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Stimulation of human B lymphocytes by Listeria cell wall fraction

LIDA IVANYI, *Department of Oral Immunology and Microbiology, Guy's Hospital Dental School, London*

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SUMMARY

Cell wall fraction of *Listeria monocytogenes* (LCWF), a B cell mitogen for mouse spleen cells, is also mitogenic for human adult and cord peripheral blood lymphocytes. Purified B-cell suspensions responded to LCWF by *in vitro* proliferation, to a similar extent as the unfractionated suspensions. Furthermore, LCWF-induced B cell differentiation into IgM-containing cells and their percentage correlated significantly with the extent of lymphocyte proliferation.

INTRODUCTION

It has been reported that a crude cell wall fraction of the Gram-positive bacterium *Listeria monocytogenes* (LCWF) was mitogenic for mouse spleen cells (Cohen *et al.*, 1975). LCWF was also shown to be a B cell adjuvant as it can stimulate B cells to develop plaque-forming cells against T-dependent antigens when T cells are severely depleted or absent (Campbell, Schuffler & Rodriguez, 1976). The chemical nature of the mitogenic substance is not known, but it has been shown to be a structure distinct from LPS (Cohen *et al.*, 1975). These authors also assayed the mitogenic effect of LCWF on human lymphocytes with negative results when harvesting cultures after 2 days of incubation. However, the optimal proliferative response to various B cell mitogens is 4–5 days of culture (Ivanyi & Lehner, 1974; Ivanyi, 1977; Brochier *et al.*, 1976). The aim of this study was to find out whether LCWF was mitogenic for human peripheral blood lymphocytes by the assessment of the lymphoproliferative response of B cells and their maturation into IgM-containing cells.

MATERIAL AND METHODS

Patients. A selected group of nineteen patients (ten male and nine female) between the ages of 20 and 30 years with gingival or periodontal disease was investigated. A control group of twelve subjects without any clinical evidence of gingival or periodontal disease was matched for age (18–30 years) and sex (six male and six female). Cord blood from eleven neonates was obtained immediately *post-partum* in heparinised bottles, care being taken to exclude contamination by maternal blood.

Mitogens. Listeria cell wall fraction (LCWF) was a gift from Dr P. A. Campbell (Division of Allergy and Clinical Immunology, National Jewish Hospital, Denver, USA) and its preparation has been described previously (Cohen *et al.*, 1975). The range of doses of LCWF tested varied from 1–500 μg/ml of culture, and the optimal concentration was 50 μg/ml of culture (Fig. 1). Pokeweed mitogen (PWM) (Grand Island Biological Co., New York) was used in a dilution of 1:100/ml of culture (Ivanyi & Lehner, 1974).

Lymphoid cell suspensions. Adult and cord lymphocytes were isolated from heparinised blood by density gradient centrifugation on Ficoll–Triosil (Ivanyi, Lehner & Burry, 1973). T cells were identified by the rosette test (E) using neuraminidase-treated sheep erythrocytes (Galili & Schlesinger, 1974). B cells were identified by surface immunoglobulin staining with fluorescein-labelled sheep anti-human immunoglobulin (Wellcome, Beckenham, UK) (Ivanyi & Lehner, 1974).

Purification of T and B cells. T cells were purified by filtration through a nylon column (Greaves & Brown, 1974). B

Correspondence: Dr Lida Ivanyi, Institute of Dental Surgery, Eastman Dental Hospital, Gray's Inn Road, London WC1X 8LD.

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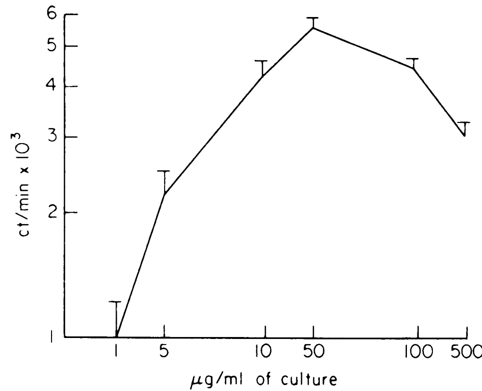

FIG. 1. Dose response to LCWF. Mean values from six subjects; ct/min = counts per min. Mean value of control cultures with saline: ct/min 707 ± 72·59.

TABLE 1. The mitogenic effect of LCWF on T or B lymphocytes

Lymphocyte donor	Cell suspension	Percentage of B cells*	Percentage of T cells†	Saline cpm‡	Mitogens PWM Ct/min‡	PWM SI§	LCWF Ct/min‡	LCWF SI§
1	T+B	13·6		1096	150,371	137·2	4055	3·7
	T	1·9		866	97,338	112·4	1039	1·2
2	T+B	11·5		469	61,673	131·5	2251	4·8
	T	1·0		523	64,433	123·2	837	1·6
3	T+B	16·2		981	50,619	51·6	5689	5·8
	T	2·8		844	43,972	52·1	1688	2·0
4	T+B		71·5	939	71,082	75·7	5915	6·3
	B		2·4	713	4919	6·9	5775	8·0
5	T+B		64·0	607	22,144	60·4	1517	2·5
	B		2·0	437	2840	6·5	1354	3·1
6	T+B		72·8	740	63,122	85·3	3996	5·4
	B		3·1	518	3677	7·1	3056	5·9

* Identified by surface immunoglobulin staining.
† Identified by E-rosette formation.
‡ Ct/min = Counts per min.
§ SI = Stimulation index.

lymphocytes were purified by eliminating E-rosettes on a Ficoll–Triosil gradient (Greaves & Brown, 1974; Galili & Schlesinger, 1974). The percentages of T cells and B cells in the purified T and B cell suspensions are shown in Table 1.

Lymphocyte cultures and DNA synthesis. Lymphocytes were cultured at a concentration of 1×10^6 cells/ml per culture for 90 hr in medium RMPI 1640 enriched with added L-glutamine (2 mmol/ml) penicillin (100 u/ml), streptomycin (100 µg/ml) and 10% human AB serum. The cultures were harvested and assayed as described previously in detail (Ivanyi & Lehner, 1970). The results were expressed as counts per min (ct/min) per 1×10^6 viable lymphocytes and as stimulation indices (SI) representing the ratio of [^{14}C]thymidine uptake between mitogen and saline stimulated cultures.

Staining for cytoplasmic IgM. Lymphocyte cultures were set up in the presence of 10% FCS (Flow) under the same conditions as described above. Sequential examination of the appearance of IgM-containing cells showed that the peak numbers were found at day 6 and this time was used routinely for harvesting the cultures. After harvesting, the cells were suspended at a concentration of 1×10^6 ml in PBS and deposited on slides using a cytocentrifuge. Staining for cytoplasmic IgM was done according to the method of Kearney & Lawton (1975) with fluorescein-labelled sheep anti-human IgM

(Wellcome) which was tested for specificity. The percentage of IgM-containing cells was assessed by counting at least 500 cells in cytocentrifuged smears. Cells with strong and weaker fluorescence were counted (Janossy *et al.*, 1976).

RESULTS

Stimulation of DNA synthesis in adult and cord blood lymphocytes by LCWF

Lymphocytes from nineteen patients with gingival or periodontal disease, lymphocytes from twelve controls with clinically healthy gingiva and cord blood lymphocytes from eleven neonates were cultured in the presence of 50 μg/ml of culture of LCWF. As no significant difference in response to LCWF was observed between patients and controls, these were treated as one group (Fig. 2). Adult peripheral

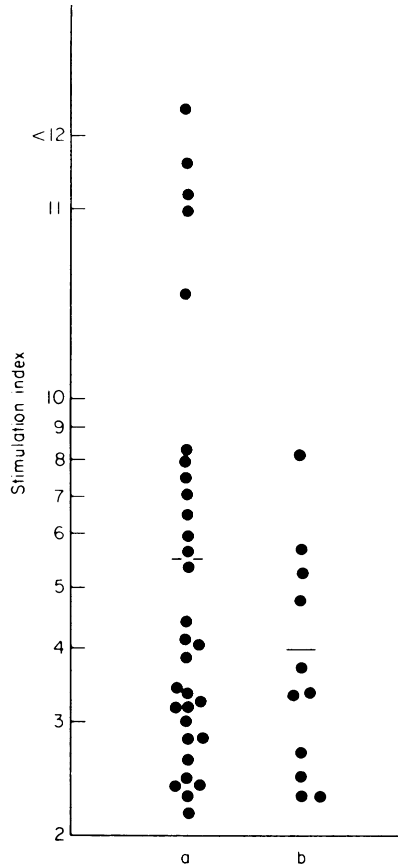

FIG. 2. The *in vitro* proliferative response of adult and cord peripheral blood lymphocytes to LCWF. (a) Adult peripheral blood lymphocytes and (b) cord blood lymphocytes. Mean ct/min of control cultures with saline were 883 ± 48·95 for adult lymphocytes and 4749 ± 485·65 for cord lymphocytes.

blood lymphocytes responded by increased DNA synthesis to LCWF (mean ct/min of 4471 ± 561 and mean SI of 5·4 ± 0·99). All lymphocyte cultures responded with a stimulation index above 2. Increased DNA synthesis was also induced by LCWF in all tested cord blood lymphocyte cultures (mean ct/min at 17,440 ± 2014 and mean SI of 3·9 ± 0·53).

The selective stimulation of T or B lymphocytes by LCWF and PWM

Untreated, and T or B purified lymphocyte suspensions were cultured in the presence of LCWF and PWM (Table 1). Untreated lymphocyte suspensions from six subjects responded by *in vitro* pro-

liferation to both mitogens (mean SI of 90·2 for PWM and mean SI of 4·7 for LCWF). The indices induced by PWM were eighteen times higher than those induced by LCWF. Purified T-lymphocyte cultures responded to PWM similarly as untreated lymphocyte cultures (mean SI of 96 and of 106) but the response to LCWF was virtually abolished (from SI of 4·8 to 1·6). In contrast, the removal of T cells did not significantly affect the stimulation induced by LCWF (mean SI of 4·7 and 5·6), whilst the response to PWM was reduced ten-fold (from mean SI of 74 to mean SI of 6·8). Nevertheless, the residual response of B cells to PWM was similar to that induced by LCWF (mean SI of 6·8 for PWM and mean SI of 5·6 for LCWF). These results indicate that LCWF is a B cell mitogen.

B cell maturation induced by LCWF and PWM

The percentages of IgM-containing cells were estimated in unfractionated lymphocyte suspensions from twenty-two subjects after stimulation with LCWF or PWM (Fig. 3). In cultures with LCWF a mean of 12% (±1·35) of recovered cells stained for cytoplasmic IgM and 28% (±2·15) of cells contained IgM after stimulation with PWM. Whilst the stimulation indices induced by PWM were eighteen times higher than those induced by LCWF, the difference in the percentage of IgM-containing cells was only two-fold. Almost all of IgM-containing cells were typical plasma cells or lymphoblasts of various sizes. Only a few of the IgM-positive cells were seen in the unstimulated cultures with saline (0·9%± 0·17) and the majority were small lymphocytes. The extent of lymphocyte stimulation was compared with the percentage of IgM-containing cells in individual subjects by Spearman's rank correlation method (Fig. 3). A significant positive correlation was found between the stimulation indices and the percentages of IgM-containing cells induced by LCWF (r = 0·92, P<0·001) and by PWM (r = 0·75, P<0·001).

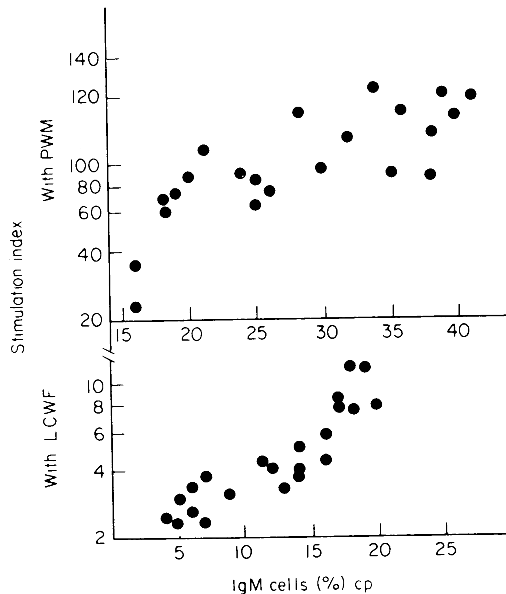

FIG. 3. Correlation between lymphocyte stimulation and the percentage of IgM containing cells induced by LCWF and PWM. IgM cells (cp) = IgM-containing cells.

DISCUSSION

The results indicate that a crude cell wall fraction of the Gram-positive bacterium *L. monocytogenes* is mitogenic for human adult and cord peripheral blood lymphocytes. Lymphocytes from all thirty-one subjects tested responded to LCWF by enhanced DNA synthesis, Similarly, cord blood lymphocytes from eleven neonates were stimulated by LCWF. Peripheral blood B-cell suspensions responded to LCWF with stimulation indices comparable to those in cultures of unfractionated (T and B) cells, whilst

the response to PWM was reduced ten times. Nevertheless, the residual response of B cells to PWM was similar to that of LWCF. These results indicate that LCWF and PWM are mitogenic for purified peripheral blood B lymphocytes. This is in agreement with the findings that human B cells from peripheral blood (Kreth & Herzenberg, 1974), spleen (Janossy & Greaves, 1975), and tonsils (Janossy *et al.*, 1976) can be stimulated by PWM in the absence of T cells. The mitogenic effect of LCWF on peripheral blood lymphocytes was comparable to the effect of an extract of *Nocardia opaca* (Brochier *et al.*, 1976). However, it has been shown that peripheral blood lymphocytes from man (Ivanyi, 1977), chicken (Weber, 1973) and rabbits (Sell & Shepherd, 1973) respond to B cell mitogens with lower stimulation indices than spleen cells. It appears that the responsiveness of the peripheral blood B lymphocytes is weaker *per se* rather than due to the weakness of the mitogen itself. Nevertheless, LCWF is apparently a more potent B cell stimulant of human peripheral blood lymphocytes than other mitogens, such as levan or dextran sulphate (Ivanyi & Lehner, 1974; Ivanyi, 1977).

We have also shown that LCWF induced B cell differentiation into IgM-containing cells. Whilst the stimulation indices induced by PWM were eighteen times higher than those induced by LCWF, the difference in the percentage of IgM-containing cells was only two-fold. The results, based on thymidine incorporation show that LCWF is mitogenic for B cells but not for T cells. Moreover, a relationship between the effect of LCWF on DNA synthesis and on immunoglobulin synthesis was suggested by several findings. The optimal dose of LCWF was the same for both activities (results not shown). The enhancement of IgM synthesis was correlated with the enhancement of DNA synthesis in individual subjects. However, the possible contribution of T cells to LCWF-induced immunoglobulin synthesis has not been investigated. It has been suggested that the B cell responsiveness to mitogenic doses of LPS is heterogenous, involving some cells which differentiate into IgM-secreting cells and others which divide but do not secrete IgM (Vitetta, Froman & Kettman, 1976). The nature of the mitogenic response may be related to the stage of maturation of the B cells as shown by Gronowitz, Coutinho & Möller (1974) in experiments with different B cell mitogens in mice. The differentiative pathway of B cells was also described in man by comparing the pattern of proliferative response of cord and maternal lymphocytes to varions B cell mitogens (Ivanyi & Lehner, 1977). As LCWF stimulation results in the appearance of a large proportion of IgM-containing cells, but in a weaker proliferative response, the responder cells might be in a mature stage of differentiation. It seems that the induction of immunoglobulin synthesis by LCWF might be a more sensitive probe than DNA synthesis for assessing the function of human peripheral blood B lymphocytes in health and disease.

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REFERENCES

BROCHIER, J., BONA, C., CIORBARU, R., REVILLARD, J.P. & CHEDID, L. (1976) A human T-independent B lymphocyte mitogen extracted from Nocarida opaca. *J. Immunol.* **117**, 1434.

CAMPBELL, P.A., SCHUFFLER, C. & RODRIGUES, G.E. (1976) Listeria cell wall fraction: A B cell adjuvant. *J. Immunol.* **116**, 590.

COHEN, J.J., RODRIGUES, G.E., KIND, P.D. & CAMPBELL, P.A. (1975) Listeria cell wall fraction; a B cell mitogen. *J. Immunol.* **114**, 1132.

GALILI, W. & SCHLESINGER, M. (1974) The formation of stable E-rosettes after neuraminidase treatment of either human peripheral blood lymphocytes or sheep red blood cells. *J. Immunol.* **112**, 1974.

GREAVES, M.F. & BROWN, G. (1974) Purification of human T and B lymphocytes. *J. Immunol.* **112**, 426.

GRONOWITZ, E., COUTINHO, A. & MÖLLER, G. (1974) Differentiation of B cells: sequential appearance of responsiveness to polyclonal activities. *Scand. J. Immunol.* **3**, 413.

IVANYI, L. (1977) The mitogenicity of dextrans for B lymphocytes in man. *Clin. Immunol. Immunopath.* **7**, 123.

IVANYI, L. & LEHNER, T. (1970) Stimulation of lymphocyte transformation by bacterial antigens in patients with periodontal disease. *Archs. oral Biol.* **15**, 1089.

IVANYI, L. & LEHNER, T. (1974) Stimulation of human lymphocytes by B-cell mitogens. *Clin. exp. Immunol.* **18**, 347.

IVANYI, L. & LEHNER, T. (1977) Interdependence of *in vitro* responsiveness of cord and maternal blood lymphocytes to antigens from oral bacteria. *Clin. exp. Immunol.* **30**, 252.

IVANYI, L., LEHNER, T. & BURRY, H.C. (1973) The response of synovial fluid lymphocytes to T and B stimulants. *Immunology*, **25**, 905.

JANOSSY, G., GOMEZ DE LA CONCHA, E., WARDAL, M.J. & PLATTS-MILLS, T. (1976) The effects of purified mitogenic proteins (Pa-1 and Pa-2) from pokeweed on human T and B lymphocytes *in vitro*. *Clin. exp. Immunol.* **26**, 108.

JANOSSY, G. & GREAVES, M.F. (1975) Functional analysis

of murine and human B lymphocyte subsets. *Transplant. Rev.* **24**, 177.

KEARNEY, J.F. & LAWTON, A.R. (1975) B lymphocyte differentiation induced by lipopolysaccharide. I. Generation of cells synthetising four major immunoglobulin classes. *J. Immunol.* **115**, 671.

KREETH, H.W. & HERZENBERG, L.A. (1974) Fluorescence-activated cell sorting of human T and B lymphocytes. I. Direct evidence that lymphocytes with a high density of membrane-bound immunoglobulin are precursors of plasmacytes. *Cell. Immunol.* **12**, 396.

SELL, S. & SHEPPARD, H.W. (1973) Rabbit blood lymphocytes may be T cells with surface immunoglobulins. *Science*, **182**, 586.

VITETTA, E.S., FORMAN, J. & KETTMAN, J. (1976) Cell surface immunoglobulin. Functional differences of B lymphocytes bearing different surface immunoglobulin isotypes. *J. exp. Med.* **143**, 1055.

WEBER, W.T. (1973) The *in vitro* response of chicken B cell populations to lipopolysaccharide. *J. Immunol.* **111**, 1277