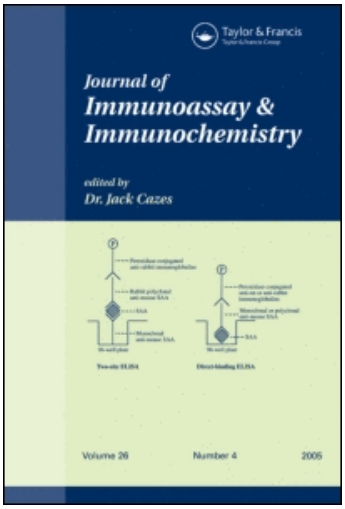

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A NEW METHOD TO DETECT DIRECTLY IN CULTURE CELL SURFACE MEMBRANE IMMUNOGLOBULINS

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ABSTRACT

An ELISA assay is described for the measurement of the smIgG. The method is based on the detection of cell-smIgG directly on the same microplate used for the culture. The cells, preincubated at 37°C for one hour, were cultured in the presence of S-ConA and serum-free medium for two days. Using this strategy, the background noise due to non specific adsorbtion of IgG to plastic wells and cytophilic antibodies was eliminated. The cells in the presence of S-ConA and serum-free medium adhered to the plastic wells, and the cell-smIgG were detected using an anti-human IgG covalently linked to alkaline phosphatase or its $F(ab')_2$ fragment. The possibility of measuring the modulation of the expression of the cell-smIgG without any additional manipulation is stressed. (KEY WORDS: ELISA, cell surface membrane immunoglobulins, modulation immunoglobulin expression)

INTRODUCTION

The expression on PBMC of the membrane surface immunoglobulins is one of the early events of the B cell differentiation.

Different methods have been developed to detect modifications in the cell expression of IgG during the complex process of lymphocyte differentiation, or as markers in the diagnosis of many disorders.

The ELISA assay has been one of the methods used to detect antibodies to cell surface antigens, both in the suspension phase (1) or with the cells adhered to plastic surfaces (2,3).

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In the present study we suggest a new and simple method to detect cell-smIgG and the modulation of its expression directly in the same microplate of culture of PBMC at an early stage of the B-cell differentiation process. Sepharose-ConA (S-ConA)(Pharmacia,Uppsala,Sweden) and culture serum-free medium allow PBMC to adhere to the wells of microplates during the incubation step. Using this system the cells do not secrete immunoglobulins into the medium, and the modulation of the expression of cell-smIgG was detected directly by an ELISA assay without any additional manipulation. The usefulness of such serum-free system could be due to increased cell density and metabolites, after addition of S-ConA and use of high cell density (4).

MATERIALS AND METHODS

Subjects

One hundred healthy men and women, aged 20 to 40 years, were examined.

Cell preparation

Peripheral blood mononuclear cells (PBMC) after centrifugation on Lympho-prep (Nyegaard et Co,Oslo,Norway), were collected from the interface,washed and resuspended in RPMI-1640 (Gibco Biocult,Grand Island,N.Y.) supplemented with 25 mM HEPES, 0.1% sodium carbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml gentamycin, 2 mM L-glutamine. The cell suspension was incubated for one hour at 37°C and then washed to remove cytophilic antibodies (5,6). Viability of isolated cells was routinely greater than 98%, as determined by Tripan Blue exclusion analysis. Cells harvested from serum-free cultures mantain a viability greater than 80-90% on day 2, and 50-60% on day 3. The use of high final cell concentration might play a critical role

in the successful serum-free system, probably by increased metabolites and cell density.

Cell surface membrane IgG detected directly in culture

Cultures were set up in triplicate in round-bottomed microtiter polystirene plates (Greiner,Nurtingen,West Germany). Each well contained 0.1 ml of cell suspension (1.6×10^6 cells/ml as a final concentration) in RPMI-1640 with or without drugs at different concentrations. S-ConA was added immediately in a further 0.1 ml volume of RPMI-1640 at a final concentration of 100 µg/ml. In each set of experiments, controls were included by adding culture medium without cells to coat the microtiter wells. Mitogens or drugs alone were also added to the wells as a control. Microtiter plates were incubated for two or three days at 37°C in a humidified atmosphere containing 5% CO_2. During the incubation step, S-ConA and serum-free medium facilitated the coating of the plastic surfaces with the cells. After 3 days of culture, the supernatants were removed and stored at -20°C until used. The cells adhering to the plastic surface were washed four times with Veronal Buffered Saline (VBS) containing 0.05% polyoxyethylene sorbitan monolaurate (Tween 20) pH 7.2, to remove the unbound cells. Washing at this and the following steps was done so that the plates were turned upside down, the content of the wells was shaken out and flushed with the washing solution. After washing, the wells were filled with 200 µl of alkaline phosphatase conjugated to anti-human IgG, affinity purified (Tago Inc.,Burlingame,CA), diluted to 10 µg/ml in VBS-Tween buffer and incubated for 2.5 hours at 37°C. The amount of enzyme-anti IgG bound to the surface IgG-bearing cells was determined using a Titertek Multiscan automatic plate reading machine (Flow Laboratories,USA).

The antibody concentration was expressed as absorbance measured at 405 nm, and was the mean of triplicate cultures ± standard deviation (s.d.). In some experiments the cells adhering to the plastic surface by S-ConA were harvested with 0.2 Mαmm, and were counted to detect the percentage of recovery (usually 30-40%). The amount of antibodies remaining in the wells after cell removal, was determined by an ELISA assay as described before. The amount of the total IgG present in the culture supernatant was determined by a standard ELISA as previously described (8,9). In other experiments, a pool of ten human sera at various dilutions was used as a control.

Identification and culture of lymphocytes

T and B cells were purified by a discontinuos density gradient of Percoll as described (10), and used for positive and negative controls. Total PBMC and PBMC from each fraction of the Percoll gradient were cultivated separately in RPMI-1640 medium. Cultures were incubated for 2 days at 37°C in a humidified 5% CO_2 atmosphere with or without PHA (10 ug/ml), and were pulsed with 5 uCi of ^{3}H-thymidine for 18 hours before termination of cultures. The cells were harvested (Cell-Harvester,Flow,USA) and incorporated radioactivity was measured. Directe immunofluorescence staining of total PBMC, B and T cells was performed using a goat anti-human IgG FITC conjugate (Tago Inc.,Burlingame,CA), with a dilution of 1:40 in the same microplate culture. The fluorescence intensity in each well of microtest tray was then detected by an inverted epi-illuminated fluorescence microscope equipped with an automated stage driver by stipping motors, photomultiplier (Zeiss) and Hewlett Packard 86 computer (11). Alternatively, analysis of B and T cells was performed using an indirect method of immunofluorescence staining.

The monoclonal antibodies used included Leu-1 (pan T), Leu-14 (B cells), HLA/DR (B cells, monocytes, T-activated) (Becton-Dickinson,CA). Lymphocytes subsets were calculated by counting the number of fluorescent cells as percentage of the total number of cells in the visible field (data not showed).

RESULTS

A schematic diagram of the method used to perform the cell-smIgG ELISA is reported in Fig.1 and described in details in the materials and methods section. The method is very simple and capable to detect surface-IgG of B cells in the same plate of culture of total lymphocytes. As shown in Table 1 total lymphocytes gave a significant detectable optical density of surface-IgG, as determined by cell-ELISA after 2 days culture. Total lymphocytes gave also good fluorescence values and they were responsive to PHA stimulation. The same results were obtained with B cells cultured alone, suggesting that during the culture of total lymphocytes in presence of S-ConA and serum-free medium, mainly B cells adhered to the microplates. In fact if T cells were cultured alone, a good PHA stimulation was still detected, although they did not show any surface-IgG by ELISA or by fluorescence. In Fig.2 is reported the influence of serum on the non specific binding of IgG to the plastic wells. If cells preincubated at 37°C were washed and then cultured in absence of serum but with S-ConA, then only cell-surface IgG were detected. No free IgG were found in the wells after cell removal or in the supernatant of cultures. But if serum was added at low concentration (0.01-0.1%), enhanced IgG were found into the wells after cell removal and in the supernatant. At higher serum concentration (1-10%) the cells did not adhere to the wells. Finally

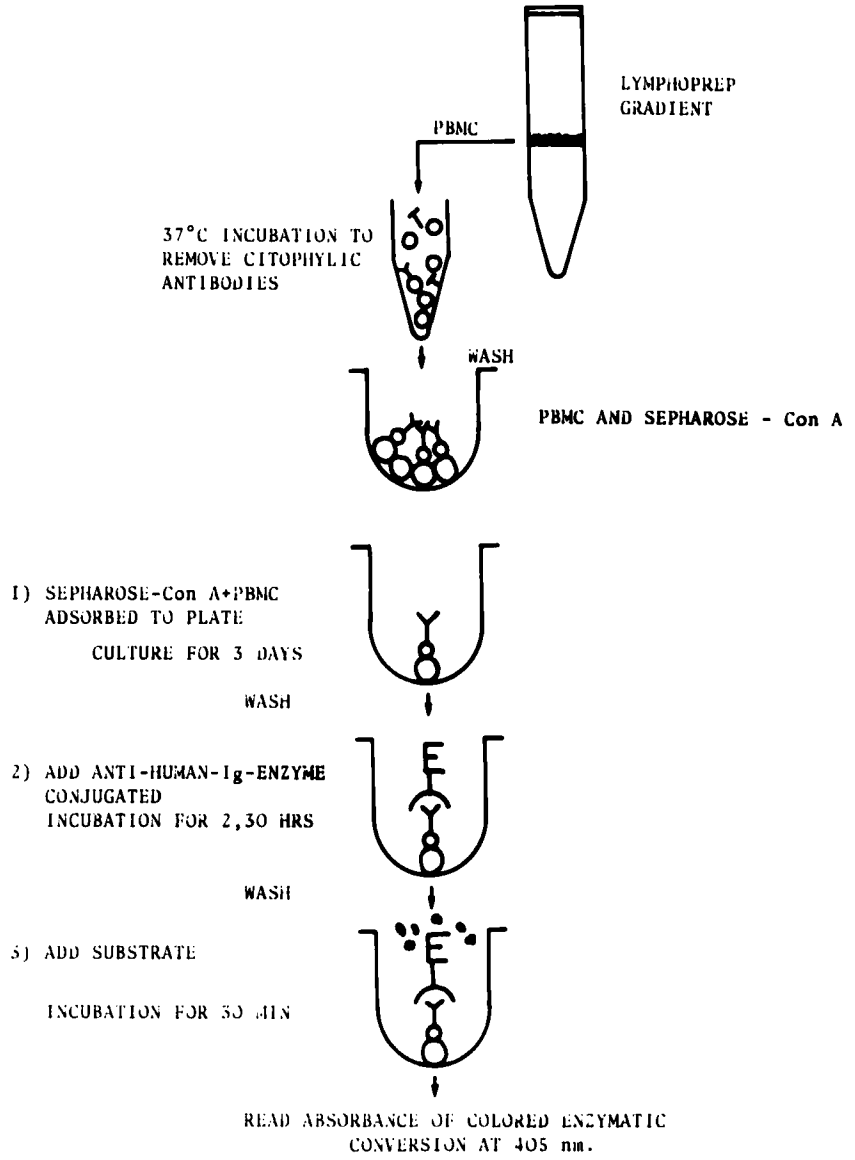

Fig.1 - Schematic diagram of the solid phase immunoenzymatic method used to detect surface membrane IgG.

TABLE 1

Behaviour of lymphocytes determined in the same culture microplate

CELLS (PBMC)	SURFACE IgG (ELISA values O.D.± s.d.)	SURFACE IgG (Fluorescence intensity*)	^{3}H-thymidine incorporation (cpm) NONE	PHA
UNSEPARATED (TOTAL)	1.800 ± 0.121	698	347±0.087	10.875±1095
FRACTION I (B Cells)	1.400 ± 0.087	500	298±0.06	6.812±0.258
FRACTION II-III (T Cells)	0.300 ± 0.10	100	953±0.21	11.196±0.754

*Fluorescence intensity measured by automated microscope equipped with computer, data given in arbitrary units.

when IgG (1-10 μg/ml) were added to the culture, they stuck to the wells and all the ELISA values were enhanced ,also after cell removal. This would suggest that the ELISA assay used was suitable to measure IgG and that using such a system, spontaneous secretion of IgG does not occur. The effect of using 100 μg/ml of S-ConA in a serum-free system is reported in Fig.3 . Preincubation for one hour at 37°C before the culture step was used to remove cytophylic antibodies. Fig.4 shows the difference of the optical density values between the first day and third day when the PBMC were cultured in serum-free medium alone or together with Betamethasone (BM) or Human Chorionic Gonadotrophin (HCG). Almost 100 human sera were analyzed and a variability was observed among the sera regarding the absolute value of optical density. Using this system the modulation of the IgG expression by BM or by HCG in a synergic effect with S-ConA was observed only

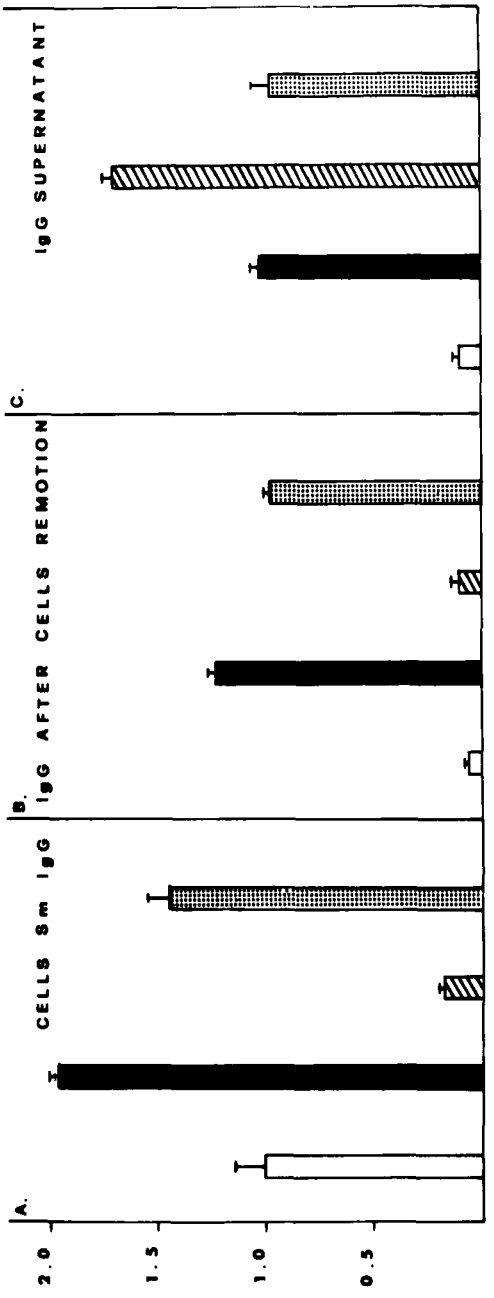

Fig.2 - Influence of serum IgG on the detection of cell-smIgG.PBMC preincubated at 37°C were cultured for 3 days with S-ConA in absence (□) or in presence of 0.01% (■) or 10% (▨) of serum or in presence of 5 µg/ml of IgG (▤). The amount of IgG was detected by ELISA on cell surface (A), on the wells after cell removal with mm (B), and in the supernatant of culture (C). Values of IgG are expressed as optical density (mean ± s.d.) of ten experiments performed in triplicate.

for the surface-IgG. No modulation was observed both for IgG detected when BM or HCG were added to the culture medium without cells. To confirm that the increased values detected by ELISA, using the anti human-IgG conjugated with alkaline phosphatase, was depending on the surface IgG and not on an aspecific binding with Fc receptors, a dose response curve of BM was carried out using anti-IgG or its $F(ab')_2$ fragment (Fig.5).

DISCUSSION

An ELISA assay designed to detect cell surface membrane immunoglobulins directly in culture, has been used to study the early steps of the lymphocyte differentiation. The absence of serum in the culture medium facilitated the adhesion of the cells to the plastic wells, while the presence of S-ConA beads increased the cell density functioning as a bridge-like connection between the cells and the plastic surface (12,13,4). The ELISA assay was capable to detect only the expression of smIgG by IgG-bearing cells since no cell growth or DNA synthesis and differentiation in antibody-secreting cells occured during the incubation time. We also showed preliminary data suggesting that the expression of smIgG can be modulated by hormones. The expression of smIgG was enhanced by BM and inhibited by HCG, and these events were different from the secretion of IgG. The method described is rapid, simple, safe and allows quantitation of the final results automatically and objectively. Radiochemicals are not required, and centrifugation of plates to wash the cells, transfer, resuspension of the cells or other manipulations or mechanical disturbance are not required. Fixatives usually used to bind the cells to the wells of microplates,that can reduce the antigenicity of cell surface glicoproteins, or can bind the IgG increasing the assay background,

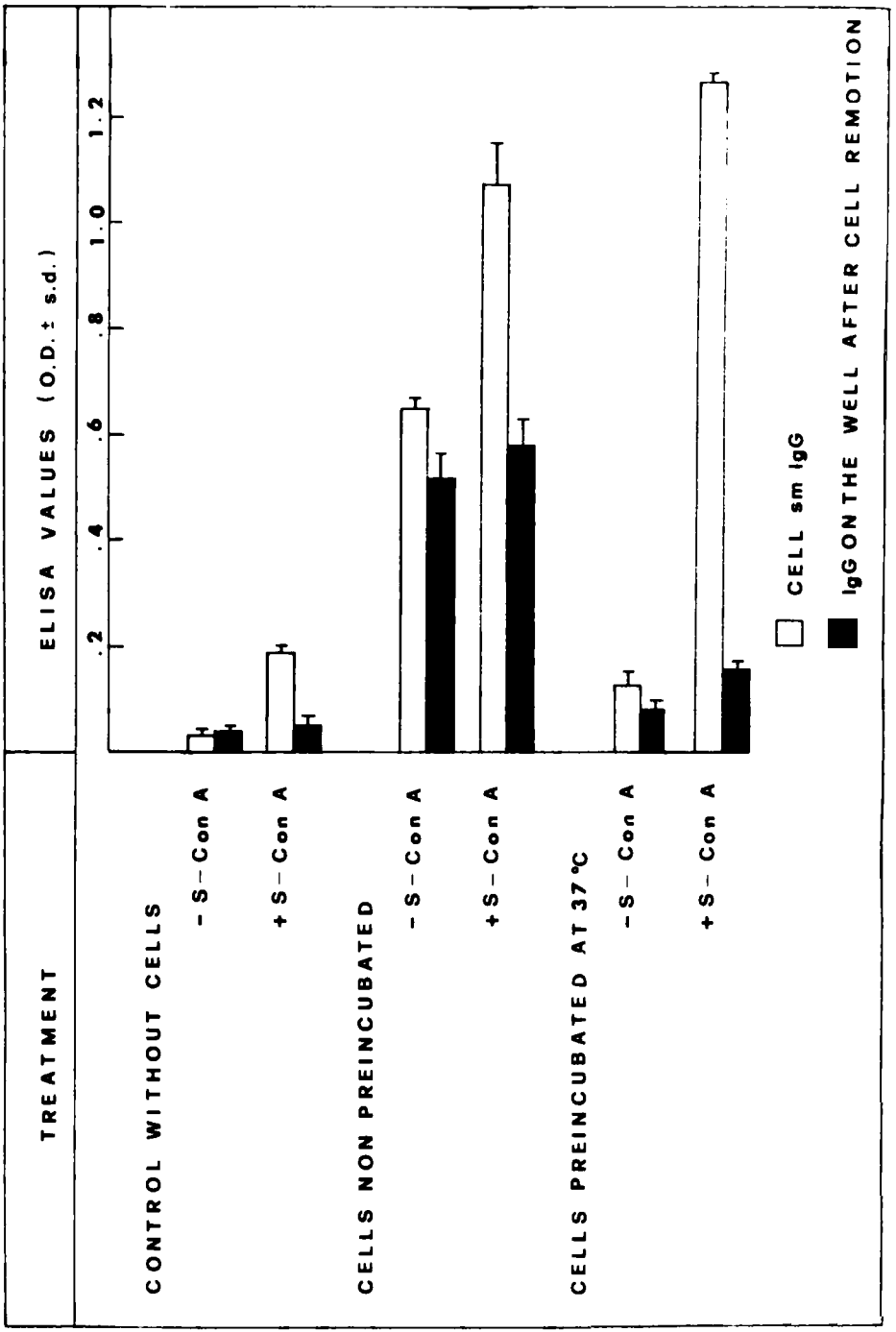

Fig.3 - The effect of S-ConA in serum-free system. PBMC (1.6×10^6 cells/ml) were preincubated at 37°C to remove cytophilic antibodies or not. PBMC were washed and cultured in presence or in absence of 100 μg/ml of S-ConA, and the antibodies were assayed on the cells (□), or in the wells after cell removal (■). As a control, S-ConA was incubated in RPMI medium without cells. The results are representative experiments from 20 normal donors. The antibody values are expressed as optical density at 405 nm as detected by ELISA, and are the mean of triplicate cultures. The s.d. did not excede 10% of the values.

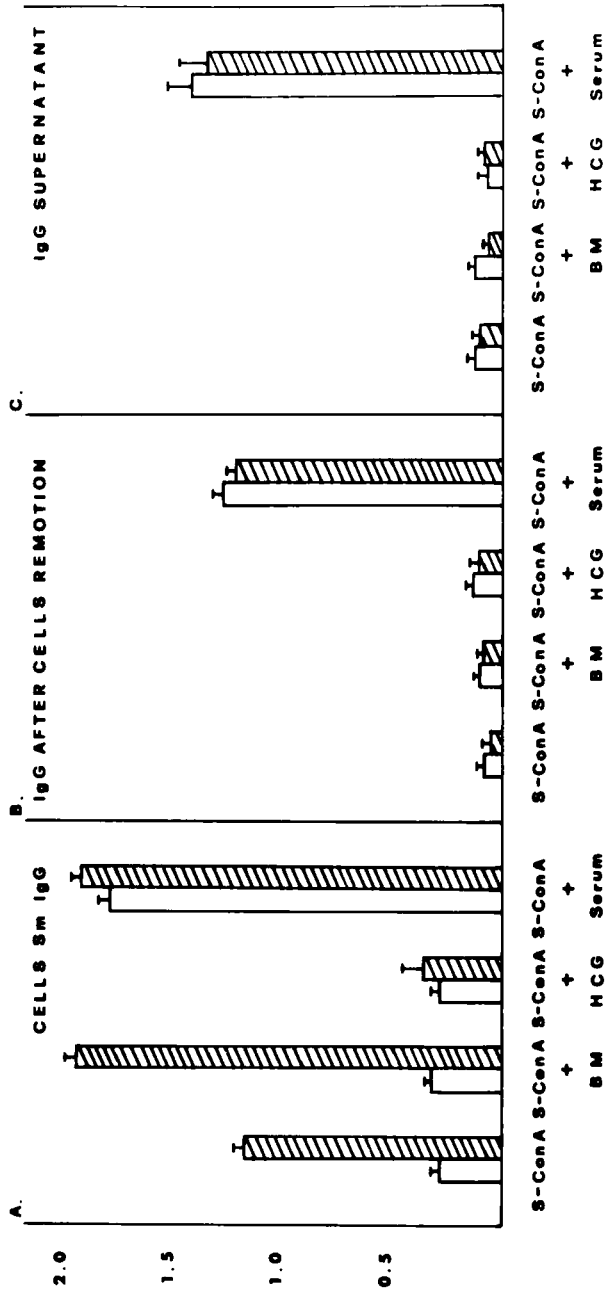

Fig.4 - Modulation of PBMC antibodies response detected on the surface of the cells (A), on the wells after cell removal (B), in the supernatant of culture (C). PBMC (1.6 x 10^6 cells/ml) preincubated at 37°C, were cultured in RPMI serum-free medium with S-ConA alone or in presence of BM 10 µg/ml, HCG 200 I.U./ml or 0.1% serum. The amount of antibodies was determined after one day (□) or three days (▨) of culture.

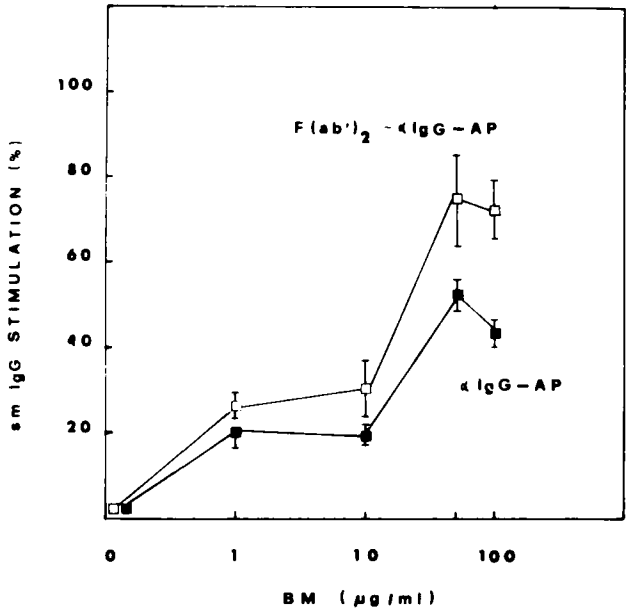

Fig.5 - Dose response curve to BM. PBMC preincubated at 37°C and cultured with S-ConA as described, in presence of increasing amount of BM (0-1-10-100 μg/ml). The O.D. values of the smIgG were determined by ELISA using alkaline phosphatase conjugated with : A) anti-IgG ; B) $F(ab')_2$ fragment anti IgG. Results are expressed as a percentage of stimulation by each BM concentration with respect to a control without BM and calculated :

$$\% \text{ smIgG stimulation} = 100 \times \frac{\text{mean O.D. of each BM conc. - mean O.D. of S-ConA alone}}{\text{mean of O.D. of S-ConA alone}}$$

or can alter the aminoacid epsilon group (gluteraldheyde), are not required as reported (1).

There are three major problems in designing a microassay to measure cell-smIgG that must be carefully considered. First, the IgG secreted can bind aspecifically to the wells of the microplates. To avoid this, usually the addi-

tion of 10% fetal calf serum in the PBMC culture medium virtually eliminated the non specific binding of IgG synthetized during the culture. This occured particularly at low serum dilutions, as suggested by other authors (3) in agreement with our results. But to avoid any possible interference by FCS that can lead to a non specific B cell activation (14), we used a system without serum and with S-ConA that not induced any response (15).

Second, the cytophylic antibodies released by the PBMC could be detected by the ELISA and so interfere with the results. To overcome this problem a preincubation step at 37°C and a washing step of PBMC before the culture was carried out (5,6). The preincubation was made in polyallomero tubes to affect only the elution of cytophilic antibodies, without remotion of adherent cells (16).

Third, the conjugate could bind to cell surface receptors. To avoid this interaction, native goat IgG anti-human IgG, which fails to bind to cell surface human Fc receptors, was used (1). In addition the smIgG on the PBMC were detected using $F(ab')_2$ fragment of goat anti-human IgG conjugated with alkaline phosphatase. The results obtained suggested that the use of the $F(ab')_2$ fragment substantially does not reduce the binding values when compared to the values obtained using the whole molecule, and this was also in agreement with the results of Morris et al.(1).

All these results suggest that using the method described is possible to detect the collaborative effect of all PBMC cultured together, focusing only on the early event of cell bearing smIgG expression, without any further manipulation of the cells.

REFERENCES

1. Morris,R.E.,Thomas,P.T.and Hong,R..Cellular enzyme-linked immunospecific assay (CELISA).I.A new micromethod that detects antibodies to cell-surface antigens. Human Immunol. 1982;5:1

2. Heusser,C.H.,Stocker,J.W. and Gisler,R.H..Methods for binding cells to plastic:Application to solid phase immunoassay for cell surface antigens. Methods in Enzymology,Academic Press Inc.,New York,1981;73:406

3. Layton,G.T. and Smithyman,A.M..A cell ELISA technique.The direct detection and semi-quantitation of immunoglobulin positive cells in 7 days lymphocyte cultures using the microtiter culture plates as the solid phase. Journal of Immunol.Methods 1983;57:37

4. Needleman,B.W. and Weiler,J.M..Human lymphocyte transformation induced by mitogen and antigens in a serum-free tissue culture system. Journal of Immunol.Methods 1981;44:3

5. Lobo,P.I.,Westervelt,F.B.and Horwitz,D.A..Identification of two populations of immunoglobulin-bearing lymphocytes in man. J.of Immunology 1975;114:116

6. Boerrigter,G.H.,Vos,A. and Scheper,R.J..Direct measurement of in vitro antibody production using an enzyme-linked immunosorbent assay. Journal of Immunol.Methods 1983;61:377

7. Engvall,E.and Perlman,P..Quantitation of specific antibodies by enzyme-linked anti immunoglobulin in antisera coated tubes. J.of Immunology 1972;109:129

8. Cocchiara,R.,Giallongo,A.,Amoroso,S.,Spina,G.,Stancampiano,R.and Geraci D..Circulating immune complexes in patients with usual interstitial pulmonary fibrosis:partial characterization and relationship with Thermoactinomyces vulgaris. Immunology 1981;44:817

9. Lems-Van Kan,P.,Verspaget,H.W.and Pena,A.S..ELISA assay for quantitative measurement of human immunoglobulins IgA,IgG and IgM in nanograms. Journal of Immunol.Methods 1983;57:51

10. Gutierrez,C.,Bernabe,R.R.,Vega,J.and Kreisler,M..Purification of human T and B cells by a discontinuous density gradient of Percoll. Journal of Immunol.Methods 1979;29:57

11. Deelder,A.M.,Koper,G.,DeWater,R.,Tanke,H.J.,Rotmans,J.P.and Ploem J.S..Automated measurement of immunogalactosidase reactions with a fluorogenic substrate by the aperture defined microvolume measurement method and its potential application to Schistosoma Mansoni immunodiagnosis. Journal of Immunol.Methods 1980;36:269

12. Sundquist,K.G.and Wanger,L..Anchorage and lymphocyte function.Contact induced augmentation of T-cell activation. Immunology 1980;42:883

13. Terukina,S.and Arai,S..Activation of human T and non T lymphocytes by Sepharose bound Concanavalin A and the differential effect of macrophages. Immunology 1981;44:215

14. Cowdery,J.S. and Steinberg,A.D..T cell mediated polyclonal B cell activation induced by cell-bound fetal calf serum. Int.Archs Allergy appl.Immunol. 1983;72:170

15. Henriksen,A.,Godal,T. and Landaas,T.O..Mitogenic effect on human lymphocytes of insolubilized anti-immunoglobulins.I.Specificity of the stimulating agent. J.of Immunology 1980;124:921

16. Treves,A.J.,Yagoda,D.,Haimovitz,A.,Ramu,N.,Rachmilewitz,D.and Fuks, Z..The isolation and purification of human peripheral blood monocytes in cell suspension. Journal of Immunol.Methods 1980;39:71