Original Article

ACTIVATION AND DIFFERENTIATION OF HUMAN CIRCULATORY MEMBRANE IgG POSITIVE B-LYMPHOCYTES INTO SECRETORY PLASMA CELLS BY BOUND CONCANAVALIN A *IN VITRO*

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ABSTRACT:

Background and objectives: *Human plasma cells are important primarily for their antibody secretion. It is of interest to understand the mechanisms involved in the activation of naïve B-lymphocytes and their differentiation into plasma cells. Homogeneous non-malignant human plasma cells can serve as experimental controls and for several other applications. Although, plasma cell populations can be obtained from multiple myeloma subjects and from primary and secondary lymphoid tissues, a simple in vitro method is useful for various applications. Here, we demonstrate a method to obtain untransformed, non-malignant human plasma cells in vitro.*

Methods: *Human Peripheral Blood Mononuclear Cells (PBMNCs) were subjected in vitro to Concanavalin A bound Agarose beads. The cultures were supplemented with various ratios of autologous heat inactivated serum and Fetal Bovine Serum (FBS). Also, cultures were supplemented with spent medium of U266, a multiple myeloma cell line, as a source of IL6 and other B Cell growth/differentiation factors. Morphological analysis, supernatant screening for IgG at various culture time intervals followed by flowcytometric analysis for enumerating plasma cells was performed. The effect on whole PBMNC culture and selective cultures of membrane IgG$^+$ cells was also studied.*

Results: *Maximum plasma cell yield was obtained when cultured for six days as total mononucleate cell population supplemented with FBS, conditioned medium and autologous serum at 5%, 20% and 20% respectively.*

Conclusions: *The conditions required are simple and apart from yielding plasma cells for many a use, the methodology can be adopted for understanding the underlying mechanisms of plasma cell activation and differentiation.*

Key Words: *Human peripheral blood, B lymphocytes, in vitro activation, Concanavalin A, plasma cells*

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INTRODUCTION:

Most *in vitro* studies of human lymphocytes have been conducted using peripheral blood owing to convenient availability and ease of handling. Although plasma cells comprise only around 0.5% of total peripheral blood mononuclear cells, obtaining a pure population of such non malignant cells can be of relevance for many an application. Studies on plasma cells have however, been limited primarily due to the difficulty in isolating the large numbers of plasma cells necessary for experiments. Pure plasma cell populations can be achieved in most cases from multiple myeloma subjects or from primary and secondary lymphoid tissues. Further, such isolations need techniques such as magnetic or flow sorting. Studies so far suggest Epstein-Barr virus transformation or other *in vitro* activation methods that are either very tedious or do not produce a pure population of plasma cells.[1, 2, 3] The use of Concanavalin A (Con A) bound to sepharose beads as a potent mitogen of human lymphocytes has been suggested before.[4] Further, plasmablasts have been seen to better differentiate if the Con A activation was supported by allogenic helper factors. [5, 6]

An earlier report that described the identification of B cell subsets, found that the small subset of B cells expressing IgG is responsible for the majority of the response to pokeweed mitogen (PWM) in an antibody-secreting cell (plaque) assay. The study also showed that these IgG-bearing cells gave rise to both IgG- and IgM-secreting cells significantly more when compared to membrane IgG negative cells. The study also suggested five day incubation for the maximal proliferative and secretory responses.[7]

The present study indicates a relatively simple method for the *in vitro* activation and differentiation of human circulatory membrane IgG positive cells into antibody secreting plasma cells using Agarose-bound Con A (CAB). Supplementation of cultures from conditioned medium of human multiple myeloma cells, U266, which secrete IL-6 apart from B cell Growth Differentiating factors, Fetal Bovine Serum and Heat Inactivated Autologous Serum (HIAS) were carried out to assess optimal support. Pure populations of non-malignant plasma cells can be utilized routinely for several applications. A few such include the understanding of the underlying mechanisms of their activation and differentiations, and also for comparative assessments of such cells to lymphoid cell malignancies. Although, morphologically and to a certain extent, physiologically, malignant and normal cells show similarities, their differences in conserved surface antigenic epitopes, gene regulations, signaling molecules and many such can be of vital use for applications such as in cancer diagnostics and therapy.

MATERIALS AND METHODS:

Human mononucleate cells from peripheral blood were isolated by density gradient centrifugation. The cells were washed thrice with Hanks balanced salt solution (HBSS). Membrane IgG $^{(+)}$ B cells were prepared for selective targeting by incubating $1x10^6$ mononucleate cells overnight at

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4^{0}C with 100ìl of the Rabbit anti-human IgG Horse Radish Peroxidase conjugate (IgG-HRP) previously diluted in HBSS. The treated cells were incubated with CAB in sodium acetate buffer for 10 minutes at 4^{0}C. Prior to usage, the beads were incubated at 4^{0}C overnight with 0.01% sodium azide and 4μl penicillin-streptomycin, washed twice with sodium acetate buffer at 700rpm for 7mins and then resuspended in 20μl of the buffer. 10μl of the Concanavalin A-agarose beads were utilized for 1x10^6 mononucleate cells. The non-target cells were removed by gentle washes at 500rpm for 5mins in 2ml sodium acetate buffer at 4^{0}C. The pellet obtained was resuspended in 4ml of RPMI 1640. This suspension was distributed as 500μl cultures into seven wells of a 24 well plate. The cultures were then further supplemented with FBS and/or heat inactivated autologous serum and/or spent medium from a confluent U266 cell culture (Table 1) and incubated for 6 days at 37^{0}C in 5% CO_2.

Table 1. The culture conditions employed for the *in vitro* activation and differentiation of naïve B-cells into IgG secretory Plasma cells. Combinations of 5% FBS, 20% CM and 20% HIAS were used.

Well no.	Vol. of matrix-cell suspension (µl)	5% FBS	20% heat inactivated autologous Serum (HIAS)	20% conditioned medium from U266 culture
1	500	Yes	No	No
2	500	No	Yes	No
3	500	No	No	Yes
4	500	Yes	Yes	No
5	500	Yes	No	Yes
6	500	No	Yes	Yes
7	500	Yes	Yes	Yes

After 6 days, the cultures were harvested, the cells and supernatants retrieved. The cells were washed thrice, subjected to intra cytoplasmic IgG staining by incubating for 1 hour at room temperature with anti human IgG-FITC conjugate in PBS-Tween. After washing thrice, the cells were analyzed by flowcytometry for enumerating plasma cells (10,000 events). All flow cytometric analyses were performed by FACS Calibur, BD Biosciences and CellQuest acquisition and analysis software. The supernatants were analyzed by Sandwich ELISA to ascertain the secretory properties of the cells for human IgG. Plates were coated with Anti human IgG and supernatants in triplicates were analyzed. For cultures that contained heat inactivated autologous serum, 3 serial dilutions were analyzed in triplicates. Essentially, the protocols adopted for the identification and enumeration of plasma cells as also the supernatant screening for IgG was adopted from a recent study that describes BCR signaling and TLR-9 expression for plasma cell proliferation.[8]

Further, the above mentioned protocols were adopted with an initial population of only membrane IgG positive B cells instead of the entire mononucleate cells from peripheral blood. These were obtained by immunopanning with Anti human IgG coated to a culture flask in sodium carbonate-bicarbonate buffer at room temperature for 5hrs. PBMNCs were incubated in 5ml of RPMI + 10% FBS overnight at 4^{o}C after washing the flask thrice in HBSS containing 0.1% Sodium azide. Non-adherent cells were removed by gentle washes by HBSS. The adherent cells were then scraped and washed thrice with wash buffer, re-suspended in 4ml plain RPMI containing 10μl of CAB for every 1x10^6 cells and 500μl of this was distributed into 7 wells each of a 24 well tissue culture plate. The cultures were then supplemented as described above and incubated at 37^{0}C for 6 days in a 5% CO_2 incubator. The harvested cells and supernatants were analyzed as described earlier. ELISA screening alone was performed for the supernatants recovered from the cultures which originally had only the immunopanned cells. All cultures were monitored for general conditions, cell health and morphology, as also numbers of cells bound to CAB on a daily basis for 6 days.

RESULTS:

The cell density as well as health of cells in both culture sets (with total PBMNCs and immunopanned cells) seemed to be higher when supplemented with heat inactivated autologous serum. It was also observed that the number of cells bound to the beads decreased markedly with the incubation period. From the third day on, maximum free cells as also healthy cells with differential morphologies were observed (Figure 1). The progressive cell detachments indicate the loss of membrane bound IgG which is indicative of the activation and differentiation of the cells during the culture. The cells and the beads are distinct owing to their size differences.[9]

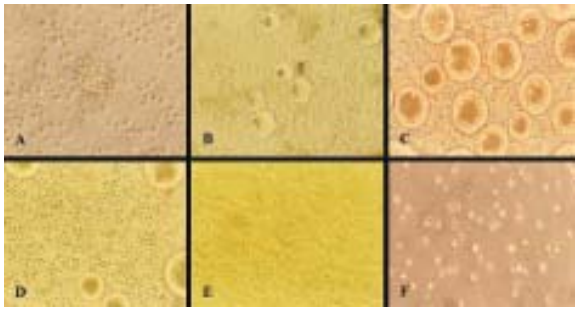

Figure 1: Cultures at various days of incubation showing progressive cell detachments to the CABs. A shows maximum attached cells to CAB at the initiation of cultures. B and C show the decreasing number of attached cells on days 1 and 2. D and E show maximal cell detachments and cultures with cells of differential morphologies on day 4. F shows cultures just before harvest, on day 6, showing highest cell densities with differential morphologies.

The total count and plasma cell enumeration as performed by flowcytometric analysis showed that maximum number of plasma cells was obtained from cultures containing all

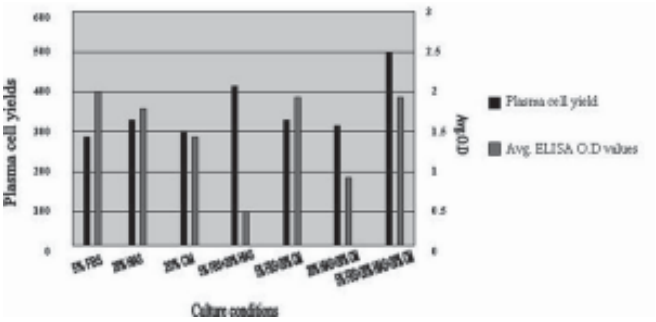

Figure 2: Yield of plasma cells and IgG quantity on harvest of whole PBMNCs under different culture conditions. Cultures with all 3 supplements showed the maximum yield of secretory plasma cells.

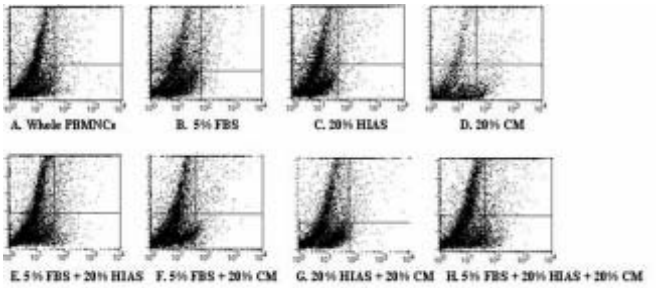

Figure 3: Cultures with all 3 supplements showing the highest yield of intracellular IgG stained cells for whole PBMNCs. The flowcytometric analysis of 10,000 events per sample show the intracytoplasmic IgG stained cells in the right top panel of each histogram; the numbers of such cells are indicated in Table 2.

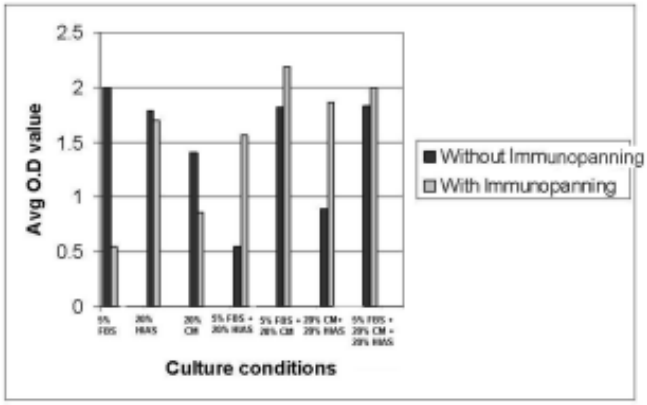

Figure 4: Maximal IgG secretion was seen in culture supernatants obtained from immunopanned cultures supplemented with 5% FBS and 20% CM. In whole PBMNC cultures, the same was observed in the presence of 5% FBS alone.

three supplements (Table 2, Figures 2 and 3). This was substantiated by the relative grading of cell densities and cell health in cultures just before harvest. Culture containing all three supplements was seen to have maximum cell density of cells in peak health. However, it was observed that cultures supplemented with 5% FBS alone gave cells with the maximal secretory property in the case of cultures containing wholePBMNCs (Figure 2). For the immunopanned cells, the IgG secretion was highest in cultures supplemented with 5% FBS and 20% conditioned medium (Figure 4).

Table 2. Effect of supplementation of whole PBMNC cultures with various combinations of FBS, HIAS and CM on cell activation and differentiation. Flowcytometric analysis revealed the increase in cell density as well as that of plasma cells in particular, 6 days after incubation.

Stage in the protocol	Culture conditions	Total cell count	No. of non-plasma cells	No. of plasma cells
Initial Sample	Whole PBMNCs	10000	9642	358
After 6 days in culture	RPMI + 5% FBS	10000	9720	284
	RPMI + 20% HIAS	10000	9673	328
	RPMI + 20% CM	10000	9706	294
	RPMI + 5% FBS + 20% HIAS	10000	9587	417
	RPMI + 5% FBS + 20% CM	10000	9660	343
	RPMI + 20% HIAS + 20% CM	10000	9688	313
	RPMI + 5% FBS + 20% HIAS 20% CM	10000	9508	499

DISCUSSION:

Studies have been carried out for the assessment of mitogenic activity of free and sepharose bound Concanavalin A. [2, 6] We have adopted in this study, different culture conditions in order to study media supplements that further enhance the mitogenic response of naïve B lymphocytes *in vitro* to CABs. Incubation of mononucleate cell population with Anti human IgG-HRP conjugate was thought of primarily to selectively target membrane IgG^+ cells to Con-A on the sepahrose beads due to their inherent affinity. The secretory properties of the plasma cells for human IgG in whole PBMNC cultures was highest in cultures supplemented with a combination of FBS and HIAS. Culture containing all three supplements was seen to have maximum cell density of cells in peak health; with such cells constituting close to 5% which is a 10 fold increase in numbers compared to their normal counts in circulation. Many studies have proven the enhanced mitogenic response of B lymphocytes in culture when present along with other cells such as dendritic cells and the T lymphocytes [10]. This could be one of the reasons for the results obtained. Another reason could be the presence of unknown activating factors in autologous heat inactivated serum. Cultures supplemented

with CM were seen to produce maximum number of plasma cells in cultures that had been subject to immunopanning. The spent medium from healthy U266 cultures contains IgE and IL-6 apart from other B cell derived growth factors that have been shown to be important for B cell activation and differentiation.[11, 12, 13] Piguet et al showed that lectins enhanced B cell proliferation and plasmablast differentiation.[14] They called this the "helper" effect and found that this was true only in cases of cultures performed at low cell concentration with suboptimal doses of lectin. The helper effect was also observed only in cultures containing a relatively low number of T blasts. This was substantiated by another study that found that Concanavalin A covalently bound to Sepharose triggered B cell preparations to higher Mitotic Factor release than either T cells or unfractionated lymphocyte suspensions.[4] This may be the reason that cells with maximum secretory potential were found in cultures subjected to immunopanning.

There were several reports of bound Concanavalin-A being an effective mitogen previously. While generative and peripheral lymphoid tissue can provide substantially large quantities of membrane IgG $^{(+)}$ cells, the significance here is that even peripheral blood which has only about 0.5% of such cells can be effectively utilized for obtaining IgG secretory plasma cells *in vitro*. It might also be important that in normal individuals, naïve B cells constitute 11 to 30% in peripheral blood whose numbers increase in certain clinical conditions.[15] Further, the protocol described is simple and can be used to study the underlying mechanisms of B cell activation and differentiation[9] apart from being utilized to obtain a population of non-malignant plasma cells for many applications.

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