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Differentiation of B lymphocytes in sheep

I. PHENOTYPIC ANALYSIS OF ILEAL PEYER'S PATCH CELLS AND THE DEMONSTRATION OF A PRECURSOR POPULATION FOR sIg$^+$ CELLS IN THE ILEAL PEYER'S PATCHES

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Summary. The ileal Peyer's patches (IPP) of sheep may be a primary lymphoid organ for B cells since they have a number of important features in common with the bursa of Fabricius of chickens. We have examined the surface phenotype of IPP cells. Approximately 90% to 95% of IPP cells are 'low sIgM$^+$'; that is, they have surface IgM, but in much smaller amounts than peripheral B cells, which are 'high sIgM$^+$'. IPP cells with sIgG or sIgA are very rare. Upon exposure to a tumour promotor, phorbol myristate acetate (PMA), *in vitro*, low sIgM$^+$ cells differentiated into high sIgM$^+$cells. The amount of Ia-like antigens on the surface also increased after PMA treatment. Approximately 5% of IPP cells bore no identifiable markers. However, these cells could also be induced into high sIgM$^+$ cells upon exposure to PMA; this may indicate the presence of precursors of sIgM$^+$ cells within the IPP.

While PNA (peanut agglutinin) binds strongly to the vast majority of IPP cells, it binds very little, if at all, to B cells obtained from the periphery, unless they have been treated with neuraminidase; this suggests that cells in the B lineage retain their PNA receptors, but that these become masked by sialic acid on mature B cells.

Abbreviations: cIg, cytoplasmic immunoglobulin; D-MEM, Dulbecco's modified Eagle's medium; FACS, fluorescence activated cell sorter; FITC, fluorescein isothiocyanate; IPP, ileocecal Peyer's patches; JPP, jejunal Peyer's patches; PMA, phorbol myristate acetate; PNA, peanut agglutinin; sIg, surface immunoglobulin; TRITC, tetramethylrhodamine isothiocyanate.

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INTRODUCTION

It has been proposed that the ileocecal or ileal Peyer's patches (IPP) are 'bursa-equivalent' lymphoid tissues in sheep (Gerber, 1979; Reynolds & Morris, 1983). Ovine IPP bear a close histological resemblance to the avian bursa of Fabricius, and the majority of cells in the IPP express surface immunoglobulins (sIg) (Reynolds, 1981; Miyasaka & Dudler, 1984). The removal of a large proportion of the IPP before birth or during the first week of postnatal life resulted in a

severe reduction in the number of circulating B cells in lambs (Gerber, 1979). Lymphopoiesis in the IPP begins during fetal life (Reynolds & Morris, 1983) and the extent of the lymphopoiesis could not be modulated by the introduction of antigens into the intestine (Reynolds & Morris, 1984). Like the bursa of Fabricius, IPP undergoes a significant degree of postnatal involution (Reynolds & Morris, 1983). With the use of an extracorporeal perfusion system, it has been shown that the extent of export of newly-formed cells from the IPP is sufficient to replace the B cell pool within 2–5 days in the peripheral blood of sheep (Reynolds & Pabst, 1984). In contrast to these IPP, jejunal Peyer's patches (JPP) of sheep bear smaller follicles separated by wide T-dependent areas, which are reminiscent of rodent Peyer's patches and may have different functions from the IPP (Reynolds & Morris, 1983).

In this paper, we will show that the dominant isotype of surface immunoglobulin on IPP cells is IgM (low $sIgM^+$) and that the amount of sIg is much less than that of B cells in the periphery (high $sIgM^+$). We will also demonstrate that these low $sIgM^+$ cells can differentiate into high $sIgM^+$ cells *in vitro* and express increased amounts of Ia-like antigens after exposure to a tumour promotor, phorbol myristate acetate (PMA), and furthermore, that cells with no detectable levels of surface immunoglobulin in the IPP (sIg^- cells) can also be induced to become high $sIgM^+$ cells in the presence of PMA, which indicates the presence of precursors of $sIgM^+$ cells within the IPP themselves. We think that these results further reinforce the notion that IPP are 'bursa-equivalent' lymphoid tissues in sheep.

MATERIALS AND METHODS

Animals

White Alpine and Black Jura lambs of both sexes aged 1–3 months old were obtained from Versuchsbetrieb Sennweid, Olsberg, Switzerland.

Reagents

Fluorescein isothiocyanate (FITC) conjugates of $F(ab')_2$ rabbit anti-sheep total-Ig, $F(ab')_2$ goat anti-rabbit Ig and $F(ab')_2$ goat anti-mouse Ig were obtained from Cappel Laboratories (Cochranville, PA). Antisera directed against sheep IgG, IgM and IgA were prepared in rabbits, made class-specific by passage through appropriate immunoadsorbent columns (Gerber, 1979) and the IgG fraction was isolated by chromatography on protein A-Sepharose CL-4B (Pharmacia, Uppsala). Fluorescein (FITC) and rhodamine (TRITC) conjugations were performed under standard conditions (Gerber, 1979). Monoclonal antibody DA6.78, which recognizes β chain of human Ia antigens, was a gift of Dr R. Palacios, Basel Institute for Immunology. Fluoresceinated peanut agglutinin (FITC-PNA) was purchased from Vector Laboratories (Burlingame, CA) and used at a concentration of 0·1 mg/ml; this had been determined by preliminary titrations of the batch (Lot no. 10501) used. Rifampicin and colchicine were obtained from Sigma (St Louis, MO) and used at concentrations of 25 μg/ml and 0·5 μg/ml respectively. Dose response experiments were performed to determine optimal concentrations (data not shown). Phorbol myristate acetate (Sigma, St Louis, MO) was incorporated into cultures at an optimal concentration of 10 ng/ml as described before (Miyasaka & Dudler, 1984).

Preparation of IPP single cell suspension

A segment of terminal ileum (10 cm long) was aseptically resected from lambs and the ileal contents were removed. The ileal segment was exhaustively rinsed in RPMI 1640 medium and non-lymphoid tissues were trimmed off with a scalpel blade. After removing the mucus from the surface of the lumen with a scalpel blade, the ileal segment was rinsed again in RPMI 1640, cut into approximately 3×2 cm pieces and placed in a petri-dish containing RPMI 1640 with 20 μg/ml DNAase (Sigma D0867). Subsequently, the IPP follicles were scraped from the segment, chopped into fine pieces with a scalpel blade and drawn in and out of a 10 ml-pipette several times to make a single cell suspension. Inclusion of DNAase in this procedure minimized cell aggregation. IPP cells were placed on a Percoll discontinuous density gradient (35%/75%) and centrifuged at 1000 ***g*** for 20 min. More than 98% of cells obtained between 35% and 75% Percoll layers were viable as assessed by the trypan blue exclusion assay. Cells were washed twice prior to use.

Cultivation of IPP cells

Cells were cultured overnight in Costar 24-well plates at a concentration of 10^6 cells/ml in 2 ml medium, i.e. D-MEM with 10% FCS, 10 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acid solution (Flow Laboratories, Rockville, MD), 100 U/ml penicillin, 100 μg/ml streptomycin, 10^{-4}M 2-ME and 0·375% sodium bicarbonate. When PMA was in-

cluded in the culture at 10 ng/ml, more than 80% of the IPP cells were viable after overnight incubation (Miyasaka & Dudler, 1984).

Isolation of B cells from lymph

This was achieved by adherence of sIg^+ cells in prescapular efferent lymph to anti-Ig coated plates (Miyasaka *et al.*, 1983). Prescapular efferent lymph was obtained following the method described by Pederson & Morris (1970).

Immunofluorescence staining and FACS sorting

IPP cells were stained and examined by a fluorescence activated cell sorter (FACS II) as described previously (Miyasaka *et al.*, 1983). The FACS was set to exclude dead cells by forward light scatter, and 2×10^4 cells were analysed. Phase- and fluorescence-microscopy on stained cell preparations were also performed to confirm the observations obtained from the FACS. For FACS sorting of 'sIg^-' cells, IPP cells were stained with FITC-$F(ab')_2$ rabbit anti-sheep total-Ig, suspended in Earle's balanced salt solution and sorted for cells without detectable levels of sIg at a rate of 3000 cells per second. The sorted cells were re-run on the FACS to examine the efficiency of the sorting and the profile of cells was shown in Fig. 4. For PNA staining, cells were incubated with 100 μg/ml of FITC-PNA for 15 min at 4° and washed twice with RPMI 1640. Cells were examined with a Leitz Orthoplan fluorescence microscope. For cytoplasmic Ig staining, cells were suspended at a concentration of 2×10^6/ml in 10% FCS in D-MEM, deposited on slides using a cytocentrifuge (Shandon Instruments, London, England), air dried and fixed for 20 min in 5% glacial acetic acid, 95% absolute ethanol, at −20°. The slides were then washed in PBS and incubated with 50 μl TRITC-rabbit anti-sheep Ig for 30 min in a humidified chamber at room temperature. The slides were washed for 5 min each in three batches of PBS at room temperature and then washed overnight in PBS at 4°. Slide preparations were mounted in veronal buffered glycerol and examined with a fluorescence microscope.

Neuraminidase treatment of B cells

B cells obtained from lymph as above were incubated with 0·002 U/ml of neuraminidase from Vibrio cholerae (Behring, Hoechst Pharma AG, Zürich) in D-MEM for 15 min at 37° and washed twice before use.

Removal of macrophages from IPP cell suspension

IPP cells were passed through a column of Sephadex G-10 (Ly & Mishell, 1974). Effluent cell population was stained for α-naphthyl acetate esterase (Yam, Li & Crosby, 1971) to examine the efficiency of the removal of macrophages.

RESULTS

Surface phenotype of IPP cells

FACS analysis of IPP cells stained with fluoresceinated anti-sheep Ig or Ig class specific antisera revealed that IgM was the dominant immunoglobulin isotype expressed on the surface of IPP cells (Fig. 1). The fluorescence profile of IPP cells stained with anti-total Ig was almost identical to that obtained by anti-μ staining, and very few sIg^+ cells were found when the IPP cells were stained by anti-γ or anti-α chain-specific serum. SIgM was detected on 85–90% of IPP cells, but the amount of sIgM was much less than that of B cells from lymph (Fig. 2). These differences were confirmed by using a fluorescence microscope. All peripheral B cells were brightly stained for sIgM (high $sIgM^+$), whereas IPP cells were very dim (low $sIgM^+$) and the fluorescence quickly faded away from the cell surface upon exposure to UV light.

Antibody DA6.78, specific for the β chain of human Ia antigens, cross-reacted with ovine B cells. It recognizes non-Ig determinants on ovine B cells, and preliminary investigations on the tissue distribution of positive cells for this antibody suggested that this antibody detects Ia-like antigens on cell surface (sIa-like antigens) in sheep. This antibody stained the majority of IPP cells (Fig. 1) but the staining intensity was noticeably less than that of peripheral B cells (Fig. 2).

As shown in Table 1, almost all IPP cells showed peanut agglutinin (PNA) binding, whereas very few sIg^+ cells obtained from efferent lymph were stained with FITC-PNA. However, when the B cells from lymph were treated with neuraminidase, conspicuous PNA binding was observed.

Increased expression of sIgM and sIa-like antigens on IPP cells after exposure to phorbol myristate acetate (PMA)

When IPP cells were cultured overnight in the presence of 10 ng/ml of phorbol myristate acetate (PMA) (a tumour promoter, known to enhance or modulate the

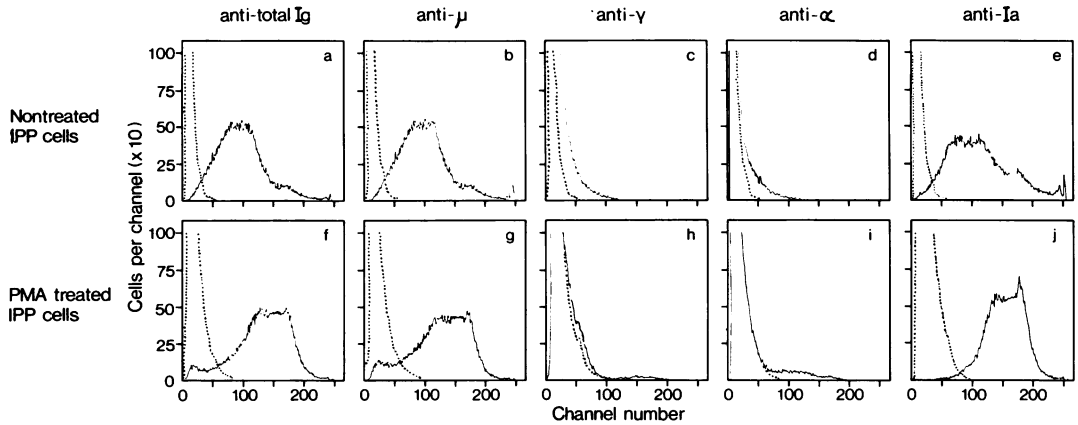

Figure 1. FACS profiles of IPP cells stained with various antibodies before and after PMA treatment. Profiles in the upper and lower rows are those of non-treated and PMA-treated IPP cells, respectively. Cells were incubated with (a) and (f), FITC-anti-sheep total-Ig; (b) and (g), anti-μ, (c) and (h), anti-γ; (d) and (i), anti-α; (e) and (j), anti-human Ia monoclonal antibody DA 6.78. For μ, γ, α staining, bound antibody was detected by reaction with FITC-F(ab')$_2$ goat anti-rabbit Ig, and for Ia staining, FITC-F(ab')$_2$ goat anti-mouse Ig was used as a second antibody. These profiles were superimposed with a fluorescence profile (- - - - -) obtained on unstained cells for direct immunofluorescence, or on cells incubated with an appropriate second antibody for indirect immunofluorescence. Logarithmic fluorescence intensity is plotted on the abscissa in arbitrary units, and linear cell frequency on the ordinate.

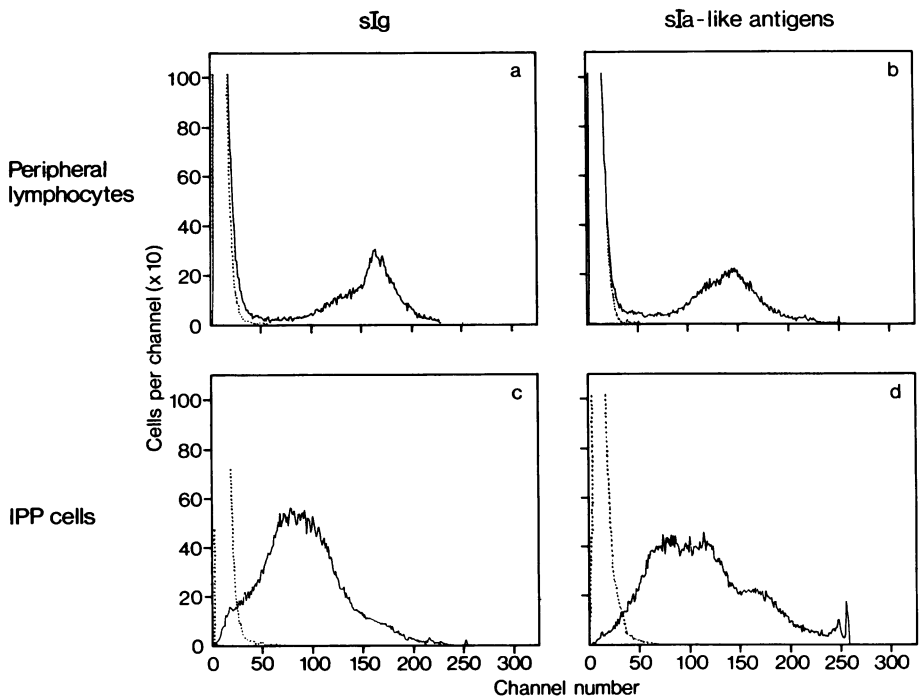

Figure 2. Comparison of sIg- and sIa-like antigens on B cells from lymph and IPP cells. Prescapular efferent lymph lymphocytes were stained for (a) sIg and (b) sIa-like antigens. IPP cells were also stained for (c) sIg and (d) sIa-like antigens under the same conditions. Logarithmic fluorescence intensity is plotted on the abscissa in arbitrary units, and linear cell frequency on the ordinate.

Table 1. FITC-PNA binding to ovine B cells

	Percentage positive cells§
IPP cells	96·8% (+++)*
B cells from lymph†	24·3% (± ~ +)
B cells from lymph treated with neuraminidase‡	100·0% (+++)

* The values in parentheses refer to the relative intensity of fluorescence observed.

† Obtained by 'Ig panning' of prescapular efferent lymph lymphocytes. More than 95% of the cells were sIg^+.

‡ Cells obtained as above were treated with 0·002 U/ml of neuraminidase for 15 min at 37° and washed twice before FITC-PNA staining.

§ The experiment was repeated twice and similar figures were obtained in both experiments.

differentiation of various cell types), the sIg expression increased substantially and the prominent increase was restricted largely to sIgM with only minimal increase in sIgG and sIgA expressions (Fig. 1). The fluorescence intensity of IPP cells exposed to PMA and stained for IgM increased to almost the same level as that of B cells from lymph (Figs 1, 2).

Exposure of IPP cells to PMA also induced an increased expression of sIa-like antigens (Fig. 1) and the extent of increase was very similar to that observed in sIg staining.

IPP cells were passed through a Sephadex G-10 column to remove macrophages, and the proportion of macrophages detected by non-specific esterase staining was reduced to less than 0·5% of the total cells in the effluent collection. However, as seen in Fig. 3, this procedure did not prevent the IPP cells cultured with PMA from expressing levels of sIg and sIa-like antigens similar to those of B cells in lymph.

The addition of anti-mitotic drugs such as rifampicin or colchicine into the cultures slightly modified the expression of sIg and sIa-like antigens on the IPP that were incubated with PMA, but the PMA-treated IPP cells still showed a noticeable increase in sIg and sIa-like antigenic determinants (Fig. 3).

Presence of sIg^- IPP cells which can express sIgM upon exposure to PMA

Whilst the majority of the cells in IPPs express sIgM as shown above, approximately 5% of IPP cells carry no identifiable surface markers. This population of cells was enriched by removing sIg^+ cells which had been stained for sIg by cell-sorting on a FACS (Fig. 4). Less than 5% of the cells obtained by this method bore detectable sIg. However, following overnight culture of these cells in the presence of 10 ng/ml of PMA, the proportion of cell population that expressed large amounts of sIgM increased to 50% to 60% (Fig. 4). When these sIg^- cells were fixed and stained with FITC-rabbit anti-sheep Ig, no cytoplasmic Ig was detected.

DISCUSSION

In this paper, we have characterized the surface phenotype of ileal Peyer's patch (IPP) cells in sheep. Ovine IPP bear characteristics compatible with those of a primary lymphoid organ for B cells. Substantial lymphoid development originates in embryonic life when there is no external antigen available (Reynolds & Morris, 1983) and the postnatal persistence of IPP is limited, i.e. they involute by 18 months after birth (Reynolds & Morris, 1983). The great majority of IPP cells are B cells (Reynolds, 1981; Miyasaka & Dudler, 1984), T cells representing less than 2% of the total population (Miyasaka *et al.*, 1983). The effect of early removal causes a drastic reduction in the number of peripheral B cells (Gerber, 1979). Although B cell-poiesis is extensive, plasmacytopoiesis *in situ* is insignificant throughout their existing period (Gerber, 1979).

The experiments presented in this paper confirm and extend our preliminary report on sIg expression by IPP cells (Miyasaka & Dudler, 1983). We have demonstrated that the majority of IPP cells bore small amount of sIgM, less than 10% being $sIgG^+$ or $sIgA^+$.

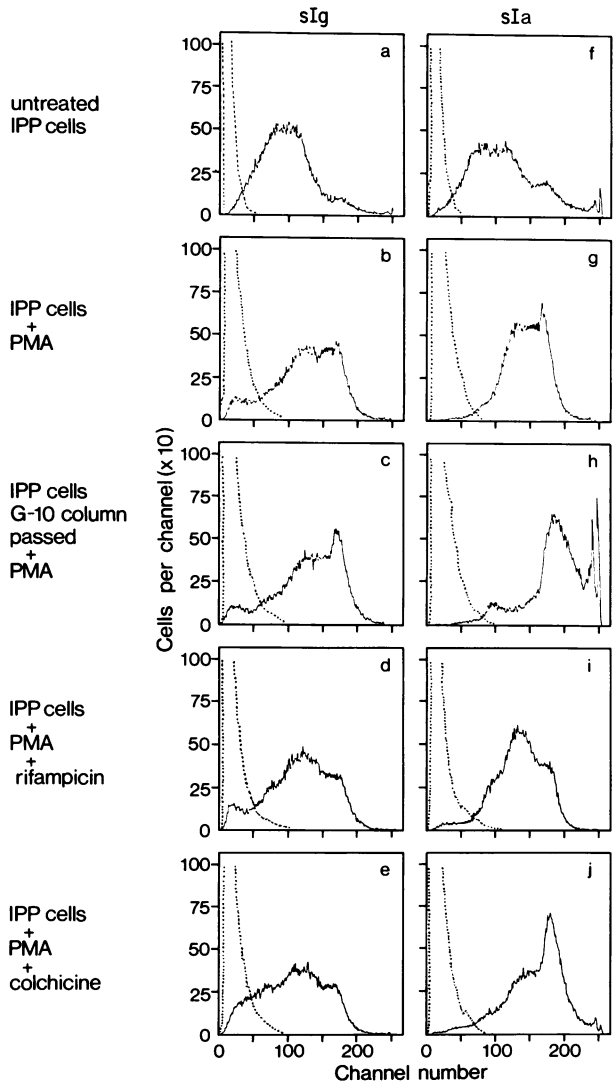

Figure 3. Effects of the removal of macrophages and of anti-mitotic drugs on the expressions of sIg- and sIa-like antigens by PMA-stimulated IPP cells. Non-cultured or cultured IPP cells were stained for sIg in profiles (a)–(e) and sIa-like antigens in profiles (f)–(j). Profiles (a) and (f), non-treated IPP cells; (b) and (g), IPP cells cultured overnight in the presence of 10 ng/ml PMA; (c) and (h), IPP cells were passed through a G-10 column and cultured with PMA; (d) and (i), IPP cells were cultured with PMA and 25 μg/ml rifampicin; (d) and (j), IPP cells were cultured with PMA and 0·5 μg/ml colchicine. Logarithmic fluorescence intensity is plotted on the abscissa in arbitrary units, and linear cell frequency on the ordinate.

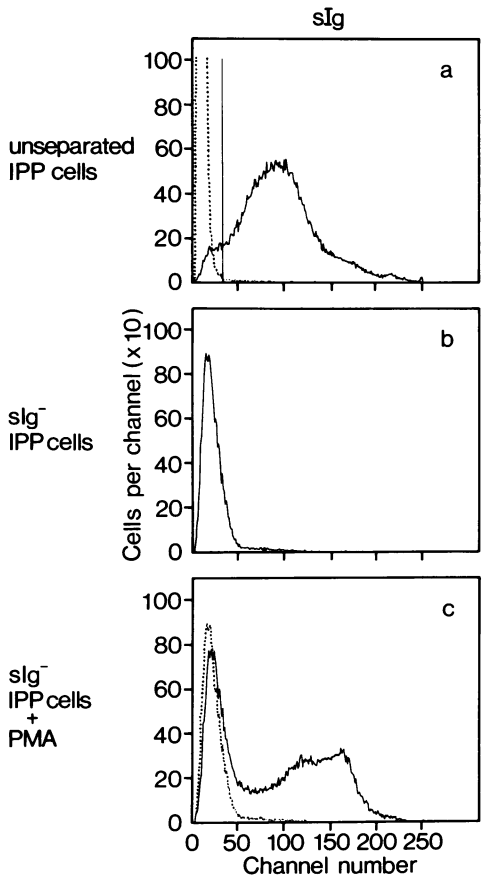

Figure 4. Appearance of sIgM$^+$ cells in 'sIg$^-$' IPP cell populations after overnight culture in the presence of PMA. FACS profile of (a) unfractionated IPP cells stained for sIgM, (b) IPP cells without detectable sIg (sIg$^-$ cells) enriched by a FACS (c) sIg$^-$ cells cultured overnight in the presence of 10 ng/ml PMA and subsequently stained for sIgM. Profile (b) is superimposed on (c) as a dotted line. Note the appearance of a high sIgM$^+$ cell population in (c).

Similar observations have been made with cells in the bursa of Fabricius of chickens (Lydgard, Grossi & Cooper, 1976) and the bone marrow of mice (Osmond & Nossal, 1974), both of which are the main site for B cell production in respective species.

The apparent dominance of sIgM in heavy-chain expression by IPP cells contrasts strikingly with the situation reported on mouse Peyer's patch cells (Butcher *et al.*, 1982) where the majority of follicular cells are apparently sIgA$^+$; only 10–20% were sIgM$^+$ or sIgG$^+$. The histology of the Peyer's patches in the mouse is quite different from that of the sheep IPP, but resembles jejunal Peyer's patches (JPP) which may have different functions from IPP (Reynolds & Morris, 1984). Work is in progress to examine the surface phenotype of JPP cells. As with the follicular cells in the mouse PP (Butcher *et al.*, 1982), almost all IPP cells showed very intense binding of galactose-specific lectin, peanut agglutinin (PNA). On the other hand, peripheral B cells showed very little, if any, PNA binding unless they were treated with neuraminidase; this suggests that cells in the B lineage in the sheep retain PNA receptors, but that these become masked by sialic acid on mature B cells.

IPP cells also bore sIa-like antigens which could be detected by a mouse anti-human Ia monoclonal antibody DA6.78. Although both sIg and sIa-like antigens were present on IPP cells at much lower levels than those found on B cells from lymph, treatment of IPP cells with PMA increased the amount of both markers per cell to the degree that was found on B cells from lymph. PMA is known to modulate or augment expression of the differentiation of various cell types (Weinstein *et al.*, 1981). We have previously shown that PMA-treated IPP cells not only expressed increased amounts of sIg, but also secreted IgM (Miyasaka & Dudler, 1984); it therefore appears that PMA induces the terminal differentiation of the IPP cells. Activation of normal and malignant B cells from other species by PMA have also been reported (Sugawara, 1982; Tötterman, Nilsson & Sundström, 1983).

Macrophages seem not to be required for the increased expression of both differentiation-associated cell markers reported here, since IPP cells passed through G-10 columns also showed similar increases in both sIg and sIa-like antigen expressions upon culture with PMA. Colchicine and rifampicin treatment had little influence on expressions of sIg and sIa-like antigens. The addition of an inhibitor of DNA synthesis, actinomycin D, in the culture killed all the cells, and we could not assess whether DNA synthesis is required for the rapid induction of both markers on the cell surface (data not shown). However, we have previously demonstrated that PMA-induced changes in the expression of surface markers by IPP cells took place without detectable increases in the cell number in culture (Miyasaka & Dudler, 1984) and, taken together, cell proliferation does not seem to be a prerequisite for this phenomenon.

PMA also induced sIg on a small population of cells which had not possessed detectable levels of sIg (sIg$^-$ cells). This cannot be easily explained by selective proliferation of cells that contaminated the sIg$^-$

population, since the proportion of cells with detectable levels of sIg in the sIg^- cell population was less than 5% of the total population, and the cultivation period was approximately 16 hr. *In vitro* induction of sIg on cells without detectable levels of sIg has been reported with bone marrow lymphocytes (Ryser & Vassalli, 1974; Hämmerling, Chin & Abbott, 1976) this has often been interpreted as an indication of the presence of B cell precursors in the bone marrow in other species. We do not know whether this sIg^- cell population observed in IPP comprises a precursor population of sIg^+ IPP cells, some of which may eventually become mature B cells in the periphery (Reynolds & Pabst, 1984), or whether they immediately become peripheral sIg^+ B cells by-passing the sIg^+ stage in IPP. The latter possibility particularly implies that the majority of IPP cells are end cells and die *in situ*, as has been believed for cortical thymocytes (McPhee, Pye & Shortman, 1979). IPP cells die quickly in *in vitro* culture (Miyasaka & Dudler, 1984), similar to cortical thymocytes, and extensive pyknotic figures are observed in IPPs *in vivo* (Reynolds, 1981). However, our preliminary experiments to examine the phenotype of migratory cells from IPP into the periphery suggest that the migrants do carry sIg, although much smaller amounts than peripheral B cells, on the cell surface (Miyasaka *et al.*, 1984), which speaks against the latter possibility.

Currently, it is a matter of speculation as to where these sIg^- cells should be placed in the maturation sequence of B cells. Available evidence, albeit indirect, suggests that cells characterized by the presence of cytoplasmic μ chains ($c\mu^+$ or cIg^+) without detectable sIg (sIg^-) are precursors of B cells (pre-B cells) in other species (Raff *et al.*, 1976; Landreth, Rosse & Clagett, 1981; Gathings, Lawton & Cooper, 1977; McElroy, Willcox & Catty, 1981). However, cIg was not detectable in sIg^- IPP cells when examined by direct immunofluorescence on fixed cells, and they seem to differ from the pre-B cells.

If it is accepted that IPP are a 'bursa-equivalent' lymphoid organ in sheep, an obvious question would be the role of bone marrow in relation to B lymphocyte production in this species. Ovine bone marrow is not very lymphocytic; unlike murine bone marrow, less than 5% of the total nucleated cells are lymphoid (M. Miyasaka, L. Dudler, J. Reynolds & D. G. Osmond, unpublished observations). Although bone marrow precedes IPP in development and persists even after IPP have involuted, it remains to be demonstrated whether or not cells which colonize and form IPP come from the bone marrow, and whether bone marrow takes over the role of IPP in B cell genesis.

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