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Human peripheral blood B cell subpopulations: surface IgD$^+$ cells respond to pokeweed mitogen with plasma cell differentiation

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SUMMARY

Human peripheral blood (PB) mononuclear cells were depleted of T cells and subsequently of monocytes by counterflow centrifugation. The resulting B cell enriched fraction contained $<3\%$ T cells, $1 \pm 1\%$ monocytes and $71 \pm 6\%$ B cells. Approximately 56% of the B cells carried surface IgM (sIgM) and sIgD, the remaining 44% carried only one isotype (IgG, IgA, IgM or IgD). Using anti-δ or anti-μ-coated Ox red blood cells (OxRBC) the B cell enriched fraction was separated into a sIgD$^+$ or sIgM$^+$ and sIgD$^-$ or sIgM$^-$ fraction. The sIgD$^+$ as well as the sIgM$^+$ fraction responded (partially) to pokeweed mitogen (PWM) with plasma cell differentiation into cytoplasmic IgM (cIgM) containing cells. Cytoplasmic IgD$^+$ cells were occasionally found in the sIgD$^+$ or sIgM$^-$ fraction, but never in the sIgM$^+$ fraction. Cells containing cIgG or cIgA were only found after stimulation of the sIgM$^-$ or sIgD$^-$ fraction, the latter also produced cIgM$^+$ cells. In a second separation step of the sIgD$^-$ and sIgM$^-$ fraction with anti-α or anti-γ-coated OxRBC it was shown that the cIgA$^+$ and cIgG$^+$ cells were derived from B cells expressing only sIgA or sIgG respectively.

In conclusion, two major B cell subpopulations can be found in PB: a sIgM$^+$D$^+$ fraction, which gives rise to only cIgM$^+$ plasma cells after PWM stimulation and a second population of B cells which express one heavy-chain isotype on their surface. These cells not only differentiate on PWM stimulation, but they also show a strong proliferative response.

Keywords human B cells counterflow-elutriator plasma cell differentiation pokeweed mitogen.

INTRODUCTION

The majority of human peripheral blood (PB) B cells coexpress surface IgM (sIgM) and sigD. These sIgM$^+$D$^+$ cells are usually referred to as virgin B cells in analogy to the mouse system (Coffmann & Cohn, 1977). B cells expressing sIgG or sigA have also been described, but only a small part of them coexpress sIgM (Vessière-Louveaux, Hijmans & Schuit, 1981; Saiki & Ralph, 1982b). B cells coexpressing sIgG and sIgA have never been found in PB, though in tonsils they can be found (Mudde, Verberne & De Gast, 1984a). PB B cells expressing only one isotype are called memory B cells, including the sIgM$^+$D$^-$ B cells (Coffmann & Cohn, 1977; Vessièrre-Louveaux, Hijmans & Schuit, 1981; Saiki & Ralph, 1982a; Gathings, Lawton & Cooper, 1977; Okumura *et al.*, 1976;

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Lucivero *et al.*, 1981; Rowe *et al.*, 1973; Kuritani & Cooper, 1982a; Knapp *et al.*, 1973; Levitt & Dagg, 1981). By in vitro stimulation with the T cell dependent polyclonal B cell activator pokeweed mitogen (PWM) B cells can be driven into plasma cell differentiation. The plasma cells obtained contain only one heavy-chain class (Saiki & Ralph, 1982a; Lucivero *et al.*, 1981; Kuritani & Cooper, 1982a; Levitt & Dagg, 1981; Saiki & Ralph, 1982b). Contradictory results have been published about the phenotype of the precursors of these different plasma cells (Saiki & Ralph, 1982a; Ault & Towle, 1981; Kuritani & Cooper, 1982b). The source of B cells is probably of great importance in studying the relationship between sIg expression and plasma cell differentiation. Ault and Towle (1981) have shown that in human spleen, plasma cells secreting IgM could be derived from $sIgG^+$ B cells and in a previous study we have shown that in tonsils $sIgM^+G^+$ B cells can differentiate into $cIgM^+$, $cIgG^+$ or $cIgA^+$ plasma cells (Mudde *et al.*, 1984a). Studies on PB non T cells indicated that $cIgG^+$ or $cIgA^+$ plasma cells were exclusively derived from B cells expressing only sIgG and sIgA respectively (Knapp *et al.*, 1973). Based on studies of Kuritani & Cooper (1982b, c) PWM was believed to be a mitogen for relatively mature ($sIgD^-$) B cells, though $sIgD^+$ cells from tonsils clearly responded to PWM with ^{3}H-thymidine uptake (Mudde *et al.*, 1984b).

In the present study we reinvestigated the target B cell population for PWM stimulation in relation to sIg expression, using highly purified PB B cells.

MATERIALS AND METHODS

Coating of ox red blood cells (OxRBC). OxRBC were coated with immunosorbent purified goat anti-human Ig, anti-human heavy-chain (μ, δ, γ, α) or anti-human light-chain (κ, λ) antibodies (Tago Inc., Burlingame, USA) using the $CrCl_3$ method (De Gast & Platts-Mills, 1979). After removing the buffy coat, 50 μl of packed OxRBC were mixed with 100 μl antiserum (1 mg/ml) and 0·8 ml saline. By using a vortex mixer 1·0 ml of $CrCl_3$ dilution was added dropwise. After 10 min the reaction was stopped with phosphate buffered saline (PBS). The coated OxRBC were washed twice with PBS and finally suspended in minimal essential medium (MEM) (GIBCO, Grand Island, NY, USA) with 5% fetal calf serum (FCS). Specificity of the anti-μ, anti-δ, anti-γ and anti-α-coated OxRBC has been reported (Mudde *et al.*, 1984a; Logtenberg & De Gast, 1981).

Cell population (a) *PB non T cells.* Heparinized, white blood cells suspensions, White blood cell suspensions, equivalent to 2·5 l blood, were obtained from healthy, adult donors undergoing leukapheresis on a Haemonetics 30 blood processor. After dilution with MEM in a 1:1 ratio, a mononuclear cell suspension was isolated by density gradient centrifugation for 20 min 1000 ***g*** at 20°C on Ficoll Isopaque, d = 1·077 (Pharmacia, Uppsala, Sweden), T and non T cells were separated using rosette formation with 2-aminoethylisothiouronium bromide hyrobromide (AET, Sigma, Chemical Co. St Louis, MO, USA) treated sheep red blood cells (SRBC) and gradient centrifugation. The non T cells were washed twice with MEM and resuspended in 10–20 ml MEM.

(b) *Counterflow centrifugation PB non T cells.* After counting in a haemocytometer between 4×10^8 and and 8×10^8 non T cells were introduced into a Beckmann J 21 C centrifuge equipped with a JE-6 elutriator rotor. Counterflow centrifugation of the non T cell suspension was performed as described by Figdor *et al.* (1981) Counterflow centrifugation was carried out under sterile conditions in isotonic PBS, containing 3·8% sodium citrate, 0·1% glucose and 0·2% bovine serum albumin (BSA). The counterflow system was cooled to 4°C. The flowrate was kept constant at 18 ml/min.

The non T cell suspension contained large numbers of blood platelets. In order to remove them the non T cell suspension was introduced at a rotorspeed of 3100 rpm. Further fractionation was accomplished by stepwise decreasing the rotorspeed to 2600, 2450, 2300 and 0 rpm. At each rotorspeed fractions with a volume of 150 ml were collected, washed twice with MEM and counted. The fractions were characterised by staining cytocentrifuge preparations for May-Gründwald Giemsa and non specific esterase.

(c) *Separation of elutriated PB non T cells on sIg.* The lymphocyte enriched 'PB non T 2600' fraction (Table 1) was mixed with anti-μ or anti-δ-coated OxRBC in a ratio of 1:30. This mixture was centrifuged for 5 min 255 ***g*** at 0°C. After resuspension the cells were separated by gradient centrifugation on Ficoll Isopaque (d = 1·077) for 20 min 1000 ***g*** at 20°C. The interface contained the

sIgM$^-$ or sIgD$^-$ cells while the pellet contained rosettes formed by the sIgM$^+$ or sIgD$^+$ cells. The fractions were washed twice with MEM, shocked with NH_4Cl, pH 7·4, during 10 min at 0°C and subsequently washed twice with ice cold MEM.

The cells of the sIgM$^-$ and sIgD$^-$ fraction, which were used for further separation, were mixed with anti-γ or anti-α-coated OxRBC respectively and the procedure as described for the separation on sIgM or sIgD was followed, resulting in four fractions (sIgM$^-$G$^+$, sIgM$^-$G$^-$, sIgD$^-$A$^+$, sIgD$^-$A$^-$).

(d) *T cell and monocyte suspensions.* PB-T cells forming rosettes with AET treated SRBC were shocked with NH_4Cl as described above. The elutriated PB non T cell fraction '2300' (Table 2) was used as monocyte suspension. Both T cell and monocyte suspensions were irradiated (2000 rad) to block any proliferation during cell culture.

Identification of sIg on B lymphocytes. sIg was detected by the rosette test. For this test, 0·1 ml mononuclear cells (3×10^6/ml) were mixed with 50 μl coated OxRBC (3×10^8/ml) and 50 μl MEM containing 4% BSA. After centrifugating this mixture for 5 min, 225 ***g*** at 0°C the pellet was resuspended on a vortex mixer. The percentage of sIg$^+$ cells was determined by counting 400 cells in a haemocytometer after addition of brilliant cresyl blue. Only cells binding three or more erythrocytes were considered positive.

Cell cultures. To study cytoplasmic immunoglobulin production induced by PWM (GIBCO, Grand Island, NY, USA), non T cell fractions were cultured in 5 ml tubes (Greiner, Mürtingen, FRG), containing 1 ml RPMI 1640 (GIBCO, Grand Island, NY, USA) to which were added 20% human AB serum, penicillin (100 iu/ml), streptomycin (100 μg/ml) and glutamine (2 mM), with 5×10^5 separated non T cells, 5×10^5 irradiated T cells and 5×10^4 irradiated monocytes. Optimal numbers of plasma cells were obtained with 50×10^6 g/ml PWM. T cells and monocytes were added, because PWM stimulation of B cells has been reported to be dependent of these cells (Janossy & Greaves, 1975; Rozenberg & Lipsky, 1979; Keighty, Lawton & Cooper, 1976; Janossy *et al.*, 1977). On day 5 of the culture period, which was optimal for the total percentage of plasma cells and cell viability, the cells were harvested. Subsequently they were counted in a haemocytometer and brought to a concentration of 1×10^6 cells/ml. Viable cells were determined using the trypan blue exclusion method.

Detection of cIg. Cells of each cultured fraction were spun down onto glass slides, air dried and fixed in cold ethanol-acetic acid. The percentage of cIg$^+$ cells was determined by immunofluorescence, using fluorescein isothiocyanate (Fitc) conjugated rabbit or sheep anti-human heavy-chain antibodies (Dakopatt, Glostrup, Denmark). At least 600 cells were counted on each slide.

RESULTS

Counterflow centrifugation of PB white blood cells

In Table 1 the results are summarized of seven experiments concerning the composition of PB non T

Table 1. Distribution of white blood cells in human peripheral blood non T cell fractions after counterflow centrifugation

Fraction	Recovered cells (%)	Cell diameter (range) (μm)	Granulocytes (%)	Lymphocytes (%)	Monocytes (%)	Esterase pos (%)
Unsep. non T	100	11·6±2·0 (7–15)	8±1	30±2	63±2	54±2
3,100 rpm* (1,540 *g*)	4±1	nd	0	93±5	7±5	8±6
2,600 rpm (800 *g*)	18±1	9·4±0·7 (7–10)	0	99±1	1±1	1±1
2,450 rpm (710 *g*)	13±2	11·2±0·9 (8–12)	5±2	52±7	43±7	41±8
2,300 rpm (630 *g*)	24±4	12·6±1·2 (9–15)	9±3	7±2	84±3	77±4
0 rpm	41±6	nd	13±3	4±1	83±2	74±2

* Flow rate 18 ml/min.
Results are presented as mean ±s.e. of seven experiments.
nd Not done.

Table 2. Surface Ig pattern of different non T cell fractions from PB

Fraction	*n*	sIg$^+$ (%)	sκ^+ (%)	sλ^+ (%)	sμ^+ (%)	sδ^+ (%)	sγ^+ (%)	sα^+ (%)
PB non T	7	77±2	62±3	53±3	34±11	21±4	60±2	37±6
PB non T '2600'	7	71±6	45±4	28±3	40±8	38±5	16±4	10±2
PB sIgD$^+$ '2600'	3	nd	nd	nd	83±5	84±4	nd	nd
PB sIgD$^-$ '2600'	3	nd	nd	nd	3±1	0	9±2	8±2
PB sIgM$^+$ '2600'	3	nd	nd	nd	96±2	nd	nd	nd
PB sIgM$^-$ '2600'	3	nd	nd	nd	2±1	14±6	18±2	12±3

Results are presented as mean ±s.d. of *n* experiments.
nd Not done.

cell fractions before and after counterflow centrifugation. Before counterflow centrifugation 63±2% of the non T cells were monocytes as determined by morphology in May-Grünwald Giemsa stained cytospin preparations. Only 30±2% of the cells were lymphocytes and 8±1% of the cells were granulocytes. On cytospin preparations the mean diameter of the cells was 11·6±2·0 μm. In the first step the platelets were removed at a rotor speed of 3,100 rpm. In the second step the rotorspeed was decreased to 2,600 rpm and at this speed an almost pure lymphocyte fraction was isolated only contaminated by 1% monocytes. The mean diameter of the cells in this fraction was 9·4±0·7 μm. At a rotorspeed of 2450 rpm an intermediate fraction containing both lymphocytes (52±7%) and monocytes (43±7%) as well as some granulocytes (52%) was isolated. The mean diameter was 11·2±0·9 μm. In the next fraction (rotor speed 2,300 rpm) the majority of the cells consisted of monocytes (84±3%), only 7±2% contaminating lymphocytes and 9±3% granulocytes were found (mean cell diameter: 12·6±1·2 μm). After irradiation (2,000 rad) this fraction was used for the additional 5% monocytes needed for optimal PWM stimulation (Rozenberg & Lipsky, 1979).

Surface Ig pattern of different non T cell fractions

In Table 2 the distribution is shown of the various heavy-chain isotypes in the different isolated non T cell fractions. The original PB non T cell fraction (before counterflow centrifugation) contained about 75% sIg$^+$ cells, 62±3% was sκ^+ and 53±3 was sλ^+. Summation of the percentage of sκ^+ + sλ^+ cells shows that cells carrying both the κ light chain and the λ light chain were present in this fraction. In the 'PB non T 2,600' fraction also 71±6% of the cells were sIg$^+$ but in this case a summation of sκ^+ + sλ cells (73±5%) equalled the percentage of sIg$^+$ cells, indicating that cells were either sκ^+ or sλ^+. The removal of monocytes from the PB non T cell fraction is accompanied by a decrease in sIgG and sIgA expression in the 'PB non T 2,600' fraction. The percentage of sκ^+ and sλ^+ cells is decreased, and now a summation sκ^+ + sλ^+ cells equals the number of sIg cells.

The results of the separations based on sIg expression of the B cells in the 'PB non T 2,600' fraction are also shown in Table 2. In these experiments the non T cells were separated into a sIgD$^+$/sIgD$^-$ fraction. The sIgD$^+$ fraction contained 84±4% sδ^+ cells and 83+4% sμ^+ cells. No stable rosettes were formed with anti-γ or anti-α-coated OxRBC. The sIgD$^-$ fraction was completely devoid of sδ^+ cells, and only 20±4% of the cells carried sIg, which was equal to the sum of the percentages of sμ^+ (3±1%), sγ^+ (9±2%) and sα^+ (8±2%) cells, indicating that in the sIgD$^-$ fraction only cells expressing one isotope at the time were present. In three other experiments the 'PB non T 2600' fractions were separated into a sIgM$^+$ and sIgM$^-$ fraction. The sIgM$^+$ fraction was extremely pure, 96±2% was sμ^+ but no stable rosettes could be found with anti-γ or anti-α-coated OxRBC. Anti-δ-coated OxRBC produced only 50% stable rosettes (data not shown). In the sIgM$^-$ fraction 42±9% of the cells were B cells carrying either sδ (14±6%), sγ (18±2%), or sα (12±3%). Only 2±1% of the cells still expressed sμ.

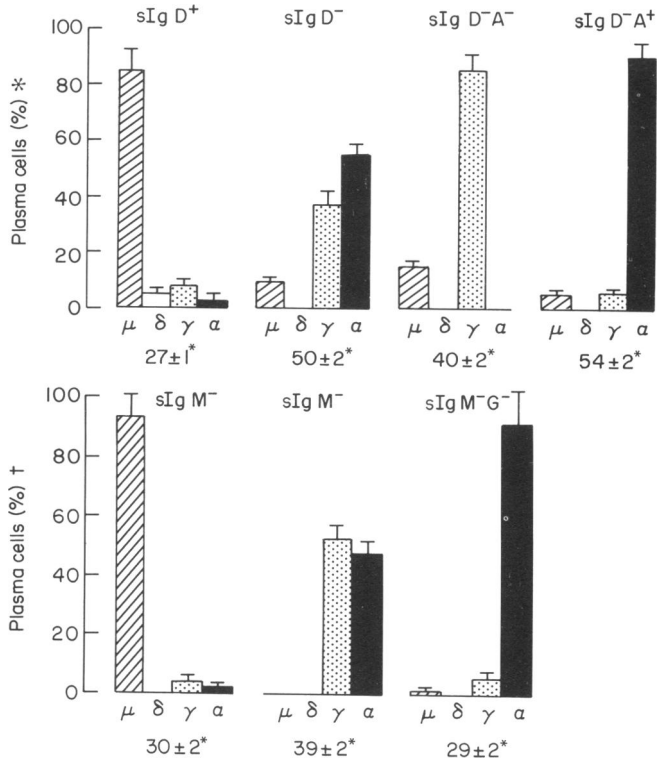

Fig. 1. Plasma cell differentiation of a separated non T cell fraction after PWM stimulation. (a) sIgD$^+$/sIgD$^-$; (b) sIgM$^+$/sIgM$^-$. Results are presented as mean ±s.d. of at least 600 counted cells. * Total percentage of plasma cells (cμ^+ + cδ^+ + cγ^+ + cα^+). † Distribution isotypes (cμ^+, cδ^+, cγ^+, cα^+), within total numbers of plasma cells (set at 100%).

Plasma cell differentiation separated B cell fractions

In Fig. 1a the results are shown of an example of experiments in which the 'PB non T 2600' fraction was separated into first a sIgD$^+$/sIgD$^-$ fraction and in a second step into a sIgD$^-$A$^-$/sIgD$^-$A$^+$ fraction and stimulated with PWM. The sIgD$^+$ fraction produced 27 ± 1% cIg$^+$ cells, most of which were cIgM$^+$ (85%). Some cIgD$^+$ (5%), cIg$^+$ (7%) and cIgA$^+$ cells (3%) were also found. The sIgD$^-$ fraction produced 50 ± 2% cIg$^+$, distributed over cIgG$^+$ cells (37%) and cIgA$^+$ (54%). No cIgD$^+$ cells were found in this fraction and only a small percentage of the cIg$^+$ cells contained cIgM (9%). After further separation of sIgD$^-$ cells into sIgA$^+$/sIgA$^-$ cells, it was shown that the cIgA$^+$ cells were only found in the sIgD$^-$A$^+$ fraction whereas the majority of the cIgM$^+$ or cIgG$^+$ cells was found in the sigD$^-$A$^-$ fraction.

The same kind of experiment was performed on sIgM$^+$ and sIgM$^-$ fractions of which the latter was separated into sIgG$^+$/sIgG$^-$ cells in a second isolation step (Fig. 1b). The sIgM$^+$ fraction produced 30 ± 2% cIg$^+$ cells of which 94% was cIgM$^+$. Cytoplasmic IgD$^+$ cells were never found in this fraction, and only very small percentages of cIgG$^+$ cells (4%) or cIgA$^+$ cells (2%) were found. In the sIgM$^-$ fraction 39 ± 2% cIg$^+$ cells were found, distributed over cIgG$^+$ cells (53%) and cIgA$^+$ cells (47%). Dependent on the donor occasionally some of the cIg$^+$ cells contained cIgD (0–5%) (data not shown). After the second step isolation it appeared that all cIgA$^+$ cells were found in the sIgM$^-$G$^-$ fraction. The sIgM$^-$G$^+$ was too small to be used for stimulation experiments.

In Fig. 2 the results are shown of the recombination experiments. In order to detect possible influences of the rosette formation or separation technique, 'PB non T 2,600' cells were separated on the basis of sIg expression and then recombined. Both the recombined and unseparated 'PB non T

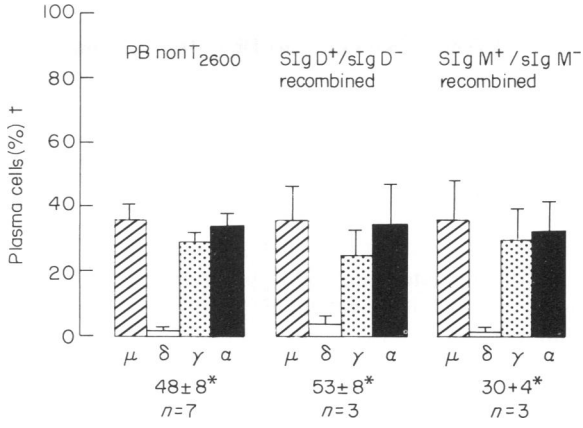

Fig. 2. Effects of sIgD and sIgM rosette formation on PWM induced plasma cell differentiation. Results are presented as mean ±s.d. of n experiments.
*Total percentage of plasma cells ($c\mu^+ + c\delta^+ + c\gamma^+ + c\alpha^+$). †Distribution of isotypes ($c\mu^+$, $c\gamma^+$, $c\gamma^+$, $c\alpha^+$), within total numbers of plasma cells (set at 100%).

2600' fraction were stimulated with PWM. Neither the separation on sIg nor the separation on sIgM significantly influenced the total percentage of plasma cells or the distribution of the different isotypes produced.

DISCUSSION

A normal PB non T cell fraction contains about 65% monocytes, which express Fc receptors for Ig molecules. Using the rosette assay for sIg detection, we found that about 75% of the non T cell expressed sIg, but many of these cells carried both the κ and the λ light chain (Table 2) indicating that randomly (passively) bound sIg was detected. Only by completely removing the monocytes from the non T cell fraction, which was done by counterflow centrifugation (2600 rpm, 18 ml/min flow rate) were we able to decrease the percentage of $s\kappa^+$ and $s\kappa^+$ cells to a level at which there no longer is an overlap of cells expressing $s\kappa$ or $s\lambda$ compared to the percentage of sIg^+ cells. As for the heavy-chain isotope expression, especially the percentage of $s\gamma^+$ and of $s\alpha^+$ cells was decreased, whereas the percentage of $s\delta^+$ and $s\mu^+$ cells was increased. In the 'PB non T 2600' fraction only lymphocytes were found, 71% of which were B cells and only 1–3% appeared to be contaminating T cells. No cells containing cIg were found, and 25% of the lymphocytes did not express sIg or a receptor for SRBC. Further characterization of the B cells in this fraction showed that about 60% of them was $s\mu^+$ or $s\delta^+$, whereas 23% expressed $s\gamma$ and about 17% expressed $s\alpha$. Only $s\mu$ and $s\delta$ could be detected on the same cell. Analogous to the mouse system these $s\mu^+\delta^+$ cells were called virgin B cells, whereas the cells expressing only 1 isotype at the time were called memory B cells (Coffman & Cohn, 1977). Therefore a separation of the PB non T cells into $s\mu^+$ and $s\mu^-$ and especially $s\delta^+$ and $s\delta^-$ cells should also separate between the virgin and memory B cells.

In the $sIgD^+$ fraction 85% of the cells also expressed $s\mu$, whereas no stable rosettes were found with anti-γ or anti-α-coated OxRBC. The same was true for $sIgM^+$ cells, except that only 50% of the cells formed stable rosettes with anti-δ-coated OxRBC. We hypothesize that this is due to steric hindrance of remaining ghosts on the $sIgM^+$ cells as discussed in detail elsewhere (Mudde *et al.*, 1984a), indicating a higher density of $s\mu$ than $s\delta$ molecules on the virgin B cells. On the other hand only few $s\delta$ cells were found in the $sIgM^-$ fraction, meaning firstly that most $s\delta^+$ cells are indeed $s\mu^+$ and secondly that there are $sIgM^-D^+$ (memory) B cells. In the $sIgD^-$ fraction only a small percentage of the cells expressed $s\mu$, confirming that most $s\mu^+$ cells also express $s\delta$ (Table 2) and also indicating the existence of $sIgM^+D^-$ (memory) B cells.

Stimulation experiments firstly learned that all isolated fractions, including the sIg(M^+)D^+ fraction, responded to PWM. This is in contrast to the results of Kuritani & Cooper (1982b), but confirms the results of Saiki & Ralph (1982a). By recombination experiments, both in the $sIgD^+$/IgD^- recombined and the $sIgM^+$/$sIgM^-$ recombined fractions, it was found that the total percentage of plasma cells, as well as the ratio between the isotypes produced were similar to the results obtained with the original 'PB non T 2600' fraction. So it was concluded that the response of $sIgD^+$ (virgin) B cells is not due to activation by the rosette formation, as may be concluded from the work of Kuritani & Cooper (1982c). Probably in their experiments addition of anti-δ-antibodies induced a proliferative signal which after PWM stimulation resulted in a memory-like proliferative response, before the cells differentiated into $cIgM^+$ plasma cells.

Secondly it was found, that the fractions containing pure (mainly virgin) B cells responded lower than the fractions composed of (memory) B cells and null cells. From these experiments it is not clear, whether null cells may in any way contribute to the response of the B cells in these fractions. However this possibility does not seem to be very obvious for two reasons: (a) Purified null cells did not respond to PWM with either proliferation or plasma cell differentiation (data not shown) and (b) Isolated $sIgD^-A^+$ fractions, containing almost pure $s\alpha^+$ (memory) B cells, showed similar responses as the $sIgD^-$ or $sIgM^-$ fractions. Since the $sIgD^-A^+$ cells (for technical reasons) were cultured with more than the usual 50% irradiated T cells, the capacity of these cells to proliferate becomes even more clear, as it was found that over 50% of the recovered cells contained cIgA. The rest of the cells appeared to be T cells (data not shown). Therefore it may be concluded that both virgin and memory B cells respond to PWM be it with different reaction kinetics. This confirms earlier experiments (Mudde *et al.*, 1984b) in which it was found that $s\delta^-$ cells showed a better proliferative response to PWM than the $s\delta^+$ cells.

The third group of data obtained from the stimulation experiments, concerns the production of different isotypes in the separated fractions. The $sIgM^+$ fraction only produced significant numbers of $cIgM^+$ cells, which were absent in the $sIgM^-$ fraction. These $cIgM^+$ cells may have derived from two different subpopulations: $sIgM^+D^+$ cells and IgM^+ (memory) B cells. Cytoplasmic IgD^+ cells were also (occasionally) found, however only in the $sIgD^+$ or $sIgM^-$ fractions, never in the $sIgD^-$ or $sIgM^+$ fractions. Therefore it was concluded that $sIgM^-D^+$ (memory) B cells after stimulation with PWM differentiate into $cIgD^+$ cells. As for $cIgG^+$ or $cIgA^+$ cells, they were both found in stimulated $sIgD^-$ or $sIgM^-$ fractions, which both contained $s\gamma^+$ and $s\alpha^+$ cells, in a second isolation step, it became clear that the $cIgG^+$ were derived from $sIgM^-D^-G^+A^-$ cells, while the $cIgA^+$ cells were derived from the $sIgM^-D^-G^-A^+$.

In conclusion we found that human PB is indeed composed of two distinct B cell subpopulations:

(a) Virgin B cells expressing both sIgM and sIgD

(b) Memory B cells, expressing one isotype at the time and comprising $sIgM^+$, $sigD^+$, $sIgG^+$ and $sIgA^+$ cells.

After PWM stimulation the response of the virgin B cells is different from that of the memory B cells, the latter not only differentiate into plasma cells but also show enormous proliferation. No class-switches were found in these *in vitro* experiments, and only those isotypes expressed on the surface of a B cell could be found in the plasma cells after PWM stimulation. Since we never found a class-switch *in vitro* during plasma cell differentiation, we hypothesize that this differentiation is a one step action for all types of B cells, whereas the maturation from virgin to memory B cell involves a class-switch from $sIgM^+D^+$ to only $sIgM^+$ or $sIgD^+$ or $sIgG^+$ or $sIgA^+$.

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