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Analysis of the antigen-induced *in vitro* differentiation of human peripheral blood B cells: Stimulation with ovalbumin induces the transition from the resting state into the excited state

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SUMMARY

Human peripheral blood B lymphocytes, when cultured for 6 days with ovalbumin in the presence of T cells and monocytes, can differentiate into plaque-forming cells (PFC) secreting small amounts of antigen-specific IgM. Phenotype analysis using Ig-isotype and B-cell specific monoclonal antibodies revealed that the PFC precursor is a $sIgM^+$ IgD^+IgG^- B lymphocyte. The PFC itself also carries the B-cell specific membrane antigens B1 and BA-1; it expresses only IgM on its surface. When apart from the antigen a Concanavalin A-induced, T-cell derived growth factor preparation was added to the cultures, we observed the disappearance of sIgM from the membrane. Furthermore, it was demonstrated by means of density gradient centrifugation that the differentiation of the PFC-precursor into a PFC is accompanied by a decrease in cellular density. Finally, it was shown using hydroxyurea as an inhibitor of DNA-synthesis, that the PFC-precursor passes several cycles of cell division during the process of differentiation into a PFC.

Keywords B-cell ovalbumin excited state

INTRODUCTION

A number of reports dealing with specific antibody production by human peripheral blood lymphocytes (PBL) *in vitro* have been published recently. Most of these data concern studies of the antigenic responses of PBL originating from donors already primed *in vivo*. Reports on the induction and regulation of a primary antibody response *in vitro* are relatively scarce (Dosch & Gelfand, 1979; Morimoto, Reinherz & Schlossman, 1981; Ballieux *et al.*, 1979; Misiti & Waldmann, 1981; Fauci & Ballieux, 1978; 1984). In previous studies we analysed some aspects of T-cell regulation in the antigen-induced B-cell response using the experimental conditions originally described by Dosch & Gelfand (1977) for the induction and measurement of antigen-specific IgM-producing plaque forming cells (PFC) in cultures (Ballieux *et al.*, 1979; Ballieux & Heijnen, 1983; Heijnen, Pot & Ballieux, 1982; Heijnen, UytdeHaag & Ballieux, 1980). However, the PFC which develop in this culture-system differ essentially from the classical PFC (Jerne, 1974), since they resemble lymphocytes rather than plasmablasts and because the plaques that are generated are rather small. Therefore we attempted to arrive at a more precise characterization of the PFC. The

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significance of our observations will be discussed in relation to T-cell regulation of human B-cell activation.

MATERIALS AND METHODS

Isolation of mononuclear blood cells (MNC). Heparinized human peripheral blood MNC were isolated by density gradient centrifugation on Ficoll-Isopaque ($\rho = 1{\cdot}077$ $\mu g/cm^3$) at 1,000*g* for 20 min. MNC were depleted of monocytes, when this cell type exceeded 15% of the total cell number, by adherence to plastic culture flasks (Falcon) at 37°C in Tris-buffered minimal essential medium (MEM-Tris, pH 7·4) supplemented with 20% (vol) fetal calf serum (FCS). The non-adherent cell fraction will be referred to as peripheral blood lymphocytes (PBL). When required, the adherent cells (AC) were harvested in MEM-Tris with a rubber policeman. PBL were depleted of T cells by two cycles of rosetting with sheep erythrocytes (SE) treated with 2-aminoethylisothiouroniumbromide (AET) (Sigma Chemical Co., St Louis, USA). These non-T cell fractions containing less than 2% SE rosette-forming cells and 40–50% sIg^+ cells will be referred to as B cells.

Cell culture conditions: activation by antigen. Isolated MNC, 10–15% of which were monocytes, were cultured at 37°C in Falcon tubes (17 × 100 mm) in air containing 5% CO_2 at a concentration of 4–5 × 10^6 cells per 5 or 10 ml culture medium. The medium consisted of RPMI-1640 supplemented with 2 mM L-glutamine/ml, antibiotics, 50 μM 2-mercapthoethanol, and 10% human AB serum absorbed with sheep erythrocytes (SE) to prevent pseudo-plaque formation (Muchmore *et al.*, 1976).

To substitute for T-helper cells, soluble ovalbumin (OA) (Sigma) specific T-helper factor ($ThF_{120}OA$) was added in an optimal concentration to B-cell cultures (10% vol). The preparation of $ThF_{120}OA$, the dose-response curve and the biological properties have been described elsewhere (Heijnen *et al.*, 1981).

For PFC induction, PBL or B cells supplemented with 10% plastic-adherent cells and $ThF_{120}OA$ were cultured with OA at the optimal concentration (3·0 μg/ml; Heijnen *et al.*, 1981). After a culture period of 6–7 days, the cells were collected, washed twice in MEM-Tris at 4°C, and assayed for the presence of PFC.

To investigate whether the OA-activated B cells proliferate, 100 μg/ml hydroxyurea (HU) was added to the cultures at different time intervals. This particular concentration is the minimal dose required for the reduction of the PFC response, but does not cause cell death when added to purified B cells, as was shown by data on cell recovery (Table 1).

*PFC assay.*The assay was performed as described by Dosch & Gelfand (1977) and by Heijnen *et al.* (1979). Briefly, monolayers of OA coated SE (Goding, 1976) were prepared after centrifugation of the erythrocytes for 5 min at 1000*g* on the bottom of Falcon microtitre plates (No. 3040), which had been pre-coated for 60 min at 37°C with poly-L-lysine (Sigma, Mr < 100,000; 100 μg/ml). Viable cultured cells were incubated for 60 min in the presence of SE-absorbed fresh guinea pig serum,

Table 1. The inhibition of the PFC response after addition of hydroxyurea (HU)

	Cell recovery (%)	PFC/10^6 ly
a. B+$ThF_{120}OA$	100±25	12±0
b. B+$ThF_{120}OA$+OA	100±0	100±0
c. B+$ThF_{120}OA$+50 μg/ml HU+OA	96±6·1	96±6·1
d. B+$ThF_{120}OA$+100 μg/ml HU+OA	87±18	9·1±0·4
e. B+$ThF_{120}OA$+150 μg/ml HU+OA	90±31	6·4±2·3

Non-T cells were cultured for 6 days with 3 μg OA/ml and 10% $ThF_{120}OA$. Data are given as percentage of two control cultures (1050 and 875 PFC/10^6 respectively).

which served as a complement source (final dilution 1:20 vol). In control experiments cultured cells or complement was omitted.

Phenotypical analysis of the PFC by depletion. Immediately prior to the plaque assay the cultured cells were depleted of cells carrying particular surface structures using the rosetting procedure described by de Gast & Platts-Mills (1979). The reagents applied were antibodies directed against human IgM, IgD, or IgG heavy chains, produced in goats (Tago Inc., Burlingham, USA) and the monoclonal antibodies (MoAb) B-1 (Coulter Electronics Ltd, England), BA-1 (Hybritech Inc., Belgium), RFA-4 (a gift from Dr G. Janossy, London), and anti HLA-DR (Becton Dickinson Inc., USA), all produced by hybridomas of murine origin. The antibodies specific for the immunoglobulin isotypes are directly coated on ox-erythrocytes (OE), whereas the cells reacting with the MoAb are depleted using OE coated with sheep anti-mouse antibodies (Becton Dickinson Inc., USA).

The effectiveness of the depletion was routinely determined by counting the percentage of the rosettes formed in control PBL and in PBL recovered after depletion and was found to be always more than 90%. This implicates that in the various experiments the depleted lymphocyte populations were contaminated with less than 0·5% $sIgM^+$, 0·5% $sIgD^+$ or 0·1% $sIgG^+$ cells.

For the depletion of cells bearing a C3 receptor by rosetting procedure SE sensitized with antibody and complement were used (EAC) (Michlmayer & Huber, 1970).

Preparation of growth factors containing supernatant derived from mitogen-activated T cells (Con A sup). The concanavalin A supernatant (Con A sup) was produced by Dr L. Aarden (Central Laboratory of the Bloodtransfusion Service, Amsterdam). Briefly PBL isolated from the blood of healthy donors were prestimulated (10^6/ml) for 24 h with phorbol myristate acetate (PMA; 1 ng/ml) followed by stimulation for 72 h with concanavalin A (Con A; 5 μg/ml). The residual Con A activity in the supernatant was abolished by the addition of α-methyl-D-mannoside (10 mg/ml; Sigma).

The activity of the Con A sup was assessed in cultures of B cells pre-activated for 24 h with formalinized *Staphylococcus aureus* Cowan I according to Wrigley & Choi (1983). The optimal factor concentration for plasma cell formation was 20% (vol).

Isolation of lymphocyte subsets by Percoll-gradient centrifugation. Cells were fractionated by discontinuous Percoll (Pharmacia, Sweden) gradient centrifugation at 1600 r.p.m. for 30 min at 12°C. Percoll gradients were made according to manufacturer's instruction. Gradients ranged between 30 and 60% Percoll ($\rho = 1{\cdot}038$–$1{\cdot}050$ determined with refractometry).

After centrifugation, the cell bands located at the interphase of two adjacent fractions were harvested and washed twice with ice-cold MEM-Tris supplemented with 2% BSA or 5% FCS before the cells were counted.

When the subsets obtained by fractionation of non-T lymphocytes were cultured, 10% plastic adherent cells and 10% $ThF_{120}OA$ were added.

RESULTS

The nature of the PFC

To demonstrate that the IgM-secreting PFC is an activated B-cell, OA-stimulated cultures of PBL were depleted by rosetting of cells carrying B-cell specific determinants. The remaining cells were tested for plaque formation. The results in Fig. 1 indicate that all PFC carry B-1 (Stashenko *et al.*, 1980) and HLA-DR and most of them the BA-1 antigens (Abrahamson, Kersey & Le Bien, 1981). This observation establishes that the PFC belongs to the B-cell lineage. In contrast, a late B-cell marker, RFA-4 (Gobbi, Caligaris Cappio & Janossy, 1983) is not expressed on the PFC surface.

The expression of immunoglobulin isotypes on the PFC

To determine whether the transformation of a PFC precursor into an IgM-secreting PFC is accompanied by a change in the phenotype, PBL were depleted of $sIgM^+$, $sIgD^+$, or $sIgG^+$ cells prior to initiation of the cultures. The remaining cells were cultured with OA under standard conditions and the PFC response was determined after 6 days. The results obtained indicate that the

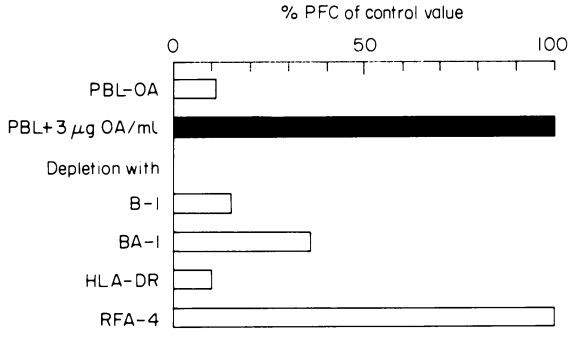

Fig. 1. Reactivity of the PFC with monoclonal antibodies. PBL were cultured with 3 μg OA ml. The cells recovered from the cultures were depleted of cells reacting with the monoclonal antibodies B-1, BA-1, RFA-4, HLA-DR or with C3 ($n=2$). The PFC response in the non-rosetting cell fractions is expressed as the percentage of the response in the non-depleted fractions (100% = 1,200–1,500 PFC 10^6 ly). Mean results are given.

PFC precursor is localized in the fraction of B cells expressing sIgM and sIgD (Fig. 1). Depletion of B cells bearing sIgG bearing sIgG did not affect the PFC response. Furthermore, the PFC-precursor cells express a surface receptor for the third complement component (C3R) and express HLA-DR backbone structures (results not shown).

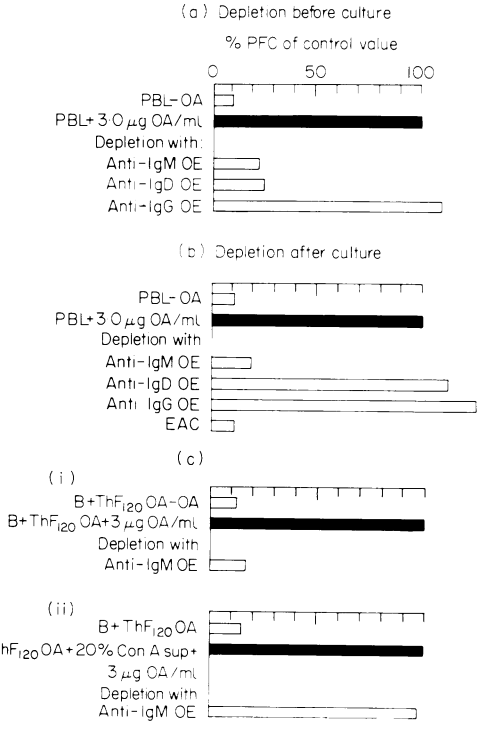

Fig. 2. Phenotypical characterization of the PFC precursor and the PFC and the reactivity of the PFC with Con A sup. PBL were depleted of cells expressing sIgM, sIgD or sIgG before (a) or after (b) culture with 3 μg OA ml and 10% $ThF_{120}OA$ ($n=4$). In a second type of experiment (c) B cells were cultured with 3 μg OA ml and 10% $ThF_{120}OA$ in the absence (i) or presence (ii) of Con A sup ($n=2$). After termination of the cultures the recovered cells were depleted of cells expressing sIgM. The PFC response in the non-rosetting cell fraction is expressed as a percentage of the response in the non-depleted fraction (100% = 1,200–1,500 PFC 10^6 ly). Mean results are given.

Table 2. Localization of PFC precursor and PFC in Percoll gradient

Interphase:	I	II	III	IV	V (control)
(a)					
45–50%	3,217	1,737	837	1,980	
50–55%		182	19		
Unfractionated	2,375	1,630	800		
(b)					
0–30%	—			375	185
30–35%	1,181			1,375	3,217
35–40%	556			—	321
40–45%	—			—	—
45–50%	—			—	—
50%	—			—	—

B cells of four donors were fractionated in a discontinuous Percoll gradient. The cells in the interphases between the 45–50% and the 50–55% density fractions were supplemented with monocytes and subsequently stimulated for 6 days with 3 μg OA/ml in the presence of 10% $ThF_{120}OA$. (a) After termination of the culture the number of PFC among 10^6 lymphocytes was determined. (b) The recovered cells of parallel cultures of the 45–50% interphase of donor I and IV were fractionated again. The number of PFC among 10^6 lymphocytes in each interphase was determined. In a control experiment non-T cells were fractionated only after termination of the culture (donor V).

The expression of immunoglobulin isotypes on the PFC

Next, we determined whether the activation of B cells with antigen in the presence of T-cell help would change the expression of surface immunoglobulin isotypes of the PFC precursor. PBL were cultured for 6 days with an optimal dose of ovalbumin and subsequently depleted of $sIgM^+$, $sIgD^+$, or $sIgG^+$ cells. As is shown in Fig. 2b, the number of PFC was considerably reduced after depletion cells expressing sIgM or C3R, whereas depletion of $sIgD^+$ or $sIgG^+$ cells had no effect. This finding implies that during differentiation the PFC lose IgD, whereas the expression of sIgM and C3R is retained. It is also clear that no novel Ig isotypes (e.g. IgG) are expressed.

Sensitivity of the PFC for polyclonal growth factors

It has been suggested on the basis of investigations in mice that after excitation by antigen in concert with T-cell help, resting B cells become sensitive to growth and maturation factors (Andersson & Melchers, 1981). This finding led us to investigate the effect of a crude supernatant derived from Con A-stimulated T cells on OA-activated B lymphocytes with respect to further maturation as reflected by a change in sIg-isotype expression of the PFC. Experiments of this type were done using cultures of purified B cells supplemented with 10% adherent cells and with OA-specific T helper factor ($ThF_{120}OA$). The results in Fig. 2c show that PFC which develop after antigenic stimulation of B cells in the presence of antigen-specific T-helper factors are $sIgM^+$ and $sIgD^-$ and $sIgG^-$, as are PFC developing in cultures of PBL (Fig. 2b). Addition of Con A sup (20% vol) to the B-cell cultures resulted in the loss of IgM from the surface of the PFC. The size of the plaques, however, did not change.

Localization of the precursor cells in a discontinuous Percoll gradient

After separation of freshly isolated PBL on the Percoll gradient, monocytes were located in the interphase of the 30–40% franction or even in fractions with a lower density. Lymphocytes were found in the interphases of the 40–50% and the 50–60% Percoll fractions. Marker analysis

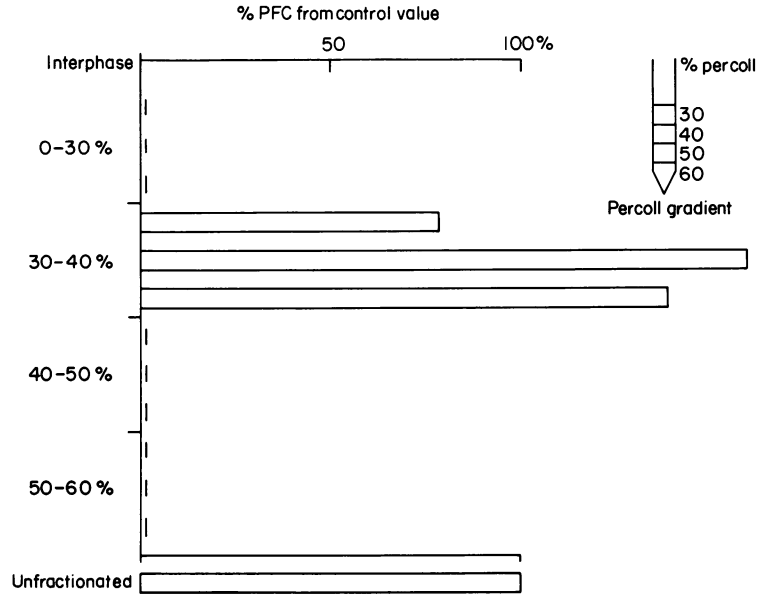

Fig. 3. Localization of the PFC in a Percoll gradient. PBL were cultured for 6 days with an optimal dose ovalbumin (3 μg OA/ml). After termination of the cultures the recovered cells were fractionated on a discontinuous Percoll gradient. The number of PFC/10^6 lymphocytes was determined in the interphase fractions. The results are expressed as the percentage of PFC among 10^6 non-fractionated lymphocytes (1,000–1,300 PFC/10^6 ly).

established that no selective distribution of T (SE rosetting) cells and B (sIg^+) cells in the gradient occurred (result not shown).

To determine the localization of the PFC precursor in the gradient, B cells isolated from peripheral blood were fractionated on a Percoll gradient. The fractions at the 45–50% (Fr.I; 80% of the cells) and at the 50–55% interphase (Fr.II) respectively (Table 2a) were cultured separately with OA and $ThF_{120}OA$ in the presence of 10% adherent cells. After 6 days of culture the formation of PFC in both fractions was assessed. The results indicate that only the B cells residing in the 45–50% interphase (Fr.I) differentiate into PFC.

Localization of the PFC in the gradient

PBL were cultured for 6 days in the presence of an optimal dose OA (3 μg/ml). After culture, the recovered cells were fractionated on a Percoll gradient. Each fraction was examined for the presence of PFC. The results show that the PFC are localized exclusively in the 30–40% interphase (Fig. 3), whereas more than 90% of the lymphocytes remain in the denser fractions, indicating that the shift in cellular density is not an artefact induced by culture conditions.

Transition of precursor PFC into PFC in relation to cellular density

To establish that PFC precursor cells really differentiate into cells of reduced density after antigenic stimulation, B cells were fractionated by Percoll gradient centrifugation. The cells at the 45–50% interphase were cultured with OA and $ThF_{120}OA$ in the presence of 10% adherent cells. After 6 days the recovered cells were fractionated on a similar Percoll gradient. The majority of the B cells could again be recovered from the 45–50% interphase; in contrast PFC were only detectable in the 30–40% interphase (Table 2b). These results clearly indicate that the transition from an unstimulated resting B cell to the state of a PFC involves a decrease in cell density.

Proliferation of the PFC precursor cell

To find out whether proliferation of the PFC precursor cell occurs during the development into an

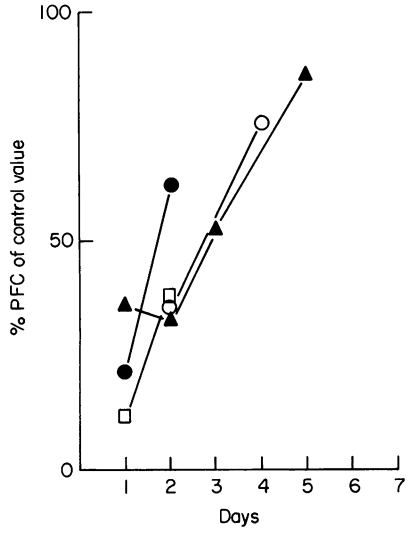

Fig. 4. Proliferation of the PFC. PBL were cultured for 6 days with 3 μg OA/ml and 5% $ThF_{120}OA$. In order to study the proliferation kinetics of the PFC precursor 100 μg/ml hydroxyurea (HU) was added to separate cultures on different days after the initiation of the culture. The PFC-response was determined after the total culture period of 6 days in all experiments. The symbols (●, ▲, □, ○) represent the results of four individual donors.

IgM-producing PFC, DNA-synthesis was blocked by HU. Addition of 100 μg HU/ml at the onset of the cultures of PBL with OA (Fig. 4) or of B cells with OA and $ThF_{120}OA$ (Table 1) led to a complete inhibition of the PFC response.

To study the kinetics of the inhibitory activity of this drug, HU was added to separate cultures of OA-stimulated PBL at day 1, 2, 4, 5 or 6. The results show that the earlier HU is added, the fewer PFC are recovered (Fig. 4).

DISCUSSION

Relatively little is known of the cellular processes occurring at the B-cell level after stimulation of human PBL with primary antigens *in vitro*. The majority of the blood B lymphocytes of human adults express surface-bound IgM ($s\mu^+$), IgD ($s\delta^+$) and receptors for the third complement component (Lawton *et al.*, 1982). Murine B cells of this phenotype were found to be small resting cells in the G_0 phase of the cell cycle (Monroe, Havran & Cambier, 1983). It has been shown that these cells can enter the G_1 and S phases after appropriate stimulation and ultimately develop into immunoglobulin secreting cells (Andersson & Melchers, 1981; Monroe *et al.*, 1983; Howard & Paul, 1983). The transition from the G_0 to the G_1 phase of the cell cycle is accompanied by the loss of sIgD, by a decrease in cellular density and by cell division (Monroe *et al.*, 1983; Howard & Paul, 1983; De Franco, Kung & Paul, 1982). It is apparent from the present results (Figs 2 and 4; Table 2) that in man, activation of $sIgM^+IgD^+$ B lymphocytes by antigen (OA) *in vitro* results in the transition of resting B cells into an 'excited' state (Andersson & Melchers, 1981) without further differentiation into high-rate secreting antibody-forming cells. This conclusion is supported by the observation that the PFC react with monoclonals with a specificity for activated B cells (manuscript in preparation). It is also in agreement with the earlier finding that the morphological and surface characteristics of the PFC resemble those of lymphocytes rather than of plasma cells (Dosch & Gelfand, 1979). The present observation that OA-activated human B cells start to proliferate within 24–48 h is consistent with previous findings in mice (Dutton & Mishell, 1967). Finally it should be

emphasized that activation of sIgG$^+$ B cells by OA does not lead to a PFC response (Fig. 1), comparing earlier observations of Dosch & Gelfand (1979).

It is generally accepted that murine B cells, once activated, are in a cycling state and express receptors for growth and differentiation factors, referred to as BCGF and BCDF (BRMF) or TRF (Andersson *et al.*, 1981; Howard & Paul, 1983; Melchers *et al.*, 1982; Schimpl & Wecker, 1975). Interaction with these non-specific, T cell-derived factors is required for the final differentiation into antibody secreting cells (Howard & Paul, 1983). The apparent block in the complete differentiation of OA-reactive human B-lymphocytes into a classic PFC in our culture system is not easy to explain. It can be excluded that the lack of complete differentiation is due to the culture system, since stimulation of PBL from a donor allergic to chicken egg ingestion led to the formation of blast-like cells, producing large OA-specific plaques (unpublished observations).

One could speculate that after stimulation with a primary antigen *in vitro* the T helper cells do not produce (any or) adequate amounts of BRMF-like factors necessary for the final maturation of the activated B cells. The results, presented in Fig. 2, show that the addition of a supernatant of Con A-activated T cells caused the loss of IgM expressed on the PFC surface but no further maturation occurred. This observation indeed suggests that in our culture system the T cells do not produce (sufficient amounts of) differentiation factor(s). However, human B cells (pre-)activated for 18 h with Sta did develop into plasma cells after addition of the Con A supernatant (data not shown). Therefore, one has to assume that the OA-activated B cells do not acquire the full spectrum of receptors for maturation factors necessary for complete differentiation.

The present study has established that antigen-activated human blood B cells proliferate repeatedly, lose IgD and undergo a decrease of the cellular density. This leads us to postulate that the PFC which appear after *in vitro* stimulation of PBL with antigens like OA, are B cells that have been induced to proliferate and that secrete IgM following passage through the cell cycle. A similar finding, made in murine B cells, has been reported by Andersson *et al.* (1977).

On the basis of the results presented in this paper one has to conclude that the antigen-induced formation of PFC in cultures of human PBL reflects an early phase of B cell activation. This implies that the antigen-specific regulations of this process by T cells, as described by us in a number of reports (Ballieux *et al.*, 1979; Ballieux & Heijnen 1983; Heijnen *et al.*, 1982; Heijnen *et al.*, 1980) is operative in the initial phase of the B-cell response.

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