordingly, we examined the effect of various T cells or factors derived from T cells or macrophages on B cells exposed to cloned PP T cells (switch T cells).

A. Studies on the Effect of T Cell and Macrophage Factors on IgA B Cell Differentiation

In our initial approach to the examination of terminal differentiation of B cells derived from PP we measured Ig production by the LPS-driven B cells co-cultured with PP or spleen cloned T cells in the presence or absence of MLN or spleen T cell factors, or macrophage factors.

Effect of Con A-Depleted IL-2, BCDF, and MF on Ig Production by LPS-Induced PP B Cells. In preliminary studies we determined the effects of various factors on LPS-induced Ig production by B cells in the absence of T cells. As indicated in Table I, IgM and IgG production by PP B cells was essentially unchanged by the addition of 10% Con A-depleted IL-2-enriched supernatant or BCDF-1 under both nonstimulated and LPS-stimulated conditions; in fact, when the concentrations of both supernatants were increased to 20%, significant suppression of Ig synthesis was sometimes observed. There was no difference between the effect of BCDF-1 and that of BCDF-2 (data not shown). Similarly, LPS-pulsed MF-1 and both LPS-pulsed and unpulsed MF-2 (at optimal concentrations) increased IgM and IgG, but not IgA (data not shown) production to a limited degree. Thus, in the absence of cloned T cells the various factors studied had very little effect on Ig production by B cells. We therefore could study the effects of such factors on B cells co-cultured with cloned T cells.

Effect of B Cell Differentiation Factor (BCDF) on Ig Production by LPS-Driven PP B Cells Co-cultured with PP or Spleen Cloned T cells. In contrast to its lack of effect on LPS-driven B cells cultured alone, BCDF (10%) had significant effects on such B cells co-cultured with cloned PP T cells. As shown in Fig. 1, B cells were driven by LPS to produce far less IgM and IgG when co-cultured with cloned PP T cells (IgM synthesis was 10-fold less) and this effect was seen both in the presence and absence of BCDF. More important, however, is the fact that IgA synthesis was modestly augmented by co-culture with cloned PP T cells and this effect was greatly magnified when BCDF was also present in the culture. Thus, both BCDF-1 (as shown in Fig. 1) and BCDF-2 (data not shown) augmented IgA synthesis from ~100 ng/culture to ~1,600 ng/culture at a 4:1 cloned T/B cell ratio. In the absence of LPS, B cell Ig synthesis in all classes was minimal and

TABLE I
Effect of IL-2 and BCDF on Immunoglobulin Production by Peyer's Patch (PP) B Cells With or Without LPS Stimulation (7-d Culture)*

Factors tested	LPS (20 µg/ ml)	Immuno- globulin secreted [‡]	Concentration (% vol/vol) of factor					
			0		10		20	
			<i>Exp. 1</i>	<i>Exp. 2</i>	<i>Exp. 1</i>	<i>Exp. 2</i>	<i>Exp. 1</i>	<i>Exp. 2</i>
IL-2-enriched supernatant (Con A-depleted)	-	IgM	480	325	565	450	395	255
		IgG	105	<32	75	<32	195	<32
		IgA	<27	27	185	251	168	113
	+	IgM	22,680	17,520	23,330	20,845	18,320	21,624
		IgG	2,737	1,919	2,965	1,805	1,624	1,830
		IgA	<27	38	115	154	113	95
BCDF-enriched supernatant (BCDF-1)	-	IgM	320	210	581	439	251	290
		IgG	140	90	178	146	145	85
		IgA	39	<27	136	216	182	91
	+	IgM	23,115	19,250	24,965	23,120	18,215	13,790
		IgG	2,029	2,290	2,380	3,132	2,064	2,360
		IgA	<27	<27	250	150	180	121

* PP B cells were cultured with and without LPS at $2.5 \times 10^5/2$ ml/culture in the presence of IL-2 or BCDF; IL-2 consists of supernatant of Con A-pulsed cells; BCDF consists of supernatants of Con A-pulsed cells absorbed with CT-6 cells; see Materials and Methods.

[‡] Expressed as ng/ml.

only modestly augmented by co-culture with cloned PP T cells and BCDF (data not shown). In all, these results indicate that B cells induced to switch to sIgA-bearing cells by switch T cells derived from PP can synthesize and secrete IgA if appropriate T cell factors are present in the culture.

A different situation is obtained when the effect of spleen cloned T cells added alone or combined with BCDF to LPS-driven B cell cultures was studied. As shown in Fig. 1, in this case IgM synthesis in cell cultures is essentially unchanged, whereas IgG synthesis was slightly decreased and IgA synthesis was slightly increased. Of note is the fact that spleen cloned T cells plus BCDF did not lead to a striking augmentation of IgA synthesis as did PP cloned T cells plus BCDF; with spleen cloned T cells (plus BCDF) IgA synthesis was ~ 200 ng/culture, whereas with PP cloned T cells (plus BCDF) IgA synthesis was $\sim 1,600$ ng/culture. Again, in the absence of LPS, the effect of spleen cloned T cells with or without BCDF was quite minimal and total Ig synthesis per culture was always low (data not shown). In summary, spleen cloned T cells do not lead to significant IgA production, even in the presence of factors that can induce terminal differentiation of IgA B cells.

Effect of Macrophage Factors (MF) on Ig Production by LPS-Driven PP B Cells Co-cultured with PP Cloned T Cells. Macrophage factors, prepared as described in the Materials and Methods, had generally marginal effects on IgM and IgG synthesis when added to LPS-driven B cells co-cultured with cloned PP T cells (data not shown). In the case of IgA production, however, the addition of MF obtained from LPS-activated macrophages (MF-1), and MF-2) led to small but

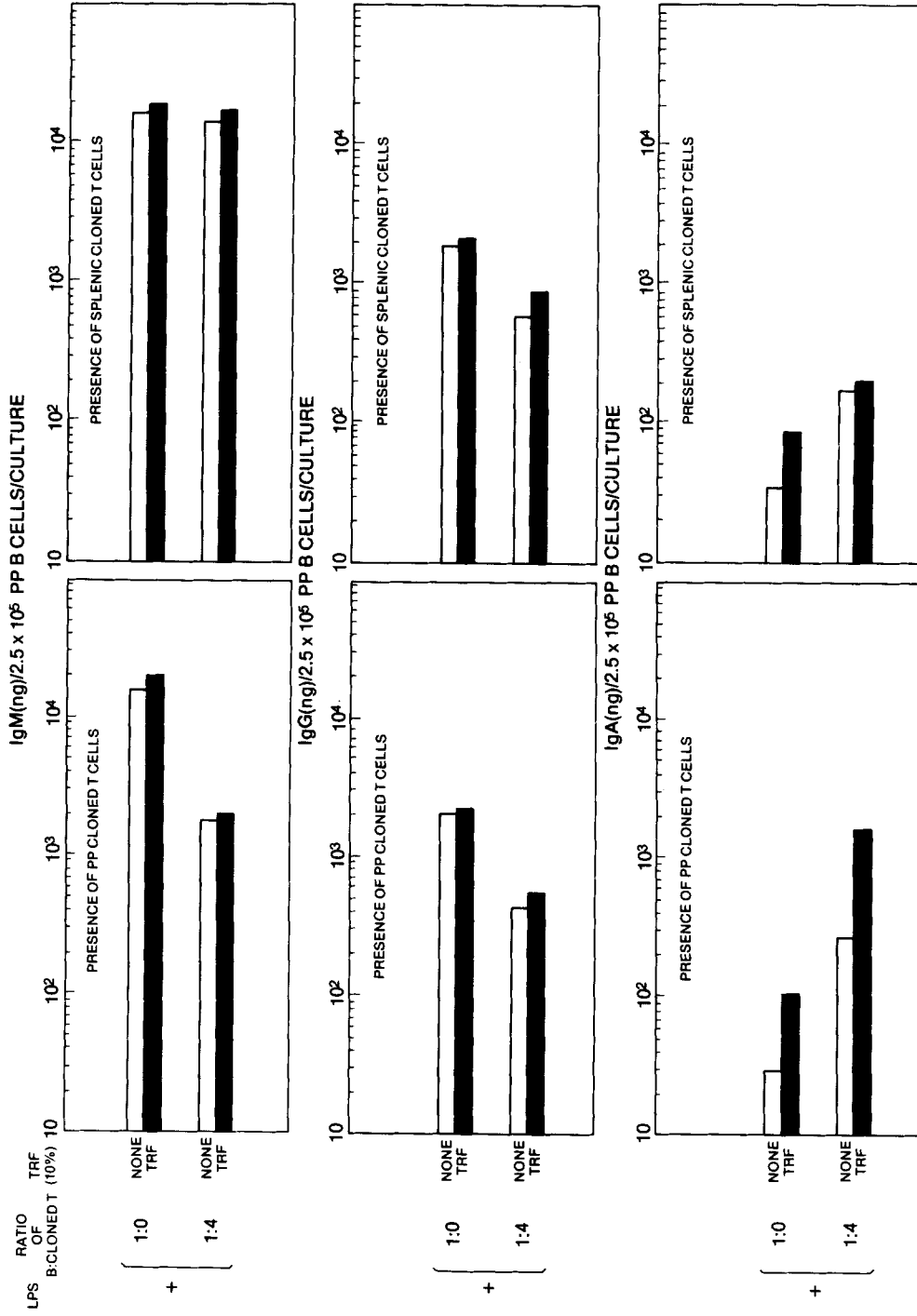


FIGURE 1. Effect of IL-2-dependent CT-6 cell-absorbed TRF (BCDF-1) LPS-driven PP B cells (2.5 x 10⁵/culture in the presence of PP or spleen (10%) (IL-1- and IL-2-depleted, lectin-free) on class-specific Ig production by cloned T cells (B/cloned T, 1:4) (cultures of 7 d).

definite, increments in IgA synthesis and secretion above that observed with B cells co-cultured with cloned PP T cells in the absence of MF (Fig. 2).

B. Studies on the Effect of Uncloned T Cells on IgA B Cell Differentiation

Our second approach to the examination of terminal differentiation of B cells exposed to cloned PP and spleen T cells was to determine Ig production by B cells cultured twice, first with cloned PP or spleen T cells in the presence of LPS to bring about switching events, and then, after preincubation, with uncloned T cells in the presence of the polyclonal stimulant, staphylococcal protein A, to bring about terminal differentiation.

Effect of Protein A on Ig Production by LPS-Driven or Undriven PP B Cells. In this experimental design the effects of T cells themselves on terminal differentiation can be determined. In initial studies purified B cells were exposed to increasing concentrations of protein A and Ig secretion was measured. As seen in Fig. 3, the optimal concentration of protein A was 250–500 $\mu\text{g}/\text{ml}$. Interestingly, there was a significant amount of IgA synthesis (average 427 ng/ml) induced by protein A (250 $\mu\text{g}/\text{ml}$) in contrast to the negligible amount induced by LPS (27 $\mu\text{g}/\text{ml}$); in contrast, IgM (average 2,489 ng/ml) and IgG (average 504 ng/ml) production was much less in protein A-stimulated cultures than in LPS-stimulated cultures (IgM 22,456 ng/ml, IgG 1,961 ng/ml). It is likely that the IgA produced in these cultures is dependent on residual T cells in the cultures which were present despite our efforts to eliminate T cells.

Ig Production by B Cells Co-cultured with Cloned PP T Cells (Plus LPS) and Then with Uncloned Spleen or MLN T Cells (Plus Protein A). In control studies, shown in Fig. 4, it is seen that when the first culture contains only B cells (plus LPS) and no cloned T cells (putative switch T cells), second culture with uncloned spleen or MLN T cells (plus protein A) does not result in changes in IgM and IgG synthesis. That is, synthesis of these immunoglobulins (Igs) was unchanged, whether or not protein A was present in the second culture. In the case of IgA, however, there was some increase in IgA synthesis when protein A was present

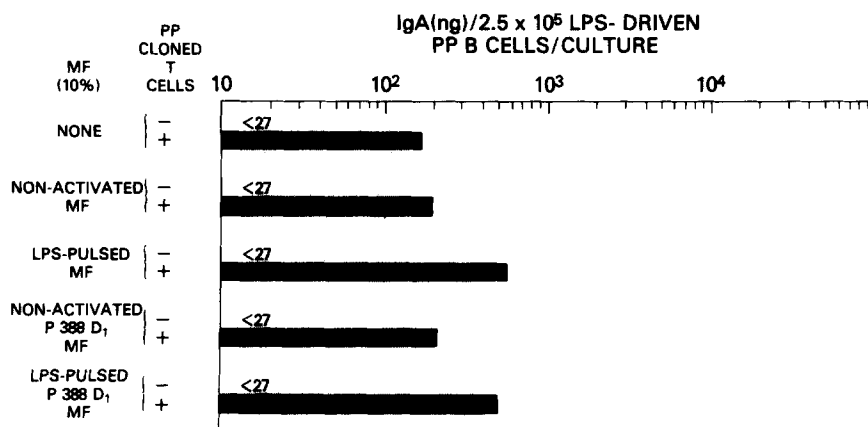


FIGURE 2. Effect of LPS-activated spleen macrophage factor (MF-1) or P388D₁-derived LPS-activated macrophage factor (MF-2) (10%) on IgA production by LPS-driven PP B cells (ng/ 2.5×10^5 /culture) in the absence or presence of PP cloned T cells (B/cloned T, 1:4) (cultures of 7 d).

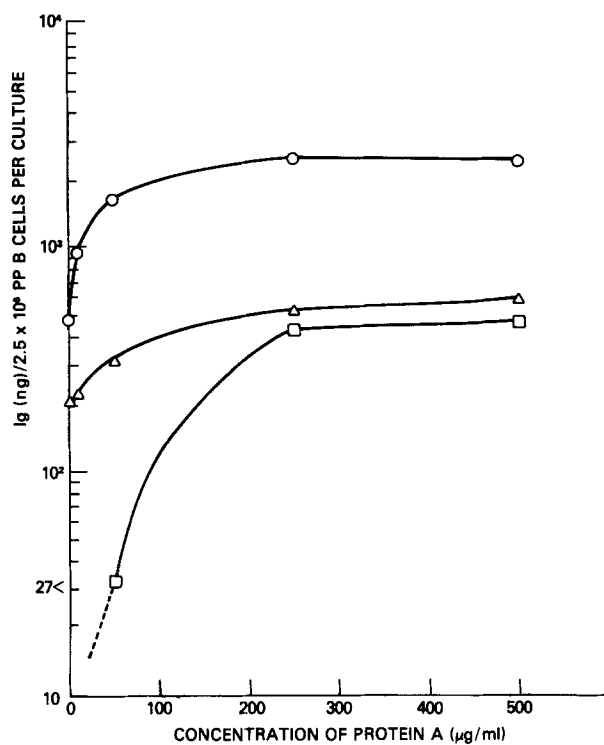


FIGURE 3. Effect of various concentrations of protein A alone on each class-specific Ig production by LPS-undriven virgin PP B cells (2.5×10^5 /culture) in cultures of 7 d. (O) IgM, (Δ) IgG, (\square) IgA.

in the second culture; however, the maximum levels of IgA synthesized were quite low and, in any case, did not exceed IgA synthesis by B cells cultured with protein A alone (see Fig. 3).

A different situation was obtained when cells were co-cultured with cloned PP T cells during the first culture (Fig. 5). Here, IgM synthesis was low in the absence of protein A during second culture and increased markedly in the presence of protein A (but never reaching levels obtained with cells not exposed to cloned PP T cells in the first culture). In the case of IgG synthesis protein A had essentially no effect on synthesis, and the level was significantly lower than that seen in cells not pre-cultured with cloned PP T cells. Finally, and most strikingly, whereas IgA synthesis was low in second cultures not containing protein A, it was markedly increased in cultures containing this mitogen. In fact, IgA synthesis under these conditions exceeded IgM or IgG synthesis. In summary of these results, it is clear that initial culture with cloned PP T cells led to a far different pattern of Ig synthesis induced by protein A in the presence of uncloned T cells: IgA synthesis was enhanced at the expense of IgM and IgG synthesis.

Ig Production by PP B Cells Co-cultured with Cloned Spleen T Cells (Plus LPS) and Then with Uncloned Spleen or MLN Cells (Plus Protein A). Parallel studies to those just described were performed with spleen cloned T cells. In this case IgM and IgG synthesis was increased by protein A stimulation in cells precultured with

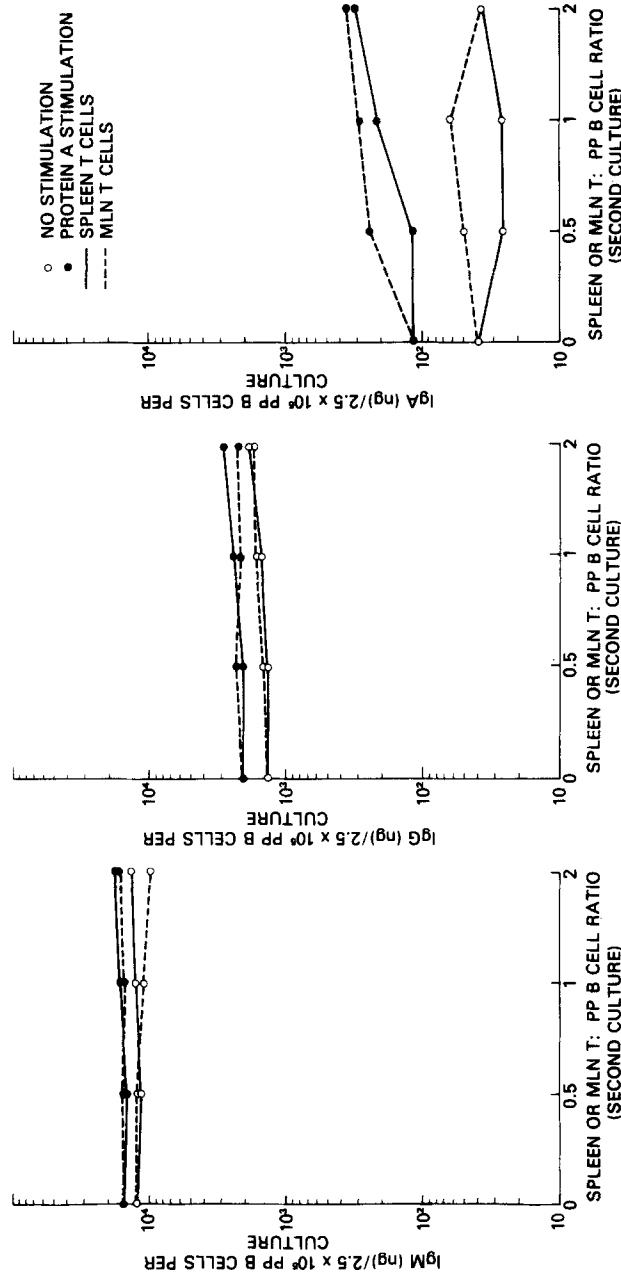


FIGURE 4. Effect of graded numbers of spleen or mesenteric lymph node T cells on each class-specific Ig production by PP B cells (2.5×10^5 /culture) precultured cells in the presence of protein A ($250 \mu\text{g}/\text{ml}$) during second culture (4 d) on with LPS, but without cloned T cells (first culture of 4 d).

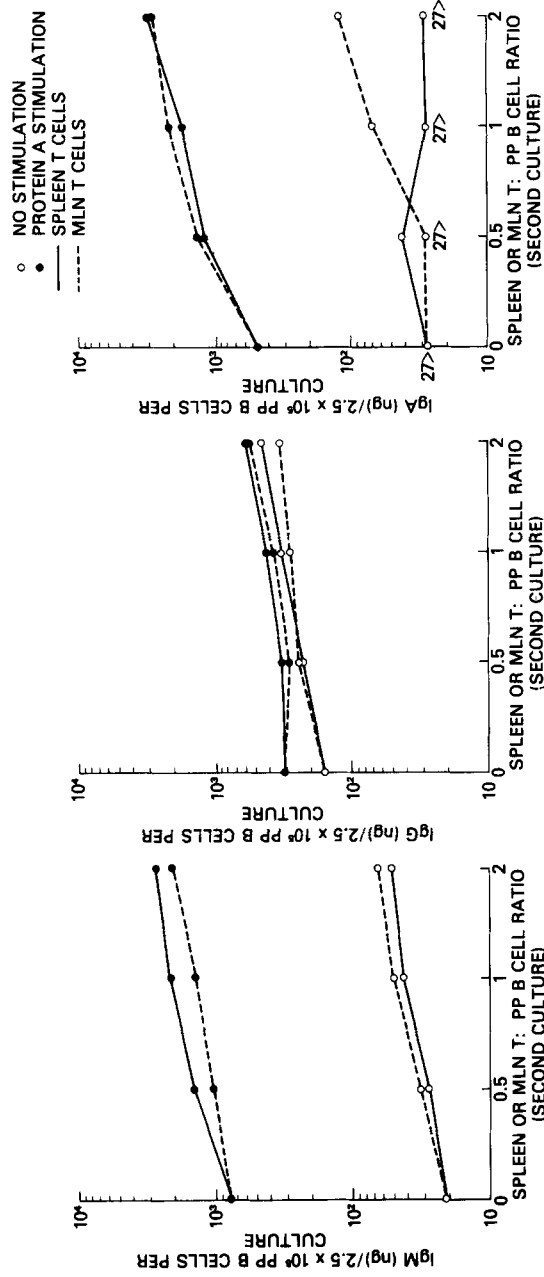


FIGURE 5. Effect of graded numbers of spleen or mesenteric lymph node T cells in the presence of protein A during second culture (4 d) on each class-specific Ig production by PP B cells (2.5×10^5 /culture) precultured with LPS cells in the presence of protein A during second culture (4 d) on each class-specific and PP cloned T cells (K-14) (B/cloned T, 1:4) (first culture of 4 d).

the spleen cloned T cells, but IgA synthesis was only minimally increased by such stimulation (Fig. 6). However, the spleen cloned T cells may have induced an intra-subclass IgG switch in that additional IgG production is observed after preculture with such cloned T cells, but not when the B cells are precultured with PP cloned T cells.

Ig Production by PP B Cells Co-cultured with Cloned PP or Spleen T Cells (Plus LPS) and then with Uncloned Spleen or MLN Lyt-1⁺ or Lyt-1⁻ T Cells (Plus Protein A). In these studies we examined which subset of uncloned spleen or MLN T cells (helper/inducer vs. suppressor) under stimulation of protein A would modulate PP B cell terminal differentiation. As indicated in Tables II and III, the T cell subset providing help for terminal differentiation of PP B cells belongs to the Lyt-1⁺ cell subset. These data also show that the major effect of protein A is to stimulate a helper T cell subpopulation, rather than a suppressor T cell inducer or suppressor T cell effector subpopulation.

C. Studies on the Class-specific Cytoplasmic Ig Expression of and Ig Production by PP B Cells Co-cultured with Cloned PP or Spleen T Cells (Plus LPS) and then Recultured in the Presence of BCDF.

In a third approach to the study of the terminal differentiation of IgA B cells we utilized a protocol that combines elements of the preceding two protocols: B cells were cultured first with (or without) cloned PP or spleen T cells (in the presence of LPS) to obtain postswitch B cells, and then cultured in the presence of BCDF (BCDF-2, 10–30% vol/vol). This protocol allowed examination of the terminal differentiation of IgA B cells in the absence of additional stimulants such as protein A. As shown in Table IV, LPS-driven PP B cells precultured with PP cloned T cells, but not with splenic cloned T cells, produced substantial amounts of IgA (average 2,224 ng/ 2.5×10^5 cells) when recultured in the presence of optimal amounts of BCDF-2 (10%). This was approximately five times the amount of IgA produced when B cells cultured with PP cloned T cells were recultured in the absence of BCDF. Finally, these results were borne out in studies utilizing cytoplasmic fluorescence as an indication of Ig synthesis (Table V). Here it was observed that B cells cultured with PP cloned T cells and then cultured with BCDF-2 resulted in a cell population containing ~33% IgA plasma cells; in contrast, cell cultured first with spleen cloned T cells did not lead to the appearance of IgA plasma cells.

Discussion

The studies described here, taken in conjunction with our previous studies, indicate that the differentiation of IgA-secreting B cells requires at least two kinds of T cells: an Ig class-specific T cell that guides B cell differentiation into an IgA pathway (a switch T cell) and a helper T cell that is necessary for terminal B cell maturation, i.e., conversion of sIgA-bearing B cells into IgA-secreting plasma cells. Evidence in support of the existence of IgA-specific switch T cells reported previously (10) can be summarized as follows. It is possible to isolate a cloned T cell population from the PP (PP cloned T cells) that, when co-cultured with sIgM-bearing B cells, reduces LPS-driven IgM and IgG secretion as well as the number of cells at the end of the culture that bear these Igs on their surfaces;

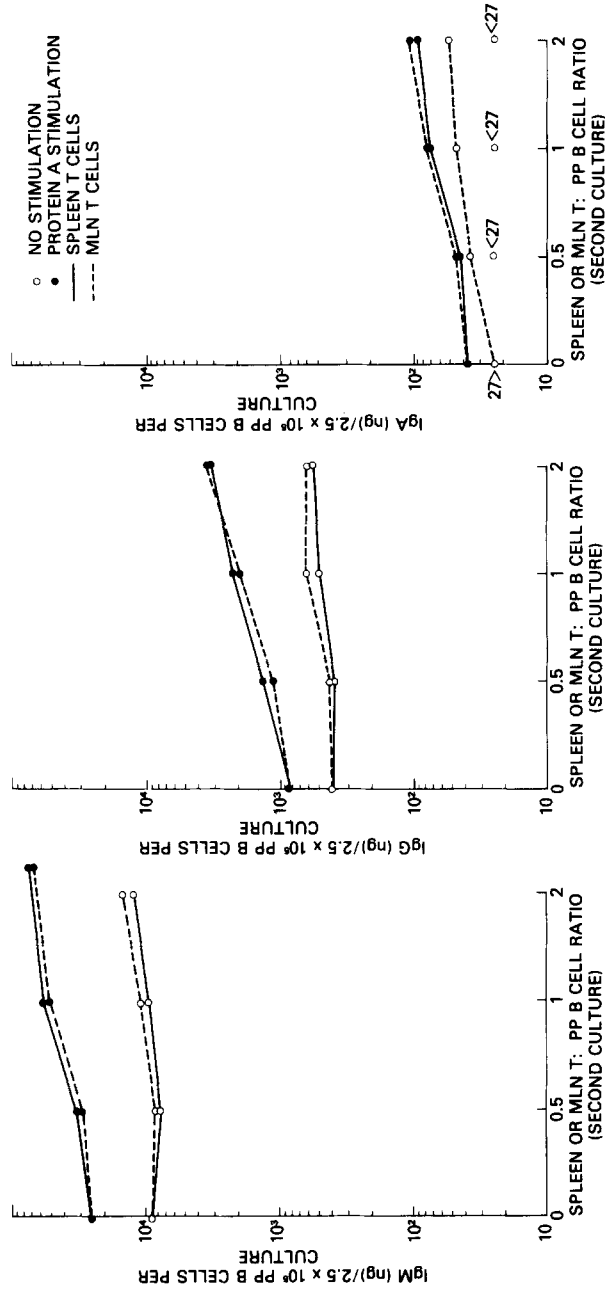


FIGURE 6. Effect of graded numbers of spleen and mesenteric lymph node specific Ig production by PP B cells (2.5×10^5 /culture) precultured with LPS and splenic cloned T cells (K-17) (B/cloned T, 1:4) (first culture of 4 d).

TABLE II

*Effect of Lyt-1⁺ (Enriched) Mesenteric Lymph Node (MLN) or Spleen T Cells on Immunoglobulin (Ig) Synthesis by Postswitch LPS-Driven Peyer's Patch (PP) B Cells Precultured With PP Cloned T Cells (K-14)**

Second culture condition			Ig secreted (ng/ml) [‡]		
T cell source	Protein A (250 µg/ml)		IgM	IgG	IgA
MLN	Unseparated	-	543	432	57
		+	1,595	406	1,823
	Lyt-1 ⁺ -enriched [§]	-	677	389	43
		+	1,939	621	2,731
Spleen	Unseparated	-	374	375	<26
		+	2,381	450	1,556
	Lyt-1 ⁺ -enriched	-	498	412	84
		+	2,585	503	2,110

* PP B cells were precultured for 4 d with PP cloned T cells at a 4:1 T/B cell ratio under LPS stimulation; postswitch PP B cells were further cultured for 4 d with MLN or spleen T cells or their subset at a 1:1 ratio.

[‡] Representative data shown here.

[§] Lyt-2⁺ T cells were depleted by treatment of unseparated T cells with anti-Lyt-2 and complement.

TABLE III

*Effect of Lyt-1⁺ (Enriched) Mesenteric Lymph Node (MLN) or Spleen T Cells on Immunoglobulin (Ig) Synthesis by Postswitch LPS-Driven Peyer's Patch (PP) B Cells Precultured With Splenic Cloned T Cells (K-77)**

Second culture condition			Ig secreted (ng/ml) [‡]		
T cell source	Protein A (250 µg/ml)		IgM	IgG	IgA
MLN	Unseparated	-	9,576	681	75
		+	14,398	2,186	38
	Lyt-1 ⁺ -enriched [§]	-	9,899	644	66
		+	12,823	2,389	131
Spleen	Unseparated	-	9,921	503	<26
		+	16,026	2,219	75
	Lyt-1 ⁺ -enriched	-	9,329	612	39
		+	19,439	3,083	86

* PP B cells were precultured for 4 d with spleen cloned T cells at a 4:1 T/B cell ratio under LPS stimulation; postswitch PP B cells were further cultured for 4 d with MLN or spleen T cells or their subset at a 1:1 ratio.

[‡] Representative data shown here.

[§] Lyt-2⁺ T cells were depleted by treatment of unseparated T cells with anti-Lyt-2 and complement.

TABLE IV
Effect of BCDF on IgA Production by Postswitch PP B Cells*

Culture conditions of Peyer's Patch B cells [‡]	IgA secreted by Post-Switch Peyer's Patch B cells (ng/2.5 × 10 ⁵ cells) with or without BCDF-2 (10%)	
	Experiment 1	Experiment 2
(LPS)	<63	<63
(LPS) + BCDF	<63	<63
(LPS + splenic cloned T cells)	<63	<63
(LPS + Peyer's Patch cloned T cells)	375	511
(LPS + splenic cloned T cells) + BCDF	<63	<63
(LPS + Peyer's Patch cloned T cells) + BCDF	1,959	2,488

* PP B cells were precultured for 4 d with spleen or PP clones T cells at a 4:1 T/B cell ratio under LPS stimulation (first culture); postswitch PP B cells were further cultured for 4 d in the presence or absence of BCDF at optimal concentration. Harvested culture supernatants were determined for secreted IgA by RIA.

[‡] Parentheses indicate first culture condition.

TABLE V
Effect of BCDF on Cytoplasmic Immunoglobulin (Ig) Expression by Postswitch PP B Cells*

Culture conditions of Peyer's Patch B cells [‡]	Percent distribution of class-specific cytoplasmic Ig-bearing post-cultured Peyer's Patch B cells	
	Experiment 1	Experiment 2
(LPS) + BCDF	(Total cell number; 7.25 × 10 ⁶ /well)	
cIgM	48.3	51.9
cIgG	23.8	27.3
cIgA	0	0
(LPS + Splenic Cloned T Cells) + BCDF	(Total cell number; 6.85 × 10 ⁶ /well)	
cIgM	42.8	50.6
cIgG	11.8	15.1
cIgA	0	0
(LPS + Peyer's Patch Cloned T Cells) + BCDF	(Total cell number; 6.26 × 10 ⁶ /well)	
cIgM	28.8	25.0
cIgG	7.9	8.7
cIgA	30.4	35.0

* PP B cells were precultured for 4 d with spleen or PP cloned T cells at a 4:1 T/B cell ratio under LPS stimulation (first culture); postswitch PP B cells were further cultured for 4 d in the presence of BCDF-2 (at optimal concentration, 10%). Viable B cells were then cytocentrifuged for enumeration of class-specific cytoplasmic Ig by immunofluorescence (see text).

[‡] Parentheses indicate first culture condition.

in contrast, co-culture of sIgM-bearing B cells with PP cloned T cells greatly increases the number of cells at the end of the culture bearing sIgA. This redirection of Ig synthesis into an IgA-dominant mode is Ig-class specific, since only sIgM-bearing cells are induced to undergo switch to sIgA-bearing cells. Furthermore, the increase in sIgA-bearing cells is not due simply to an expansion of the small number of sIgA-bearing cells normally generated in LPS-induced cultures since isolated sIgA-bearing B cell populations are not expanded by exposure to PP cloned T cells (i.e., there is no PP cloned T cell-induced increase in sIgA-bearing cells over that brought about by LPS alone).

Evidence in support of the existence of helper cells necessary for induction of terminal differentiation of sIgA-bearing cells that are the products of the switch process (the postswitch B cell) is provided in the present work. Here we show that (a) addition of T cell or macrophage factors to LPS-induced postswitch cells (particularly T cell factors) can act on the postswitch T cells to induce terminal differentiation; (b) addition of fresh uncloned T cells (appropriately stimulated with staphylococcal protein A) and (c) addition of BCDF to cultures of postswitch B cells can act on the postswitch B cells to induce terminal differentiation. These three methods of bringing about terminal differentiation and IgA production in LPS-induced, postswitch B cells warrant further discussion.

Terminal Differentiation of Postswitch B Cells Induced by T Cell and Macrophage Factors. In recent years evidence has accumulated that a variety of soluble factors derived from either T cells or macrophages are important in B cell activation, proliferation, and differentiation. These factors include IL-1, a macrophage-derived factor necessary to T cell production of IL-2 (21, 30) as well as TCGF (IL-2) and "BCGF" (19, 27) necessary for T cell and B cell proliferation, respectively. In addition, T cells produce BCDF, a factor that can be differentiated from those mentioned above, which is important in terminal differentiation of B cells (19, 20, 23, 27). In the present studies, the data reported were obtained with the supernatant of macrophage-depleted rat spleen cells pulse-activated with Con A and subsequently absorbed with cells (CT-6) that have a high affinity for IL-2. This supernatant was devoid of IL-1, functionally active Con A, and IL-2; it did, however, contain BCDF, as indicated by its ability to induce terminal B cell differentiation. Significantly, BCDF-containing supernatant had little effect on LPS-induced IgM and IgG production by postswitch B cells in the presence or absence of cloned T cells. In contrast, it had a considerable effect on IgA production: when B cells were cultured with LPS and the BCDF-containing supernatant, very little IgA was produced; however, when PP cloned T cells were also present in the culture, then the BCDF-containing supernatant could act on the B cells to induce substantial amounts of IgA. These studies show that the differentiating effect of BCDF was dependent on the switch state of the B cells: when switch to IgA did not occur, no significant terminal differentiation to IgA-producing plasma cells was seen. The co-cultures of B cells with cloned T cells derived from spleen, a condition not productive of sIgA-bearing cells, did not lead to IgA synthesis in the presence of BCDF-containing supernatant. Thus, again, B cells must be switched by an appropriate (class-specific) T cell if IgA differentiation is to occur.

Macrophages may also produce factors (MF) that induce B cells to produce Ig

that are independent of T cell factors (14–16). Thus, Butler et al. (15) have demonstrated that MF derived from P388D₁, a macrophage cell line similar to the cells used as a source of MF in these studies, can enhance B cell Ig production, even in the absence of T cells. MF prepared from LPS-activated normal macrophages or P388D₁ macrophages did indeed enhance IgA synthesis in LPS-induced B cells co-cultured with cloned PP T cells. However, this effect, while consistent, was marginal. Furthermore, it is at least theoretically possible that the MF was acting by inducing residual T cells present in the culture to produce BCDF.

In summary of the studies performed with T cell and macrophage factors, it is clear that BCDF-enriched supernatants and possibly LPS-activated macrophage factors can cause B cells to undergo terminal differentiation. If the B cells are co-cultured with T cells capable of bringing about IgA switches, then the B cells will produce IgA. It is also clear that the B cells need a terminal differentiation signal provided by the factors, i.e., exposure to LPS, and IgA-specific switch T cells are not sufficient for IgA production. Nevertheless, there is no evidence of class specificity in the terminal differentiation signal: all B cells were terminally differentiated in proportion to the number of class-specific cells present after the switch process.

Terminal Differentiation of Postswitch B Cells Induced by Activated T Cells. The co-cultured T cells activated by staphylococcal protein A with B cells precultured with LPS and switch T cells provided the most dramatic evidence that T cell help is necessary for terminal differentiation of postswitch sIgA-bearing B cells. In these studies we showed that T cells from two different sources, spleen and mesenteric lymph node, have a striking ability to cause LPS-induced B cells precultured with PP cloned T cells (but not spleen cloned T cells) to become IgA-producing cells. As in the case of the cells cultured with T cell and macrophage factors, IgA production does not occur in cells not exposed (in this case pre-exposed) to the switch T cells. The activated (uncloned) T cells appear to act on a B cell population to induce terminal differentiation on the already isotype-determined cells.

Terminal Differentiation of Postswitch B Cells Induced by BCDF. Since protein A itself causes some baseline IgA secretion, we carried out an additional experiment in which B cells were sequentially cultured (as in the second protocol) but the second culture was performed in the presence of BCDF rather than uncloned T cells stimulated by protein A. Here cells were precultured with cloned T cells and LPS and then cultured with BCDF alone. Under these conditions baseline IgA secretion was low in the cultures of B cells that had not been pre-exposed to Peyer's patch cloned T cells. As expected, we found that BCDF induces terminal maturation of IgA-committed postswitch PP B cells (cells pre-exposed to PP cloned T cells and LPS) to IgA-secreting plasma cells. In contrast, B cells precultured with spleen cloned B cells were not induced to produce IgA under the influence of BCDF.

Terminal Differentiation of Postswitch B Cells into IgM- and IgG-Producing B Cells. The effect of uncloned T cells on IgM and IgG production by LPS-induced B cells precultured with the cloned PP and spleen T cells is also of some interest. In the case of IgM production, B cells precultured with PP cloned T

cells increase IgM synthesis when cultured with uncloned T cells and staphylococcal protein A. This could indicate that the already switched B cell population contains residual sIgM-bearing B cells still capable of terminal differentiation into IgM plasma cells, i.e., cells that escape the switch process and yet are not terminally differentiated by LPS alone. Finally, in the case of IgG production by postswitch cells, we see that B cells switched by PP cloned T cells showed no increment in IgG production by addition of uncloned T cells and protein A. This is in keeping with the fact that cells have switched out of the IgG pathway of differentiation entirely. On the other hand, B cells switched by splenic cloned T cells do show enhancement of IgG synthesis by addition of uncloned T cells and protein A. This, together with our previous observation (10, 11) that the spleen cloned T cells induce an increased number of sIgG-bearing cells (but a decrease in IgG-secreting cells), is evidence that the spleen cloned T cells can function as intra-subclass switch T cells.

The data reported here are summarized in Fig. 7. Here we see that switch T cells specific for IgA are located preferentially (but perhaps not exclusively) in PP and influence development of B cells at this site. Having been exposed to switch T cells, the B cells migrate to other lymphoid tissues where they come under the influence of maturation factors produced by helper T cells and/or macrophages (directly or acting through helper T cells). At this point, cells differentiate into IgA-producing plasma cells. The initial intrapatch T cell step appears to be class-specific; the terminal differentiation may also be class-specific, but this remains to be proven.

Summary

Our previous studies indicated that cloned T cells obtained from Peyer's patches (PP) (Lyt-1⁺, 2⁻, Ia⁺, and H-2K/D⁺) evoked immunoglobulin (Ig) class switching of PP B cells from sIgM to sIgA cells *in vitro*; however, these switch T cells could not in themselves provide optimal help for the differentiation of postswitch sIgA-bearing PP B cells to IgA-secreting cells. Thus, in the present report we described studies focused on mechanisms regulating terminal differentiation of the postswitch PP sIgA-bearing B cells. First, to explore the effect of T cell-derived B cell differentiation factor(s) (BCDF) and macrophage factor(s) (MF) on the terminal maturation of PP B cells, LPS-stimulated PP B cells were co-cultured for 7 d with cloned T cells in the presence or absence of the above factors. In the absence of PP cloned T cells the BCDF and MF had only a modest effect on IgA production, whereas in the presence of PP, but not spleen cloned T cells, IgA production was increased. Next, to investigate the effect of T cells derived from a gut-associated lymphoid tissue (GALT), mesenteric lymph nodes (MLN), as well as from spleen on terminal differentiation of postswitch sIgA PP B cells, LPS-driven PP B cells were precultured with the cloned T cells to induce a switch to sIgA, and subsequently cultured with MLN or spleen T cells or a Lyt-2⁺-depleted T cell subset in the presence of a T-dependent polyclonal mitogen, staphylococcal protein A. Alternatively, in the second culture period BCDF alone was added, instead of T cells and protein A. Here it was found that B cells pre-exposed to switch T cells from PP, but not spleen, were induced to produce greatly increased amounts of IgA in the presence of protein A and T

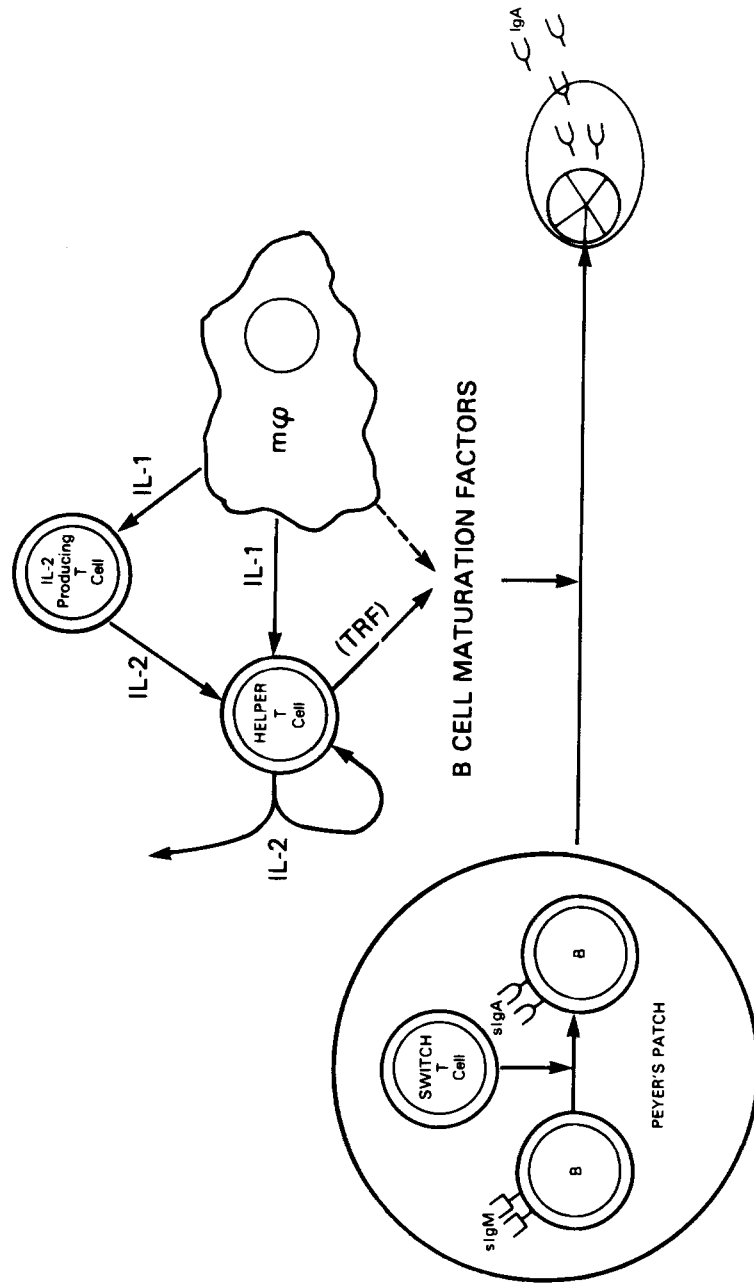


FIGURE 7. Proposed mechanisms, regulating terminal differentiation of postswitch sIgA-bearing PP B cells induced by switch T cells. B cell maturation (differentiation) factors derived from helper T cells and macrophages appear to play a key role of the maturation to IgA-secreting plasma cells.

cells or a Lyt-2⁺-depleted T cell subset as well as in the presence of BCDF alone. Furthermore, in the presence of BCDF alone many B cells expressed cytoplasmic IgA. These observations strongly support the view that the terminal differentiation of postswitch sIgA B cells is governed by helper T cells and macrophages, or factors derived from such cells. Such cells or factors do not affect preswitch B cells.

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