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STIMULATION OF HUMAN LYMPHOCYTES BY B-CELL MITOGENS

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SUMMARY

B-cell mitogens stimulate human peripheral blood lymphocytes in the following order of effectiveness: levan, lipopolysaccharide, dextran. The incidence and magnitude of the response was increased in patients with chronic gingival and periodontal disease. Whereas in 45% of healthy subjects levan yielded a stimulation index >3, patients manifested an increased response according to the severity of the disease, reaching 75% in the advanced form of periodontitis. Levan and LPS stimulated spleen lymphocytes to yield higher stimulation indices than peripheral blood lymphocytes. The *in vitro* responses to these mitogens were abolished by the removal of B lymphocytes or by the depletion of phagocytic cells. It is now evident that a protein component of *Veillonella alcalescens* stimulates T lymphocytes, whereas lipid A fraction of lipopolysaccharide from the same organism stimulates B lymphocytes.

INTRODUCTION

Lipopolysaccharides (LPS) from Gram-negative bacteria are mitogenic *in vitro* for B lymphocytes from mice and other species (Stobo, Rosenthal & Paul, 1972; Gery, Krugel & Spiesel, 1972; Greaves & Jannossy, 1972; Weber, 1973; Sell & Sheppard, 1973). It has also been reported that levan and dextran are B-cell mitogens in mice (Coutinho & Möller, 1973).

The results of lymphocyte stimulation by B-cell mitogens in man are on the other hand inconclusive; Peavy, Adler & Smith (1970) reported that human lymphocytes were not stimulated *in vitro* by LPS, while others have described low but significant stimulation (Oppenheim & Perry, 1965; Eisen, Lyle & Parker, 1973). However, the class of human lymphocytes which are stimulated by LPS has not been investigated. To our knowledge there is no report so far on lymphocyte stimulation by levan and dextran in man.

We have reported recently, that ultrasonicates of some oral Gram-negative bacteria, such as *Veillonella alcalescens*, and bacterial dental plaque induced *in vitro* stimulation of lym-

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phocytes from patients with periodontal disease (Ivanyi & Lehner, 1970, 1971). As the ultrasonicates used in our study contain LPS and bacterial dental plaque contains levan and dextran, the aims of this investigation were to find out: (a) if LPS were responsible for the lymphocyte stimulation induced by ultrasonicates of *Veillonella alcalescens* in patients with periodontal disease; (b) if LPS, levan and dextran are B-cell stimulants in man.

MATERIALS AND METHODS

Patients

A selected group of sixty-one patients (thirty male and thirty-one female), 20–52 years of age were examined and classified according to severity of the disease by Russell's periodontal index (PI) into three groups: chronic gingivitis (G) (PI = 0·2–1·0); mild and moderate periodontitis (MP) (PI > 1·0–4·0) and severe periodontitis (SP) (PI > 4·0). A control group of twenty-eight subjects without any clinical evidence of gingival or periodontal disease (PI < 0·2) was matched for age (19–50 years) and sex (thirteen male and fifteen female).

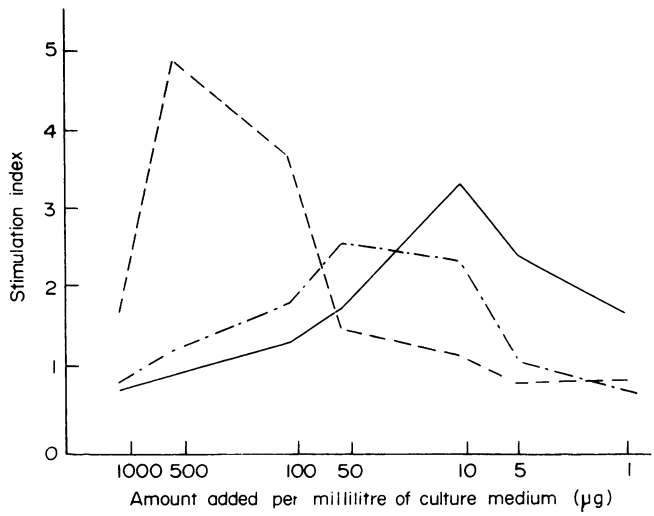

FIG. 1. Dose response to LPS, levan and dextran in five patients with periodontal disease. (——) LPS. (– – –) Levan. (– · – ·) Dextran.

Preparation of stimulants

Veillonella alcalescens ultrasonicate was prepared as described previously (Ivanyi & Lehner, 1970) and 0·1 ml of the optimal dilution 1:4 (v/v) of antigen was added to lymphocyte cultures. *Veillonella* sonicate, treated with pronase according to Bona *et al.* (1972), was used in the same dilution as described above. Lipopolysaccharides (LPS) from *Veillonella alcalescens* were prepared as described previously (Ivanyi, Lehner & Burry, 1973). Levan from *Corynebacterium levaniformis* NC BI 9659 and dextran from *Leuconostoc mesenteroides* BI 355 were gifts from Dr J. G. Howard (Wellcome Research Laboratory). The range of doses of the three putative mitogens tested varied from 1 μg to 1000 μg and the optimal concentrations were found to be 10 μg of LPS, 500 μg of levan and 50 μg of dextran per

culture (Fig. 1). The preparations of LPS were also treated by alkaline hydrolysis as described by Andersson *et al.* (1973) and the preparations were used at an optimal concentration of 10 μg per culture. Preservative-free PPD was used at a concentration of 10 μg per culture. Phytohaemagglutinin (PHA) (Wellcome Reagents) was diluted 1:100 and pokeweed mitogen (PWM) (Grand Island Biological Company, New York) was used in dilutions of 1:10; 0·1 ml of each stimulant was added to the cultures.

Lymphoid cell suspensions

Human lymphocytes were isolated from heparinized blood by density gradient centrifugation using a mixture of 'Ficoll–Triosil' as described previously (Ivanyi, Lehner & Burry, 1973). In some experiments lymphocytes were treated with colloidal iron particles to remove most of the phagocytic cells before centrifugation on 'Ficoll–Triosil' gradient (Tebbi, 1973). By this procedure the contamination of lymphocytes with up to 10% of other cells was decreased to less than 0·4% and this was tested by staining the cells for nucleoli with Toluidine Blue at pH 5·1 (Smetana, 1961).

Human spleens were obtained from the cadavers up to 8 hr after death and lymphocytes were separated by mechanical dispersion and density gradient centrifugation on 'Ficoll–Triosil'.

Removal of B lymphocytes on anti-immunoglobulin coated columns

Double layer columns were prepared by a modification of the method of Wigzell, Sundquist & Yoshida (1972). Diakon beads MG 101 (Imperial Chemical Industries) were coated with human immunoglobulin (Ig) and then equilibrated with an excess of sheep anti-human IgG serum (Wellcome Reagents). B lymphocytes were specifically retained on the columns and this was determined by immunofluorescent staining of the effluent cells; less than 4% of the lymphocytes in the effluent showed membrane fluorescence with anti-human Ig. The membrane fluorescence technique was carried out according to the method of Fröland & Natvig (1972) as described previously (Ivanyi, Lehner & Burry, 1973).

Lymphocyte transformation test

After lymphocyte separation by 'Ficoll–Triosil', cultures were prepared, harvested and evaluated as described in detail previously (Ivanyi & Lehner, 1970). The results of lymphocyte stimulation are presented in terms of the stimulation index (SI), as the ratio of [^{14}C]thymidine uptake between antigen and saline-stimulated cultures and also as c.p.m./10^6 live lymphocytes placed in the cultures.

RESULTS

Comparison of lymphocyte stimulation induced by LPS, levan and Veillonella sonicate in healthy controls and in patients with periodontal disease

Lymphocytes from healthy controls, from patients with gingivitis and mild periodontitis (G-MP group) and from patients with severe periodontitis (SP group) were cultured in the presence of LPS, levan and *Veillonella* sonicate (Table 1). The results were classified according to the values of SI into four groups: negative (SI < 1·5), mild (SI > 1·5–3·0), moderate (SI > 3·0–5·0) and strong (SI > 5·0) stimulation. More than 30% of patients from both G-MP and SP groups responded to LPS by moderate or strong stimulation and over 60%

TABLE 1. Comparison of lymphocyte stimulation induced by LPS, levan and *Veillonella* sonicate in patients with periodontal disease

Group	Number of subjects	Saline		SI	LPS			Levan			*Veillonella* sonicate		
		C.p.m.	Mean		Number of subjects	%	Mean	Number of subjects	%	Mean	Number of subjects	%	Mean
Controls	28	1235	64·11	<1·5	18	64·5		7	25		16	57·1	
				1·5–3·0	6	21·5	1·9	8	28·6	3·0	12	42·9	1·4
				3·0–5·0	2	7	0·30	10	35·7	0·32	0	0	0·08
				>5·0	2	7		3	10·7		0	0	
G-MP	48	1278	48·57	<1·5	11	22·9		1	2		0	0	
				1·5–3·0	21	43·7	2·9	18	37·5	4·4	13	27	4·6
				3·0–5·0	11	22·9	0·26	17	35·4	0·44	18	37·5	0·32
				>5·0	5	10·5	$P<0·05$*	12	25·1	$P<0·05$	17	35·5	$P<0·01$
SP	13	1387	80·79	<1·5	1	7·7		0	0		4	30·8	
				1·5–3·0	7	53·8	3·0	3	23	5·0	9	69·2	1·7
				3·0–5·0	4	30·8	0·44	4	30·8	0·67	0	0	0·12
				>5·0	1	7·7	$P<0·05$	6	46·2	$P<0·01$	0	0	$P<0·02$

* Student's *t*-test was applied to compare G-MP and SP groups with the control group.

of patients from the G-MP group and 70% of patients from the SP group responded to levan with a SI over 3·0. In the control group moderate and strong stimulation by LPS was observed only in 14% of cases but 45% of controls were stimulated by levan. Lymphocyte stimulation induced by LPS and levan was significantly increased in the G-MP ($P<0·05$) and SP group ($P<0·05$–$0·01$) as compared with the control group.

Lymphocyte stimulation induced by *Veillonella* sonicate showed the same results as described previously (Ivanyi & Lehner, 1970, 1971; Ivanyi, Challacombe & Lehner, 1973) with negative or mild stimulation in both the control and SP groups and moderate or strong stimulation in the G-MP group. There was no clear correlation between stimulation induced by *Veillonella* sonicate and by LPS in individual patients. Lymphocytes from patients with the highest response to LPS were virtually not stimulated by *Veillonella* sonicate.

TABLE 2. Comparison of lymphocyte stimulation induced by dextran, LPS and levan

Subjects	Saline (c.p.m.)	Stimulation index		
		Dextran	LPS	Levan
LS	1432	3·0	1·8	6·1
PR	1200	1·5	2·0	3·0
DK	936	2·4	3·3	8·5
BD	1000	3·1	3·4	4·0
PP	879	2·7	5·3	2·5
ML	1390	3·2	3·7	2·8
DE	1110	3·0	2·0	5·6
FA	958	0·9	0·9	3·0
SC	1162	1·0	1·4	2·2
Mean stimulation index		2·3	2·6	4·2

TABLE 3. The effect of removal of phagocytic cells on lymphocyte stimulation induced by LPS, levan and dextran in three patients with periodontal disease

Lymphoid suspension	Saline		Stimulation index			
	C.p.m.	Mean	LPS (mean ± s.e.)	Levan (mean ± s.e.)	Dextran (mean ± s.e.)	*Veillonella* (mean ± s.e.)
Untreated	1099	97·0	4·6 ± 1·56	5·2 ± 0·64	3·3 ± 0·67	6·7 ± 0·65
Treated with iron particles	930	70·48	2·0 ± 0·46	1·9 ± 0·23	1·6 ± 0·17	2·2 ± 0·08

Mitogenic potency and macrophage dependence of dextran, LPS and levan

The extent of lymphocyte stimulation induced by dextran, LPS and levan was compared in nine subjects (Table 2). The results show that levan induced the highest stimulation with a mean SI of 4·2, whilst dextran and LPS stimulated lymphocytes to a smaller extent with a

mean SI of 2·3 and 2·6. The effect of removal of phagocytic cells on lymphocyte stimulation induced by LPS, levan and dextran was studied in three patients (Table 3). The removal of most phagocytic cells greatly decreased the lymphocyte response to LPS, levan, dextran and *Veillonella*.

TABLE 4. Comparison of lymphocyte stimulation induced by modified and untreated stimulants

Stimulants	Number of patients	C.p.m.	Mean	SI (mean)	Student's *t*-test
Saline	18	1279	66·80		
Veillonella	8	7182	806·42	5·6±0·66	
Veillonella treated with pronase		2115	262·15	1·5±0·11	$P<0·001$
LPS	10	5495	737·69	4·8±0·77	
LPS treated with alkali		1891	316·66	1·7±0·33	$P<0·01$

TABLE 5. The effect of removal of B lymphocytes on lymphocyte stimulation induced by various B or T stimulants

Subject number	Lymphocytes passed through anti-immunoglobulin column	Percentage of immunoglobulin-bearing lymphocytes	Saline (c.p.m.)	Stimulation index				
				Levan	LPS	Dextran	*Veillonella*	PHA
1	−	29	1390	4·8	3·7	3·1	6·2	352·1
	+	3·5	1416	0·9	0·8	0·7	6·3	369·4
2	−	22	976	6·5	5·4	2·9	5·9	147·8
	+	2	763	1·5	2·1	0·9	4·2	200·1
3	−	14	848	3·0	2·8	2·1	3·7	335·8
	+	0	762	1·0	1·6	0·5	4·2	381·4
4	−	13	920	4·6	2·6	2·4	5·4	243·5
	+	0·5	440	1·3	0·9	0·6	8·2	326·7
5	−	11	910	5·0	2·7	3·1	6·7	382·1
	+	0	815	2·1	1·5	1·6	7·4	409·4

The effect of modified and untreated stimulants

Treatment of *Veillonella* sonicate with pronase fully abolished stimulation of lymphocytes from eight patients. This difference between stimulation induced by untreated and by pronase-treated *Veillonella* sonicate was statistically significant (Table 4). The results suggest that a protein is the antigenic component in *Veillonella* ultrasonicate concerned with specific stimulation of lymphocytes. Alkali-treated preparations of LPS were used to determine whether lipid A was responsible for the mitogenic activity on human lymphocytes (Table 4).

The stimulation induced by LPS was abolished by alkaline hydrolysis of LPS. These results imply that lipid A is the active component of LPS in stimulating human lymphocytes.

Removal of B lymphocytes on anti-immunoglobulin coated columns

The results of stimulation by levan, LPS, dextran, *Veillonella* and PHA of anti-immunoglobulin column-purified T-lymphocyte suspensions are shown in Table 5. Untreated lymphocyte suspensions from five patients responded well to all five stimulants. The stimulation induced by *Veillonella* sonicate and by PHA was not significantly affected by the removal of B lymphocytes. On the other hand, removal of B lymphocytes abolished the stimulation induced by dextran in all five patients and stimulation induced by levan and LPS was abolished in four out of five patients. Two patients responded by only mild stimulation after B-cell removal (patients numbers 2 and 5 with an SI of 2·1 to levan or LPS). The results suggest that levan, LPS and dextran are B-cell stimulants in man, whereas *Veillonella* sonicate is predominantly a T-cell stimulant. The magnitude of stimulation induced by B-cell mitogens was not dependent on the percentage of immunoglobulin bearing B lymphocytes in the suspension, as the SI in patients numbers 1 and 5 were comparable, although the percentages of B lymphocytes present in these two suspensions were different (29% and 11%).

TABLE 6. Stimulation of lymphocytes from human spleens by various stimulants

Age of spleen donor	Saline (c.p.m.)	Stimulation index					
		PHA	PWM	*Veillonella*	PPD	Levan	LPS
58 years	1331	120·0	207·5	4·6	7·2	18·2	11·4
5 months	870	24·7	35·0	0·8	1·0	5·5	3·7

Stimulation of lymphocytes from human spleens

Since stimulation of peripheral blood lymphocytes by levan and LPS was significantly increased in patients compared with the controls, the mitogenicity of levan and LPS was explored further by testing the response of human spleen lymphocytes (Table 6). Spleen lymphocytes from a 58-year-old donor responded well to both T and B stimulants and to PWM; the responses induced by levan and LPS were greatly increased with a SI of 18·2 for levan and 11·4 for LPS, when compared with those elicited in peripheral blood lymphocytes. Spleen lymphocytes from a 5-month-old donor responded again well to levan and LPS, with SI similar to those found in peripheral blood lymphocytes. However, their response to PHA and PWM was low and they failed to respond to *Veillonella* and PPD. The results suggest that levan and LPS stimulate spleen lymphocytes to a greater extent than peripheral blood lymphocytes.

DISCUSSION

The results demonstrate that levan, LPS and dextran induce a proliferative response in human lymphocytes. These three stimulants differ, however, in their mitogenic potency and in their optimal concentration. Levan induced the highest stimulation, while LPS and dex-

tran were stimulatory to a lesser but significant extent. The optimal concentrations (per 1 ml of culture medium) were 500 μg for levan, 50 μg for dextran and 10 μg for LPS, which are 2–20 times lower than those claimed to be optimal for stimulation of mouse spleen lymphocytes (Coutinho & Möller, 1973).

The stimulation induced by levan, LPS and dextran was dependent on the presence of phagocytic cells. Our results agree with those of Eisen, Lyle & Parker (1973) who have shown that purification of lymphocytes on nylon columns abolished lymphocyte stimulation induced by LPS. However, they are inconsistent with the views of Andersson, Sjöberg & Möller (1972) that B-cell mitogens can trigger lymphocytes directly in the absence of macrophages.

B cell-depleted lymphocyte cultures mostly failed to respond to all three mitogens as compared with untreated (mixed T and B) lymphocyte cultures. The presence of residual immunoglobulin-bearing lymphocytes (up to 3·5%) in some of the effluent suspension was not reflected in the stimulation indices to these mitogens. Consequently, a very slight response of T lymphocytes to LPS and levan cannot be entirely excluded.

Spleen lymphocytes from a 5-month-old child responded well to both LPS and levan, but not to specific antigens, PPD and *Veillonella*. Furthermore, human spleen lymphocytes responded with higher stimulation indices than peripheral blood lymphocytes, and the values were comparable with the response of mouse spleen cells. Similar comparative data have been reported in chicken and rabbits (Weber, 1973; Sell & Sheppard, 1973).

Peripheral blood lymphocytes from patients with periodontal disease responded significantly better to levan and LPS than lymphocytes from healthy controls. The reason for this is unclear at present, but at least two alternative mechanisms should be considered. We have suggested recently that LPS stimulates a subpopulation of human B lymphocytes, which can be found at sites of chronic inflammation, such as the synovial fluid from patients with rheumatoid arthritis (Ivanyi, Lehner & Burry, 1973). It is therefore possible that this subclass of B lymphocytes accounts for the increased response to levan and LPS of peripheral blood lymphocytes from patients with periodontal disease. Furthermore the number of B lymphocytes with complement receptors are increased at sites of chronic inflammation (Pincus, Bianco & Nussenzweig, 1972). It has also been demonstrated in mice, that the majority of LPS-stimulated blast cells form rosettes via the C3 receptor sites (Gormus, Crandall & Shands, 1974). Since periodontal disease is a chronic inflammatory disease, the increased response to LPS and levan of peripheral blood lymphocytes from patients compared with controls might be interpreted as an increased number of C3 receptor-bearing lymphocytes in peripheral blood.

An alternative mechanism is suggested by our finding that stimulation of B lymphocytes by mitogens is macrophage-dependent. It is possible that processing of LPS, levan and dextran by phagocytic cells would cause release of an endogenous mitogen, as a common mediator of lymphocyte stimulation. Consequently, greater stimulation indices may result from a higher (or more active) proportion of macrophages in the spleen and in the peripheral blood of patients with periodontal disease.

It is significant that bacterial dental plaque consists of large numbers of micro-organisms and their products, of which LPS, levan and dextran are quantitatively important. Thus dental plaque in man is a focus in which T cell-dependent antigens co-exist with B cell mitogens. The latter have an adjuvant effect (Allison & Davies, 1971) and may contribute to the persistent broad spectrum immune responses of the host (Lehner *et al.*, 1974).

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