

# Production and Secretion of Immunoglobulins by *in Vitro*-Activated Human B Lymphocytes

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Activation induced by pokeweed mitogen in cultures of mononuclear cells from human blood was followed sequentially by simultaneous quantitation of live cells, thymidine incorporation, cells displaying cytoplasmic IgM, IgG, IgA or IgD, cells secreting IgM, IgG or IgA and cumulated IgM secretion. Maximal cellular activity was found after 7 days of culture, with means of 16000 IgM-, 20700 IgG- and 9900 IgA-secreting cells per 10<sup>6</sup> originally cultured cells. The cumulated IgM secretion after 21 days of culture averaged 10400 ng per 10<sup>6</sup> originally cultured cells. A close correlation was found between the number of IgM-secreting cells and the cumulated IgM secretion.

*Key words:* B lymphocytes; immunoglobulin production; plaque-forming cells.

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*In vitro* studies on the generation of immunoglobulin-containing cells (ICC) (12), of plaque-forming cells (PFC) (3) or on immunoglobulin production (10) have been employed in order to gain insight into the function of human B lymphocytes and the cellular interactions that modulate their activation. As emphasized by Nespoli et al. (8) the information obtained by the simultaneous application of several functional assays can be of value for example in the investigation of immune deficiencies and autoimmune diseases.

In the present experiments selected parameters of B lymphocyte function were studied sequentially in cultures of human mononuclear cells (MNC) stimulated with pokeweed mitogen (PWM) during a culture period of 21 days.

## MATERIAL AND METHODS

### *Mononuclear cells*

Heparinized blood from healthy volunteers was separated on Lymphoprep<sup>®</sup> (Nyegaard, Oslo, Norway). The MNC of the interphase were washed three times in RPMI 1640 (GIBCO, Paisley, Scotland) supplemented with penicillin (500 IU/ml), streptomycin (500 µg/ml), glutamine (0.8 mM) and 5% foetal calf serum (FCS) (GIBCO).

### *Culture conditions*

Culture medium was RPMI supplemented with 10% FCS, penicillin, streptomycin, glutamine

and nystatin (20 IU/ml). MNC were cultured in flasks (Falcon No. 3013, Oxnard, California),  $5 \times 10^6$  cells in 10 ml. The cultures were incubated in a humidified atmosphere with 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ . PWM (GIBCO) was used at a final dilution of 1:1250.

Cultures were maintained for up to 21 days. In the main experiments, the medium was changed on days 3, 5, 7, 11, 14 and 18. Five ml of the medium were removed and replaced with an equal amount of fresh medium with or without PWM.

#### *Proliferative capacity and recovery of MNC*

On the day of harvest, MNC were resuspended in the medium and triplicate samples of 200  $\mu\text{l}$  cell suspension were incubated with 0.05  $\mu\text{Ci}$   $^{14}\text{C}$ -thymidine (specific activity  $> 50 \text{ mCi/mmol}$ ) for 6 h at  $37^\circ\text{C}$ . Cells were harvested on glass fibre filters and thymidine incorporation was assessed by liquid scintillation counting. Viability was estimated by nigrosin exclusion.

#### *Assay for PFC*

Immunoglobulin-secreting cells were detected by a reverse PFC-technique, essentially as described by Hammarström et al. (5), using sheep red blood cells (SRBC) conjugated with protein A from *Staphylococcus aureus* (SPA) as target cells (4). In brief, one part of SPA (Pharmacia, Uppsala, Sweden) 0.5 mg/ml in saline, one part of SRBC (washed 3 times in saline) and 10 parts of chromium chloride (1% w/v aged stock solution diluted 1:150 in saline) were mixed and incubated for 1 h at  $30^\circ\text{C}$ , washed once in saline and twice in Minimum Essential Medium (MEM) (Flow, Irvine, Scotland) ( $4^\circ\text{C}$ ). These SPA-conjugated SRBC were stored in liquid nitrogen (1).

For quantitation of PFC, 100  $\mu\text{l}$  of MNC (washed once in MEM) in a concentration suitable for plaque counting, 50  $\mu\text{l}$  SPA-SRBC diluted 1:6 in MEM and kept on ice, 25  $\mu\text{l}$  rabbit anti-human IgM, IgG or IgA (DAKO Immunoglobulins, Copenhagen, Denmark) diluted 1:30 in MEM and 25  $\mu\text{l}$  guinea pig complement (Flow) absorbed twice with SRBC, diluted 1:4 in

MEM and kept on ice, were mixed with 800  $\mu\text{l}$  Noble Agar (DIFCO, Detroit, Michigan) 5 mg/ml supplemented with DEAE-dextran (Pharmacia) 0.45 mg/ml at  $45^\circ\text{C}$ . From this mixture,  $3 \times 200 \mu\text{l}$  were placed on lids for microtiter plates (Falcon No. 3041) and a cover slip ( $25 \times 50 \text{ mm}$ ) was layered on each drop. The preparations were incubated for 4 h at  $37^\circ\text{C}$  and PFC were counted at 10 times magnification using indirect light. Immunoglobulin class specificity of the PFC assay had been assured by a pseudo-plaque procedure in which droplets of serially diluted, purified preparations of human monoclonal IgM, normal IgG or monoclonal IgA were placed on agar prepared as described above but without MNC. PFC were expressed per  $10^6$  originally cultured MNC.

#### *Assay for ICC*

Cytocentrifuged smears were kept at  $-80^\circ\text{C}$ . The smears were fixed with 5% glacial acetic acid in 95% ethanol, washed 3 times in phosphate buffered saline (PBS) and stained with fluorescein isothiocyanate-conjugated anti-IgG or -IgD, and secondly with rhodamine-conjugated anti-IgM or -IgA (DAKO Immunoglobulins) for 30 min at room temperature, washed 2 times in PBS and mounted in PBS/glycerol (1:2). ICC, defined as cells showing bright cytoplasmic fluorescence, were counted in a Leitz Orthoplan fluorescence microscope equipped as described (11). Monospecificity of the conjugates had been ensured by testing on monoclonal bone marrow plasma cells (6). ICC were estimated per  $10^6$  cells present in the culture.

#### *Determination of IgM in culture medium and in cell pellets*

All samples were kept at  $-20^\circ\text{C}$ . IgM concentrations were estimated by an enzyme immunoassay (2).

Pellets containing  $0.5 \times 10^6$  cells were thawed and dissolved in 1% Triton X-100® prior to determination of the IgM concentration. This concentration of Triton X-100 did not interfere with the IgM assay.

### Statistics

All results are given as mean  $\pm$  standard error of the mean (SEM). The test for correlation was performed by computing the Spearman rank correlation coefficient ( $r_s$ ) and testing the obtained value by the Hotelling-Pabst test (two-tailed).

## RESULTS

### *Initial experiments: kinetics of the PWM-induced activation*

In order to reveal the course of activation of B lymphocytes in the chosen culture system, PFC, ICC, thymidine incorporation and cell recovery were determined day by day for a period of 13 days. Thirteen identical cultures were prepared, and each day one culture was terminated and examined. In these experiments, no change of medium took place. PFC appeared on day 4 in cultures stimulated by PWM and rose to a maximum on day 7 (Fig. 1), followed by a rapid decline. No difference in kinetics was observed between the generation of IgM-, IgG-, and PFC (Fig. 1). The numbers of PFC found in unstimulated cultures never exceeded the values found with freshly isolated MNC.

The frequency of cells displaying cytoplasmic IgM, IgG or IgA in PWM-stimulated cultures reached a maximum around day 6 and remained at a constant level for several days (Fig. 2). Cells with cytoplasmic IgD were found in very small numbers only. In unstimulated cultures the numbers of ICC were low throughout the culture period. In the experiment shown in Fig. 2 the total number of ICC on day 6 was approximately 30 times greater in cultures exposed to PWM than in unstimulated cultures.

Thymidine incorporation increased in stimulated cultures from day 3 and reached a maximum on day 6. This was reflected by a rise in the number of live cells from a mean of approximately 50% of originally cultured MNC on day 4 to a mean of 120% on day 7.

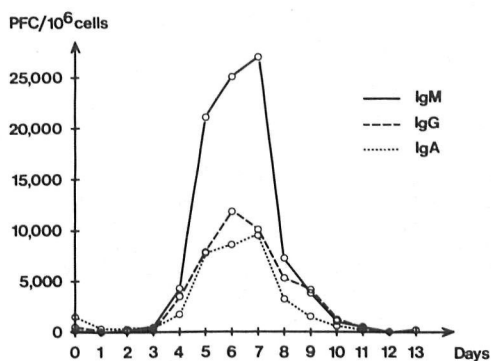


Fig. 1. Day-to-day observations on plaque-forming cells (PFC) in PWM-stimulated cultures: IgM-, IgG- and IgA-secreting cells/ $10^6$  originally cultured MNC. Data from a representative experiment are shown.

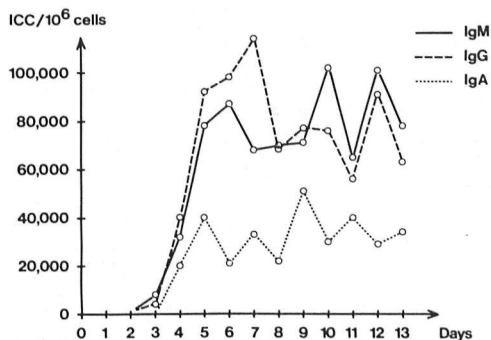


Fig. 2. Day-to-day observations on immunoglobulin-containing cells (ICC) in PWM-stimulated cultures: Cells with cytoplasmic IgM, IgG or IgA/ $10^6$  MNC. Data from the experiment shown in Fig. 1 are given.

### *Comparison of parameters of activation*

In order to compare parameters of B lymphocyte activation, cultures of MNC from eight normal donors were studied. In these cultures, the medium was changed on day 3, 5, 7, 11, and 18, as described in Material and Methods.

**Immunoglobulin-secreting cells.** The activity of freshly isolated MNC was low. Means of  $61 \pm 17$  IgM-secreting PFC/ $10^6$  MNC,  $149 \pm 68$  IgG-secreting PFC/ $10^6$  MNC and  $552 \pm 224$  IgA-secreting PFC/ $10^6$  MNC were found.

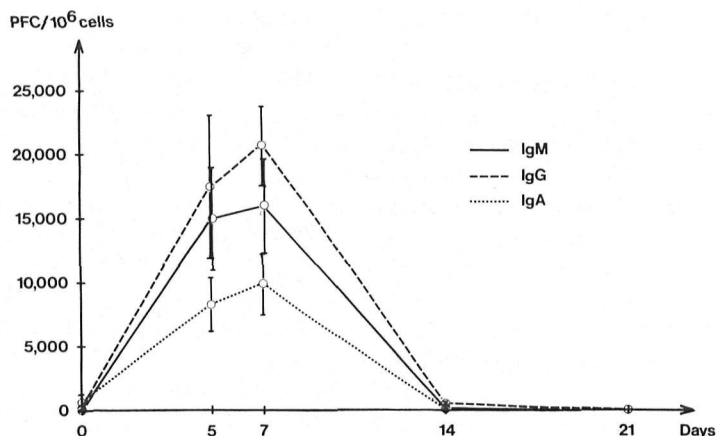


Fig. 3. Plaque-forming cells (PFC) in PWM-stimulated cultures with change of medium: IgM-, IgG- and IgA-secreting cells/10<sup>6</sup> originally cultured MNC.

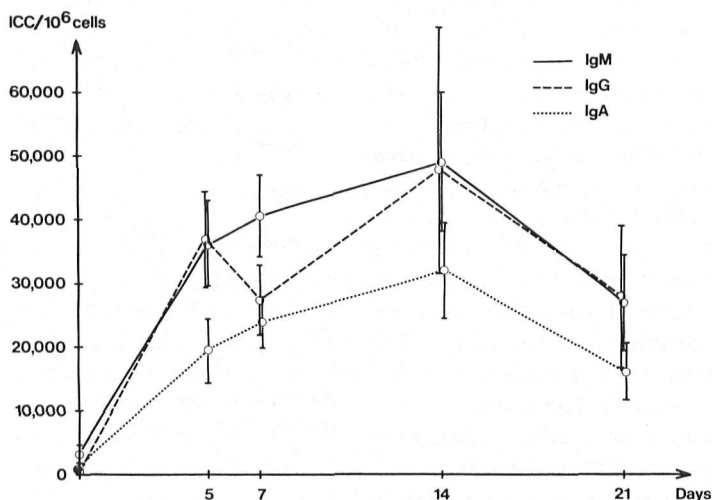


Fig. 4. Immunoglobulin-containing cells (ICC) in PWM-stimulated cultures with change of medium: Cells with cytoplasmic IgM, IgG and IgA/10<sup>6</sup> MNC.

In unstimulated cultures the numbers of PFC were maximal on day 5 with a mean of  $533 \pm 292$  IgM-secreting PFC and  $241 \pm 60$  IgG-secreting PFC/10<sup>6</sup> originally cultured MNC. The numbers of IgA-secreting cells in unstimulated cultures never exceeded the values obtained with freshly isolated MNC.

In PWM-stimulated cultures a significant induction of PFC had occurred by day 5 (Fig. 3).

*Immunoglobulin-containing cells.* Among freshly isolated MNC, we found a mean of  $3200 \pm 1020$  IgM-containing cells/10<sup>6</sup> MNC,  $600 \pm 400$  IgG-containing cells/10<sup>6</sup> MNC,  $800 \pm 583$  IgA-containing cells/10<sup>6</sup> MNC, whereas no IgD-containing cells were demonstrated.

In unstimulated cultures the frequencies of cells displaying cytoplasmic IgM or IgA were maximal on the 7th day of culture with a mean

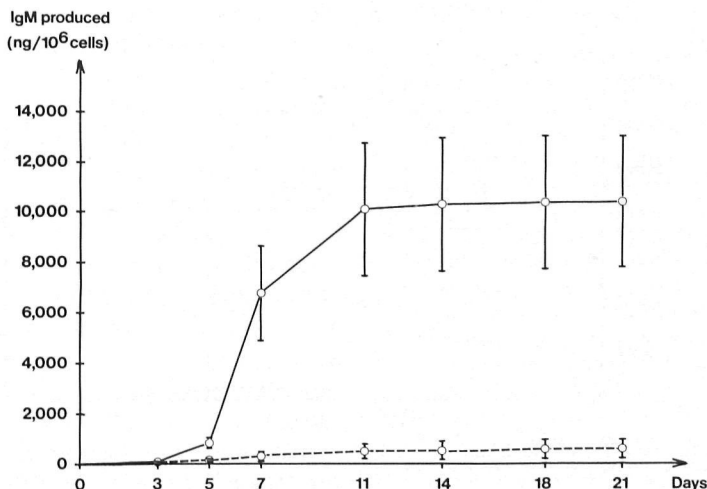


Fig. 5. Cumulated IgM production, expressed in ng/10<sup>6</sup> originally cultured MNC, in cultures with change of medium. ○---○: Unstimulated cultures. ○—○: PWM-stimulated cultures.

number of  $6571 \pm 1378$  cells with cytoplasmic IgM/10<sup>6</sup> MNC and  $4143 \pm 911$  cells with cytoplasmic IgA/10<sup>6</sup> MNC. The frequency of cells displaying cytoplasmic IgG was maximal on the 14th day of culture with a mean of  $3143 \pm 228$  ng/10<sup>6</sup> MNC in stimulated cultures and  $1534/10^6$  MNC. Cells showing cytoplasmic IgD were most frequent on the 5th day of culture, mean  $714 \pm 1890/10^6$  MNC.

In PWM-stimulated cultures, the frequency of cells with cytoplasmic IgM, IgG or IgA increased 7–15 times in comparison to unstimulated cultures (Fig. 4). The frequencies were maximal on day 14. Few cells displayed cytoplasmic IgD; a maximal number of  $813 \pm 1067/10^6$  MNC was found on the 5th day of culture, which means that PWM did not stimulate the formation of such cells.

**IgM production.** In cultures exposed to PWM approximately 18 times more IgM was produced during the culture period of 21 days than in unstimulated cultures. The cumulated amount of IgM secreted in PWM-stimulated cultures was low before day 5 of culture but increased considerably from the 5th to the 11th day. Thereafter, virtually no IgM was produced (Fig. 5).

The amount of IgM detected in solubilized cell pellets showed less difference between stimulated and unstimulated cultures. Little IgM

was present in cell pellets from PWM-stimulated cultures as compared to the amount found in the culture media. Maximal values were found on the 7th day of culture with a mean of  $638 \pm 228$  ng/10<sup>6</sup> MNC in stimulated cultures and  $381 \pm 229$  ng/10<sup>6</sup> MNC in unstimulated cultures.

**Proliferation.** Thymidine incorporation was maximal in both PWM-stimulated (Fig. 6) and unstimulated cultures on day 7, on which approximately 6 times more thymidine was taken up in stimulated than in unstimulated cultures.

The recovery of MNC in the PWM-stimulated cultures was rapidly declining after 7 days of culture, and on day 21 no live cells were found (Fig. 6). On this day the number of dead cells present on the average equalled 76% of the originally cultured MNC.

**Correlations.** The correlations between IgM-, IgG- and IgA-PFC, IgM-, IgG- and IgA-ICC, cumulated IgM production, thymidine incorporation and recovery in PWM-stimulated cultures were investigated. The values obtained on day 5, on day 7 and the maximal values obtained throughout the culture period were used. The parameters were compared two by two. A close correlation was observed on day 7 between the number of IgM-PFC and the cumulated amount

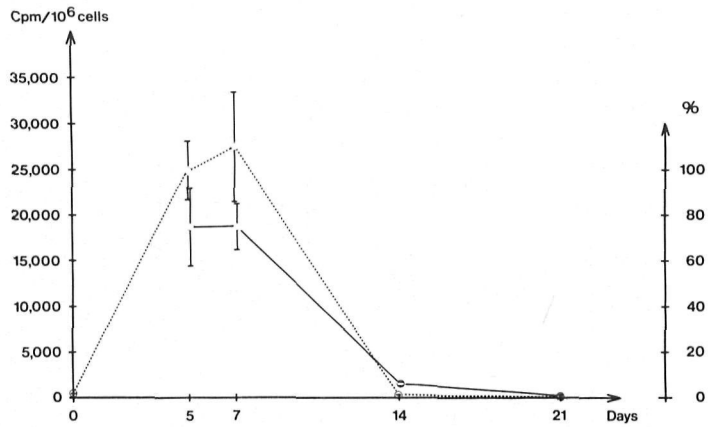


Fig. 6. Cellular kinetics during PWM-stimulation. ○-----○: Thymidine incorporation expressed as counts per min/10<sup>6</sup> originally cultured MNC (left axis). ○——○: Number of live cells, in per cent of MNC originally cultured.

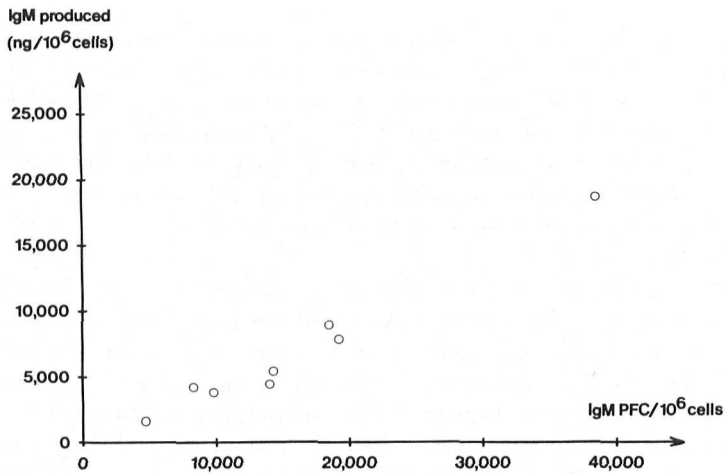


Fig. 7. Correlation between cumulated IgM production (ng/10<sup>6</sup> originally cultured MNC) and number of IgM-secreting cells (plaque-forming cells/10<sup>6</sup> originally cultured MNC) on day 7 in PWM-stimulated cultures with change of medium.

of IgM secreted into the medium ( $r_s = 0.93$ ,  $P < 0.01$ ) (Fig. 7). These parameters did not, however, correlate on day 5, when the number of IgM-secreting cells was high, while the amount of IgM secreted to the medium was still low. A close correlation ( $r_s = 0.90$ ,  $P < 0.01$ ) was also found between the maximal values of IgM-PFC and cumulated IgM production found in the course of each experiment.

All other constellations of the parameters

studied revealed less significant and in most cases weak correlations.

## DISCUSSION

These studies provide no evidence of a switch during B lymphocyte activation, from the production of one class of immunoglobulins to the production of another. Simultaneous appear-

ance of IgM-, IgG- and IgA-containing cells and, a little later, of IgM-, IgG- and IgA-secreting cells was observed, and the ratios between cells producing the individual immunoglobulin classes were not altered during the response.

Cellular activity reached a maximum after about a week of culture. During the next week activity declined and few live cells were present after 14 days. In the cytocentrifuged smears used for quantitation of ICC it was impossible to distinguish between cells that were alive when the slides were prepared, and cells that were dead. It must be kept in mind that the values obtained on day 14 and later for this particular parameter reflect observations on dead cells.

The amount of IgM secreted into the medium during the first 7 days of culture was of the same magnitude as found by others (7–10). The bulk of the cumulated output was produced between the 5th and the 11th day of culture, and contrary to the finding of McLachlan et al. (7), we did not observe any significant production of IgM during the third week of culture.

In spite of the similarities in the course of the different parameters, they did not numerically correlate very well. The only significant correlation was found between the two assays that were based on the actual secretion of immunoglobulins: the number of IgM secreting PFC and the cumulated IgM production. It thus appears that different aspects of B lymphocyte function may be elucidated by the techniques employed, and that the inclusion of several of these therefore would be of value in studies on regulatory mechanisms and in investigations on diseases characterized by abnormal B lymphocyte function.

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