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THE SPECIFICITY OF SERUM FACTORS IN LYMPHOCYTE TRANSFORMATION IN PERIODONTAL DISEASE

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SUMMARY

Peripheral blood lymphocytes from patients with periodontal disease were stimulated *in vitro* by sonicates of *Veillonella alcalescens* in the presence of autologous and substituted sera which were either untreated or absorbed with *Veillonella* cells. A significant positive correlation was found between the level of lymphocyte stimulation and IgM class of haemagglutinating antibody titres in patients with mild periodontitis (MP) ($P<0{\cdot}01$) but not in those with gingivitis (G), severe periodontitis (SP) or in the control group (C). Absorption of autologous serum with a suspension of *Veillonella* cells resulted in a depression of lymphocyte stimulation by *Veillonella* antigen in G or MP patients, but had no effect on lymphocyte stimulation in SP patients. Furthermore, absorption with *Veillonella* abolished the stimulating activity of substituted G or MP sera on lymphocyte cultures from SP patients and the inhibitory activity of SP sera on lymphocyte cultures from G or MP patients. Serum absorption was specific, as it did not affect the mitogenic response of lymphocytes to *Actinomyces viscosus*, PPD or PHA. The data is interpreted in terms of modulation of lymphocyte responses *in vitro* by stimulating or inhibitory antibodies.

INTRODUCTION

It has been reported that *Veillonella alcalescens*, other Gram-negative bacteria and *Actinomyces viscosus* can induce specific lymphocyte transformation in patients with gingivitis and mild periodontitis but not in those with severe periodontitis (Ivanyi & Lehner, 1970). The inhibition of lymphocyte transformation in the advanced stage of the disease was ascribed to a serum inhibitory factor (Ivanyi & Lehner, 1971). This was established by its suppressive effect on *Veillonella* induced stimulation of autologous lymphocytes as well as of lymphocytes from patients with gingivitis or mild periodontitis. Removal of the inhibitory factor in serum restored the *in vitro* stimulation of lymphocytes from patients with severe periodontitis. Furthermore, their lymphocytes when cultured in the presence of foetal calf serum revealed strong cytotoxic and migration inhibitory activity (Ivanyi, Wilton & Lehner,

1972). Sera from patients with the early stages of periodontal disease showed a stimulatory factor which promoted the response of sensitized lymphocytes (Ivanyi & Lehner, 1971). This factor enhanced transformation of lymphocytes from patients with severe periodontitis but not from controls that lacked sensitized lymphocytes.

The aims of this study were to determine the specificity of these factors and to investigate the possibility that they are antibodies.

MATERIALS AND METHODS

Patients

A selected group of fifty-six patients (thirty male and twenty-six female) 18–53 years of age, were examined clinically and radiologically 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) (P > 1–4) and severe periodontitis (SP) (PI > 4) (Ivanyi & Lehner, 1970). A control group of ten subjects without any clinical evidence of gingival or periodontal disease (PI < 0·2) was matched for age (18–50) and sex (four male and six female).

Preparation of antigens

Veillonella alcalescens and *Actinomyces viscosus* sonicates were prepared as previously described (Ivanyi & Lehner, 1970) and 0·1 ml of 1:2 (v/v) diluted antigen was added to lymphocyte cultures. Preservative-free PPD was used at a concentration of 100 μg/ml and 0·1 ml of the antigen was added per culture. Phytohaemagglutinin (PHA: Wellcome Reagents) was used as a positive control with lymphocytes from all subjects in dilutions 1/100 (v/v).

Lymphocyte transformation test

Lymphocyte cultures were prepared, harvested and evaluated by methods described in detail previously (Ivanyi & Lehner, 1970). The results were expressed in terms of the stimulation index (SI) as the ratio of ^{14}C-thymidine uptake between antigen and saline-stimulated cultures.

Sera used in lymphocyte cultures

Human serum was separated from blood which was collected on the same occasion as that for lymphocytes. Lymphocyte cultures were set up in a 20% serum concentration as which follows: (a) autologous serum from the lymphocyte donor (b) sera from patients with gingivitis or mild periodontitis had both a high antibody titre (5–8) and high SI (> 6·0) with autologous lymphocytes (G or MP sera) (c) sera from patients with severe periodontitis (SP sera).

Absorption of sera with Veillonella cells

Formalin-inactivated *Veillonella alcalescens* cells were washed six times in saline. The cell sediment after the last wash was resuspended at a concentration of 5 mg (dry weight) per 1 ml of the sera stated above. The suspension was incubated at 37°C for 1 hr, centrifuged and the serum was removed and passed through a 0·45 μm Millipore membrane filter.

The effect of serum absorption with *Veillonella* cells was studied in lymphocyte cultures

with autologous or reciprocally substituted sera: (a) cultures of SP lymphocytes with G or MP sera, (b) cultures of G or MP lymphocytes with SP sera. After absorption with *Veillonella* cells all sera were tested by passsive haemagglutination against *Veillonella* antigen. Almost all sera were found to be negative except for a few high-titred sera which had a residual titre of $\log_2$ 1.

Passive haemagglutination test

Haemagglutinating antibodies were assayed by a modification of the micromethod of Sever (1962). Sheep red cells (5%) were mixed with an equal volume of *Veillonella* antigen (dilution 1:5) and sensitized for 1 hr at 37°C. After washing 4 μl of 1·25% of sensitized cells were added to 50 μl of serially diluted sera. Cells were allowed to settle for 4 hr at room temperature and the agglutinating end point was determined. The controls consisted of unsensitized cells added to serially diluted samples and sensitized cells in diluent alone. The results were expressed as the mean $\log_2$ reciprocal titre of two separate estimations. Foetal calf serum was included in every test as a negative control and a serum shown to contain antibodies was included in every test as a positive control. The specificity of the reaction was confirmed by haemagglutination inhibition, using a 1:5 dilution of *Veillonella* antigen added to serial dilutions of the test sera before addition of the sensitized cells. Complete inhibition was observed in all instances.

Characterization of antibodies

Stepwise DEAE-cellulose chromatography (Levy & Sober, 1960) modified as described by Lehner (1969) was used to separate the sera of ten patients and four normal controls into the major immunoglobulin classes. The fractions resulting from the application of the three buffers were concentrated to the original volume of serum by positive pressure dialysis and then each assayed for antibody activity by the passive haemagglutination test. When tested by the immunoelectrophoretic and double diffusion in agar techniques fraction I contained only IgG, fraction II mainly IgA with some IgG, and fraction III IgM with a trace of IgG.

RESULTS

Quantitative relationship between SI and antibody titres

The results of lymphocyte transformation revealed the same pattern as described previously; lymphocytes from patients with gingivitis (G group) and mild periodontitis (MP group) responded to stimulation by *Veillonella* antigen with mean SI values of 4·2 and 6·5, while in patients with severe periodontitis (SP group) stimulation was only observed in two patients and the mean SI was 1·4. Lymphocytes from control subjects were not stimulated by *Veillonella* (mean SI 1·0; Fig. 1).

Haemagglutinating antibody titres to *Veillonella* antigen varied between $\log_2$ reciprocal 3 and 10, but did not differ significantly from one group of patients to another, or when these were compared with the controls. The mean $\log_2$ antibody titre was 4·5 for the control group, 5·2 for the G group, 6·2 for the MP group and 5·9 for the SP group. A positive correlation was found between the level of lymphocyte stimulation and antibody titres to *Veillonella* antigen in patients with mild and moderate periodontitis ($P<0·01$). The highest stimulation indices were found in cultures from patients with the highest serum antibody

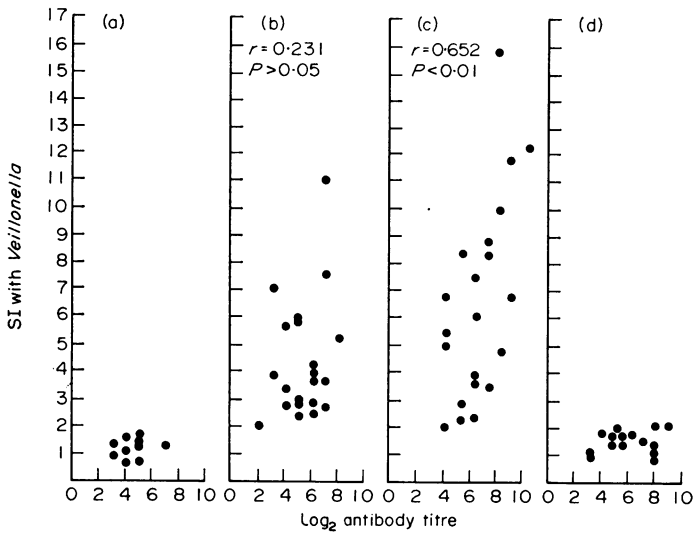

FIG. 1. Quantitative relationship between SI and antibody titres to *Veillonella* antigen. (a) Control. (b) Gingivitis. (c) Mild and moderate periodontitis. (d) Severe periodontitis. SI = stimulation index.

TABLE 1. The results of substitution of sera in cultures of lymphocytes from patients with gingivitis, mild or moderate periodontitis stimulated by *Veillonella alcalescens*

Lymphocyte donor with gingivitis, mild or moderate periodonitis	Serum concentration (%)	Sera					
		Autologous		G or MP		Control	
		Cpm	SI	Cpm	SI	Cpm	SI
H.V.	20	4314	3·9	3289	3·3	2658	2·1
S.T.	20	2634	3·7	2592	3 2	1833	2 0
W.I.	20	2753	3·6	1959	2·6	1537	1·8
M.A.	20	2528	4·4	1876	3·0	1035	1·5
C.A.	20	2325	4·9	2286	4·5	ND	
	10*	902	1·9	739	1·7		
J.O.	20	2432	3·8	2304	3·2	ND	
	10*	1152	1·8	1152	1·4		

* 10% = 10% tested serum supplemented with 10% FCS.
G or MP = serum from patients with gingivitis or mild periodontitis.
All substituted sera (G or MP or Control) had antibody titres $\log_2$ 5–7.
ND = not done.

titres. However, in patients with gingivitis a correlation between SI and antibody titre was not found ($P>0{\cdot}05$). The stimulation indices in both control and SP groups were near to 1·0, irrespective of the antibody titre (Fig. 1).

Lymphocytes from patients with mild periodontitis were stimulated by *Veillonella* antigen in the presence of homologous G or MP serum almost as effectively as in the presence of autologous serum of the same antibody titre (5–7; Table 1). Sera from control subjects were less or non-stimulatory, although their antibody titre was the same (5–7) as that of the autologous or homologous G or MP sera. The highest SI were found in cultures with 20% concentration of autologous or homologous G or MP serum (Patients C.A., J.O.; Table 1).

Characterization of antibodies

Assay of the fractions from DEAE–cellulose chromatography showed that haemagglutinating antibodies to *Veillonella* sonicate resided predominantly in the IgM class (Table 2).

TABLE 2. Haemagglutinating activity of fractions of human sera separated by DEAE–cellulose chromatography

Sera	Fractions		
	I (IgG)	II (IgA–IgG)	III (IgM)
Patients			
1 G	1*	0	7
2 G	0	1	5
3 G	0	0	2
4 G	0	0	5
5 MP	1	0	8
6 MP	1	0	8
9 MP	0	0	5
8 SP	0	0	3
9 SP	3	0	7
10 SP	3	2	7
Controls			
1	0	0	3
2	0	2	5
3	0	0	3
4	1	0	7

* Titres expressed as $\log_2$ reciprocal.
G = gingivitis.
MP = mild periodontitis.
SP = severe periodontitis.

A low titre of antibody of the IgG class was found in about half of the subjects. Two patients (Nos 9 and 10) with the highest antibody titres in the IgG fraction belonged to the SP group. Three sera showed low antibody titres in the IgA fraction, but this may have been due to the contaminating IgG. Separation of two sera by gel filtration on Sephadex G–200 resulted in the isolation of IgM fractions which were not contaminated by IgG. Haemag-

glutinating antibody activity was found only in the IgM fractions, thus confirming the results with DEAE–cellulose chromatography fractions.

The effect of absorption of autologous serum with Veillonella cells on lymphocyte stimulation

Absorption of autologous serum with the suspension of *Veillonella* cells resulted in a depression of SI by more than 1·5 in most of the patients with gingivitis and mild or moderate periodontitis; the largest fall in SI was 10·0 (Fig. 2). On the other hand absorption of autologous serum had no effect on lymphocyte stimulation by *Veillonella* antigen in patients with severe periodontitis or in the control group. Hence absorption with *Veillonella* cells has effectively removed the 'stimulating' activity of autologous sera on lymphocyte cultures from patients with gingivitis and mild or moderate periodontitis, but has failed to remove the 'inhibitory' serum activity on lymphocytes from patients with severe periodontitis.

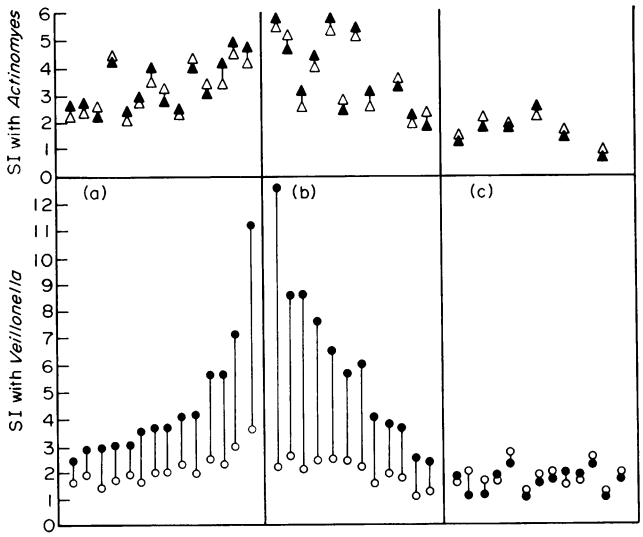

FIG. 2. The effect of absorption of autologous serum with *Veillonella* cells on lymphocyte stimulation. (a) Gingivitis. (b) Mild and moderate periodontitis. (c) Severe periodontitis. (●, ▲) Untreated serum. (○, △) Absorbed serum. SI = stimulation index.

The specificity of absorption with *Veillonella* cells was clearly shown, since the absorbed serum did not alter the lymphocyte response to an unrelated antigen, *Actinomyces viscosus*.

The effect of substituted sera which were untreated or absorbed, on lymphocyte stimulation

The stimulating activity of G or MP sera on lymphocytes from patients with severe periodontitis was decreased or abolished in the presence of absorbed serum (Table 3). The inhibitory activity of SP sera on lymphocytes from patients with gingivitis and mild periodontitis was abolished by pre-treatment of the sera with *Veillonella* cells (Table 4). Although lymphocyte stimulation by the unrelated antigen PPD was slightly decreased in four out of seven patients in the presence of SP serum compared with autologous serum, absorption of the sera with *Veillonella* cells had no effect when compared with stimulation in the presence of untreated SP serum. PHA induced lymphocyte transformation was not

TABLE 3. Substitution of autologous serum by G or MP serum in lymphocyte cultures from patients with severe periodontitis: all cultures were stimulated with *Veillonella* antigen

Lymphocyte donors with severe periodontitis	Antigens	Sera: Autologous Cpm	Autologous SI	G or MP: Untreated Cpm	Untreated SI	G or MP: Absorbed with *Veillonella* cells Cpm	Absorbed with *Veillonella* cells SI
O.S.	*Veillonella*	890	1·0	3519	4·1	941	1·3
	Saline	890		858		723	
M.U.	*Veillonella*	1549	2·1	3059	4·6	1451	2·1
	Saline	737		665		690	
W.A.	*Veillonella*	1504	1·6	2917	3·3	1700	2·0
	Saline	940		883		850	
H.E.	*Veillonella*	624	1·2	1432	2·4	900	1·8
	Saline	520		596		500	

G or MP = sera from patients with gingivitis or mild periodontitis.
SI = stimulation index.

TABLE 4. Substitution of autologous serum by SP serum in lymphocyte cultures from patients with gingivitis or mild or moderate periodontitis

Lymphocyte donors with gingivitis or mild or moderate periodontitis	Stimulation index: *Veillonella* AU	*Veillonella* SP	*Veillonella* Absorbed SP	PPD AU	PPD SP	PPD Absorbed SP	PHA AU	PHA SP	PHA Absorbed SP
O.K.	3·8	1·4	3·0	8·0	6·1	6·0	209·0	183·4	203·6
O.K.	3·8	1·7	2·9	8·0	6·7	6·4	212·0	204·6	189·3
G.R.	4·3	1·5	2·2	7·7	8·9	8·4	267·4	281·1	255·8
L.E.	2·5	0·9	2·4	19·9	14·7	14·0	144·5	126·7	140·0
D.A.	2·9	1·6	2 0	10·7	11·5	12·0	139·4	148·7	156·8
D.A.	2·9	1·3	2·1	10·5	10·3	9·7	121·0	116·0	110·7
B.R.	6·8	1·2	2·3	8·0	6·8	6·7	ND	ND	ND

AU = autologous serum.
SP = serum from patients with severe periodontitis untreated.
Absorbed SP = serum from patients with severe periodontitis absorbed with *Veillonella* cells.

significantly affected by the substituted sera. Thus absorption of SP sera with *Veillonella* cells removed their inhibitory activity on *Veillonella*–induced lymphocyte stimulation, when tested with lymphocytes from G or MP patients (Table 4), but not with autologous SP lymphocytes (Fig. 2).

DISCUSSION

Lymphocyte transformation with *Veillonella* antigen depends not only on the presence of specifically sensitized lymphocytes, but also on stimulating factors present in sera from patients with gingivitis or mild periodontitis (Ivanyi & Lehner, 1971). G or MP sera also restore the response of lymphocytes from patients with severe periodontitis to *Veillonella* antigen *in vitro*; this effect appears similar to that of 'deblocking antibodies' from the sera of immunized animals, which can counteract the effect of 'blocking' sera on lymphocyte mediated cytotoxicity against tumour cells *in vitro* (Hellström & Hellström, 1970; Bansal & Sjögren, 1971). The present evidence shows that the lymphocyte stimulation enhancing factor from G or MP sera was specifically removed by absorption of serum with *Veillonella* cells and therefore it can be ascribed to antibodies. The positive correlation between stimulation indices and antibody titres in patients with mild periodontitis suggests that the haemagglutinins which belong predominantly to the IgM class were effective in enhancing the *in vitro* lymphocyte response. However, sera from control subjects did not promote lymphocyte stimulation in spite of the fact that they contained haemagglutinating antibodies, although these were of slightly lower titres than in patients. Interpretation of this is not clear at present and depends on testing pure IgM fractions from G or MP sera on lymphocyte stimulation *in vitro*. The mechanism for enhancement is poorly understood; it is possible that enhanced lymphocyte stimulation by antigen–antibody complexes operates via macrophage-dependent antigen processing (Oppenheim, 1972). It was shown that passively administered antibody has the ability to enhance the delayed type of hypersensitivity reactions (Liew & Parish, 1972). Furthermore, passively administered IgM antibodies enhanced the antibody response to sheep erythrocytes *in vivo* (Henry & Jerne, 1968; Dennert, 1971).

A consistent depression of lymphocyte transformation in autologous serum in patients with severe periodontitis has been ascribed to a serum inhibitory factor (Ivanyi & Lehner, 1971). Absorption of the antibodies present in SP sera failed to restore the response of autologous lymphocytes from patients with severe periodontitis. However, in the serum substitution experiments using lymphocytes from G or MP patients the inhibitory activity of SP serum was abolished by pretreatment with *Veillonella* cells. These apparently conflicting results might be explained by assuming the presence of 'blocking antibodies' not only in the serum, but also on the surface of lymphocytes from patients with severe periodontitis. Removing 'blocking antibodies' from the sera alone cannot restore the response of lymphocytes, because their receptors are still blocked. This would account for the finding of 'blocked' lymphocytes in SP patients even in the presence of absorbed SP serum. 'Deblocking antibodies' from the sera of G or MP patients may effectively compete and replace the 'blocking antibodies' or antigen–antibody complexes from the surface of SP lymphocytes and thus render them responsive to *Veillonella* antigen.

Serum factors blocking *in vitro* assays of cell-mediated immunity were demonstrated in various human and animal models of allograft, tumour and autoimmunity (Hellström &

Hellström, 1970; Hellström, Hellström & Allison, 1971; Jagarlamoody *et al.*, 1971; Glancy, 1972). It was reported recently that the immunosuppressive effect of sera which were capable of enhancement of tumour growth *in vivo* and of inhibition of lymphocyte mediated cytotoxicity *in vitro* was mediated not by free antibody but rather by antigen–antibody complexes affecting the lymphocytes directly (Klein, 1971); similar mechanisms may play a role in the present system. Antigen–antibody complexes cytophilic for lymphocytes have been demonstrated some time ago (Uhr, 1965). Although the prevailing evidence shows a binding of such complexes to B lymphocytes (Basten, Sprent & Miller, 1972; Harding *et al.*, 1971; Paraskevas *et al.*, 1972) there is also some experimental evidence for antibodies cytophilic for T cells (Modabber & Coons, 1972; Fakhri & Hobbs, 1972). The latter possibility is of considerable interest in view of the present data and this will be further explored.

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