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Mitogenic stimulation of malignant B cells

CHRONIC LYMPHOCYTIC LEUKAEMIA: RELATIONSHIP BETWEEN STIMULATION AND SURFACE PHENOTYPE

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SUMMARY

Peripheral blood lymphocytes (PBL) from 14 patients with chronic lymphocytic leukaemia (CLL) were stimulated with pokeweed mitogen (PWM), formalinized *Staphylococcus aureus* (Sta) and a combination of both mitogens. The leukaemic B cells were characterized by rosetting techniques (using mouse erythrocytes and complement coated erythrocytes) and immunofluorescence for membrane bound immunoglobulin (mIg). No clear correlation between phenotype and the reactivity with PWM could be found. Results of stimulation with Sta however indicate that lymphocytes carrying membrane bound IgM (mIgM) and IgD (mIgD) and the receptor of the third complement component (C3R) can be induced to differentiate into immunoglobulin (Ig) containing cells. Addition of PWM to these cultures often enhanced this response. Some leukaemic B cells are able to differentiate after challenge with the appropriate stimulus.

INTRODUCTION

Mitogens are useful tools for investigating functional aspects of peripheral blood lymphocytes (PBL). Stimulation with the mitogens phytohaemagglutinin (PHA) and concanavalin A (Con A) results in T cell activation, whereas B cells respond only marginally. On the other hand, B cells can be stimulated by pokeweed mitogen (PWM) (Friedman, Breard & Chess, 1976), lipopolysaccharide (LPS) (Levitt, Duber-Stull & Lawton, 1981), a water soluble extract from *Nocardia opaca* (NWSM) (Bona *et al.*, 1974) or live Epstein-Barr virus (EBV) (Rosen *et al.*, 1977). The last three mitogens may activate B cells independently of T cell helper function; B cell stimulation with PWM however is highly T cell-dependent (Keightley, Lawton & Cooper, 1976; Janossy *et al.*, 1977; Gmelig-Meyling, UytdeHaag & Ballieux, 1977). In contrast to normal peripheral blood B lymphocytes, stimulation of B cells from patients with chronic lymphocytic leukaemia (CLL) is reportedly difficult. This might be the result of a functional impairment of the T lymphocytes, or of the leukaemic cells or of both cell types. Deficient helper T cell function (Chiorazzi *et al.*, 1979) in combination with excessive suppressor T cell function (Kay, 1981) in CLL patients have been described. In some cases the addition of irradiated allogeneic T cells or T cell factors could substitute for the lack of autologous helper T cell function, resulting in the restoration of the response to PWM (Fu *et al.*, 1978; Robert, 1979; Nowell *et al.*, 1981). Culturing of leukaemic B cells with EBV does not generally lead to proliferation and immunoglobulin (Ig) secretion (Robert, Bird & Möller, 1979), suggesting the occurrence of intrinsic B cell defects in CLL or the absence of appropriate target cells for EBV infection.

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Formalinized staphylococcus bacteria strain Cowan I (Sta) have been described to activate normal B cells in a T cell-independent way (Forsgren, Svedjelund & Wigzell, 1976; Sirianni *et al.*, Pryjma *et al.*, 1980; Schuurman, Gelfand & Dosch, 1980). Recently Saiki & Ralph (1981) reported an enhanced response of normal blood B lymphocytes after concomitant stimulation with Sta and PWM. This prompted us to study PBL from patients with CLL by stimulation with PWM, Sta and the combination of these two mitogens. The results indicate that leukaemic B cells can be stimulated by Sta and even better by Sta in combination with PWM.

The population of B cells reactive to Sta seems to be characterized by the presence of membrane bound IgM (mIgM) and IgD (mIgD) and receptors for the third complement component (C3R) at the cell surface.

MATERIALS AND METHODS

Lymphocytes were isolated from heparinized blood from patients with CLL by Ficoll-Isopaque centrifugation (d = 1·077 g/ml). T cells were enumerated by binding of sheep red blood cells (Es), and subpopulations of B cells were demonstrated by rosetting assays using mouse red blood cells (Em) (Gupta *et al.*, 1976) and complement coated erythrocytes (EAC) (Bianco, Patrick & Nussenzweig, 1970). Membrane bound immunoglobulins (mIg) on peripheral blood lymphocytes were determined using affinity-purified FITC- and TRITC-labelled antibodies, as described by Gathings, Lawton & Cooper (1977).

Cells ($0{\cdot}8 \times 10^6$) stimulated with the mitogens Sta, PWM and the combination of Sta + PWM were cultured in flat bottomed plates (Falcon) in RPMI 1640 supplemented with penicillin, streptomycin and glutamine (2 mM) at a cell density of 1×10^6 ly/ml and a final volume of 200 μl per well. All cultures contained 10% fetal calf serum (FCS) (Flow, batches 2906011 or 29051106). When used separately, PWM (Gibco, batch 15K0201) and Sta (a Sta suspension kindly provided by R.K.B. Schuurman) (Schuurman *et al.*, 1980) were added in 106 μg/ml and a 1:300 final dilution respectively. These concentrations were found to be optimal when tested on PBL from normal donors (data not shown). The undiluted Sta stock suspension was kept at −80°C. A working suspension, diluted 1:20 with RPMI 1640 was stored at −20°C in small aliquots until use. The Sta were added to the cultures after vigorous resuspension. The final Sta concentration in the culture medium amounted to $7{\cdot}5 \times 10^6$ Sta/ml. Differentiation of B cells was established by measurement of the generation of plasmablasts after 5 days of culture. Plasmablasts were detected by immunofluorescence on fixed cytocentrifuge preparations after incubation with the appropriate fluorescinated antibodies as described by Gathings *et al.*, (1977). For specific TRITC- and FITC-excitation (HBO100) the filter combination K and N_2 were used in a Ploemopak II set up.

RESULTS

Unseparated peripheral blood lymphocytes from 14 patients suffering from CLL were cultured using Sta, PWM and the combination of these mitogens. The responses to mitogenic stimulation *in vitro* as well as the results of marker tests (Es; Em; EAC; mIg) are presented in Table 1. The percentages of cells forming rosettes with Em varied between 3 and 87%. The majority of the neoplastic B cells having the surface phenotype IgM + IgD without other heavy chains, were positive for the complement receptor (> 50%). The percentage of cells rosetting with Es (T cells) in all patients was 21% at most. No correlation between the percentage of T cells in the individual patients and the PWM-induced reactivity of the leukaemic B cells could be noticed, however. For instance the B cells of patient Cor could not be stimulated by PWM, in spite of the relative high percentage of T cells present. On the other hand a percentage of less than 5% was sufficient to help in this response (e.g. patient Ve). No correlation between cell surface characteristics and the PWM induced activation of the neoplastic B cells could be found.

In the stimulation of the leukaemic B cells with Sta, a correlation with the phenotype of these cells emerges. Common features of the responding B cells may be the concomitant presence of

Table 1. Lymphocyte marker studies and cell differentiation data

	Cell surface markers of the PBL*				Mitogen stimulation‡		
	% Es	% Em	% EAC	% mIg†	PWM§	Sta¶	PWM + Sta**
Kr	8	55	61	80 ($\mu\delta\lambda$)	4,500 $\mu\lambda$ 5%	47,500 $\mu\lambda$ 26%	56,375 $\mu\lambda$ 51%
Ho	14	63	68	70 ($\mu\delta\lambda$)	35,750 $\mu\lambda$ 16%	4,120 $\mu\lambda$ 4%	104,625 $\mu\lambda$ 45%
vM	4	3	52	70 ($\mu\delta\kappa$)	neg††	30,000 $\mu\kappa$ 75%	45,000 $\mu\kappa$ 90%
dB	6	55	48	75 ($\mu\delta\kappa$)	375 $\mu\kappa$ 0·5%	37,500 $\mu\kappa$ 26%	32,000 $\mu\kappa$ 21%
Ev	2	87	72	73 ($\mu\delta\kappa$)	3,000 $\mu\kappa$ 1%	200 $\mu\kappa$ 0·5%	1,000 $\mu\kappa$ 1%
Cor	20	52	83	80 ($\mu\delta\lambda$)	neg	2,000 $\mu\lambda$ 2%	8,250 $\mu\lambda$ 3%
Ri	21	68	82	54 ($\mu\delta\lambda$)	7,000 $\mu\lambda$ 7%	12,000 $\mu\lambda$ 10%	233,100 $\mu\lambda$ 63%
Li	12	71	90	80 ($\mu\delta\lambda$)	266,250 $\mu\lambda$ 42%	240,000 $\mu\lambda$ 61%	321,666 $\mu\lambda$ 49%
Ve	3	16	86	90 ($\mu\delta\lambda$)	73,500 $\mu\kappa$ 19%	4,800 $\mu\kappa$ 3%	75,000 $\mu\kappa$ 19%
vR	18	24	48	70 ($\mu\delta\kappa$)	neg	neg	neg
St	3	16	86	90 ($\mu\delta\kappa$)	neg	neg	neg
Col	18	79	19	69 ($\mu\alpha\delta\kappa$)	neg	neg	neg
Kn	2	5	61	70 ($\mu\kappa$)	2,250 $\mu\kappa$ 1%	neg	neg
Br	5	36	83	80 ($\gamma\kappa$)	neg	neg	neg

* The percentage of the cells bearing the indicated marker in respect to the total PBL.

† Characterization of the heavy and light chain isotype on the surface of > 97% of the total B cell population.

‡ Absolute number of plasmablasts containing the indicated isotype and light chain. The percentual yield is calculated from the total amount of recovered cells after 5 days of culture.

§ PWM = 106 μg/ml

¶ Sta = 1:300 diluted stock suspension.

** Sta + PWM = 1:300 diluted stock suspension Sta together with 13 μg PWM per ml.

†† neg = no plasmablasts present.

The fluorescence pattern after stimulation was compared with a control, containing the same cells and medium, without the mitogen(s).

No correlation between patient's treatment and the stimulating capacity of the leukaemic B cells could be noticed.

membrane bound IgM and IgD and the C3 receptor. Within the group of patients bearing this phenotype (11 patients) the cells of nine patients could be stimulated with Sta and the combination of PWM + Sta. Cells with another phenotypic expression could not be induced to differentiate by Sta.

The Ig present in the cytoplasm of the majority of the plasmablasts (> 97%) after stimulation and the Ig present on the membrane of the unstimulated PBL carry the same light chain type. In addition all plasmablasts are only IgM positive, showing the monoclonal nature of the differentiating cells.

DISCUSSION

Sta has been described to activate B cells in a T cell-independent way (Forsgren *et al.*, 1976; Siranni *et al.*, 1979; Pryjma *et al.*, 1980; Romagnani *et al.*, 1980a); Schuurman *et al.*, 1980). It has been suggested that an interaction of protein A, present on the Sta membrane, and lymphocyte membrane protein(s) are necessary for the induction of lymphocyte activation (Romagnani *et al.*, 1981b). In agreement with this is the capacity of protein A coated Sepharose beads to stimulate lymphocyte proliferation (Forsgren *et al.*, 1976). Romagnani *et al.*, (1980b) described the ability of protein A to bind with membrane IgM, IgD and IgG molecules present on human tonsillar B cells. The same group (Romagnani *et al.*, 1981a) also showed that IgM/IgD and IgG bearing blood lymphocytes from CLL patients could bind to protein A coated human red cells, probably through a protein A–mIg interaction. The cells isolated with this rosetting technique were able to proliferate after Sta stimulation.

Studies on the proliferation of normal peripheral blood lymphocytes induced by Sta, performed by Schuurman *et al.*, (1980) showed that the target in this response is included in the C3 receptor bearing cell population; 60% of these cells are mIgM/mIgD positive B cells. Both these and our results suggest that the target in the Sta-induced stimulation is a C3 receptor, mIgM/mIgD positive B cell. Recently Saiki & Ralph (1981) showed that normal peripheral blood B lymphocytes can be effectively stimulated by PWM and Sta. The combination of these two mitogens gave considerable better results compared to stimulation with either mitogen separately. Our present data on leukaemic B lymphocytes are compatible with their original observation on normal PBL. The number of Ig containing monoclonal plasmablasts and both the fluorescence intensity and Ig secretion after stimulation with Sta+PWM were increased as compared with the exclusive stimulation with Sta or PWM.

A new finding in this study is that the responsiveness to Sta and Sta+PWM correlate completely, suggesting that in both cases the same cells are stimulated.

Kuritani & Cooper (1982) showed that among the B lymphocytes in normal blood only the IgM^+ IgD^- cells are stimulated by PWM. Our findings on leukaemic B cells may be at variance with their results working with normal B cells. In the present study the cells of seven patients carrying the phenotype IgM, IgD, C3R could be stimulated with PWM. It could be possible, however, that within the monoclonal cell population differences in maturation stage exist and the PWM stimulated a minor subpopulation bearing the phenotype IgM^+ IgD^- $C3R^+$. Suggestive in this respect is patient Kn whose B cells can only be stimulated by PWM. The findings of Kuritani & Cooper (1981) and Saiki & Ralph (1981) that T cell factors can substitute for PWM only to some extent, combined with our results, leads to a hypothesis on the working mechanism of the concomitant stimulation of PWM and Sta. The neoplastic B cells (IgM^+ IgD^+ $C3R^+$) are triggered by Sta and the cells are induced to differentiate. During this process the B cell becomes susceptible to PWM activation. Besides this B cell stimulative capacity, PWM activates residual T cells which offer help in the B cell differentiation.

The results presented here indicate that neoplastic B cells react similar to Sta stimulation as their physiological counterparts. Addition of low concentrations of PWM, often, enhances this response. Thus, the data provided from proliferation and differentiation studies performed with monoclonal lymphoid cells, can be used in the formation of a model related to the functional aspects of normal human B cells.

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