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T cell proliferative responses to molecular fractions of periodontopathic bacteria

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SUMMARY

Soluble antigenic preparations of *Veillonella parvula* and *Bacteroides gingivalis* were separated by SDS–PAGE and used after electroblotting and solubilization for *in vitro* lymphocyte stimulation in 13 patients with severe periodontitis and 12 controls. The cellular responses of controls and patients to *V. parvula* antigens were represented by four main proliferation-inducing fractions with 74–66, 52–46, 22–19 and 12 kD mol. wt. These fractions induced slightly enhanced DNA synthesis in lymphocytes from eight patients who failed to respond to whole antigenic extract. Lymphocyte samples from *Veillonella* whole extract unresponsive patients were also examined for *in vitro* proliferation by *B. gingivalis* fractions. Almost all stimulatory activities could be classified into five regions of 84–74, 35–31, 28–25, 17–15 and 12 kD.

Keywords molecular fractions periodontopathic bacteria T cell repertoire periodontal disease lymphocyte proliferation

INTRODUCTION

Dysfunctions of immune responses of various degrees of specificity frequently occur in the advanced stages of chronic inflammatory diseases. It has been reported previously that young adults and adult patients with severe progressive periodontitis manifested low levels or complete failure of proliferative responses of both peripheral blood and gingival lymphocytes to periodontopathic organisms (Ivanyi & Lehner, 1970; Ivanyi, Challacombe & Lehner, 1973; Baker *et al.*, 1978; Ivanyi, 1980; Ivanyi, Topic & Lydyard, 1981; Stashenko *et al.*, 1983, 1985; Seymour, Cole & Powell, 1985). The results were attributed to CD8 lymphocyte-mediated immunoregulatory suppression activated by either *Veillonella parvula* (Ivanyi, 1986) or *Actinobacillus actinomycetemcomitans* (Shenker & DiRienzo, 1984). The present study analyses the specificity of polyclonal T cell proliferative responses in patients with severe progressive periodontitis and controls. Soluble antigenic preparations of *V. parvula* and *Bacteroides gingivalis* were separated on the basis of molecular weight within the range of 10–100 kD by SDS–PAGE and were used after electroblotting and solubilization for *in vitro* lymphocyte stimulation (Abou-Zeid *et al.*, 1987). The aims were to investigate the most frequent immunogenic fractions, and to find out whether separated fractions are able to stimulate responses of lymphocytes which fail to proliferate in the presence of whole extracts.

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MATERIALS AND METHODS

Selection of subjects

Blood samples were obtained from 13 patients with severe periodontitis (SP) and from 12 control subjects (Table 1). The SP group contained patients exhibiting generalized alveolar bone loss with multiple vertical osseous defects (Mouton *et al.*, 1981). The control group was chosen on the basis of probing depth measurement of $\leqslant 3$ mm and no evidence of inflammatory periodontal disease other than mild gingivitis. All controls were matched for age and sex.

Bacterial strains and growth conditions

The bacterial strains were *B. gingivalis* W50 and *V. parvula* ATCC 17745. The cells were grown by batch culture to stationary phase at 37°C in an atmosphere of 70% N_2, 20% H_2 and 10% CO_2 in a complex medium as described previously (Farida *et al.*, 1986).

Preparation of sonicated extracts

The cells were harvested by centrifugation (10 000 ***g*** for 10 min), resuspended in 20 ml phosphate-buffered saline (PBS) followed by ultrasonic disruption at maximum output (7A at setting 8; Dawe Soniprobe, London, UK) for 16 min. During sonication the cells were kept at 4°C and a 2-min cooling period followed each 2-min burst of sonication (Farida *et al.*, 1986). Cell disruption was confirmed by microscopy. Cell debris was removed by centrifugation (15 000 ***g*** for 5 min) and the total protein content of the supernatant was determined using a Lowry protein estimation.

Antigenic fractions

Sonicated extracts of *V. parvula* or *B. gingivalis* (100 μg/gel) were applied, using a blank comb with a single reference well for mol. wt. markers (Sigma Chemical Co., Poole, UK). Protein bands were separated using SDS–PAGE (12% w/v acrylamide in the running gel and 4·8% in the stacking gel) under reducing conditions at 15 mA per gel for 50 min in a Minigel electrophoresis system (SE 250 Hoeffer Scientific Instruments, San Francisco, CA). Both bacterial extracts contained several protein constituents, apparent after staining with Coomassie blue.

The proteins were electrophoretically transferred to nitrocellulose paper in the TE22 Apparatus (Hoeffer Scientific Instruments) using 0·02 M Tris–glycine buffer (pH 8·3) containing 20% methanol for 1 h at 50 V. Nitrocellulose membranes (7·5 × 40 mm) were cut into 20 horizontal sections each 2-mm wide, corresponding to 12–92-kD fractions. Strips were then solubilized as described previously (Abou-Zeid *et al.*, 1987) by incubation and intermittent mixing for 1 h with 500 μl DMSO, and nitrocellulose particles were precipitated by the addition of 500 μl sterile 0·05 M carbonate–bicarbonate buffer while vortexing the mixture vigorously. The nitrocellulose particles were suspended in 0·5 ml of RPMI 1640 (Flow Laboratories, Rickmansworth, UK), after removal of DMSO by repeated washing and centrifugation with PBS. The emulsified fractions from 16 replicate nitrocellulose membranes were pooled, and aliquoted at −20°C for use in proliferation assay. Molecular weights were assigned to fractions using markers (Sigma) that were stained following separation with amido black.

Lymphocyte proliferation assays

Lymphocyte blastogenesis was assessed in a micro-culture system as described previously (Ivanyi, 1986). Briefly, mononuclear cells were obtained from peripheral blood by separation on Ficoll–Hypaque density gradient and washed twice in medium TC199. Cells were suspended (2×10^5/well) in 96-well microtitre trays in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U of penicillin, 100 μg of streptomycin per ml, and 10% autologous serum; 20 μl of the 20 separate fractions from gels loaded with 100 μg sonicated bacterial extracts were added to each well at the initiation of these cell cultures. After 6 days of incubation, the cultures were pulsed with 1·0 μCi of ^{3}H-thymidine (^{3}H-TdR; Amersham International, Amersham UK. Specific activity 25 Ci/mmol) for 8 or 16 h and harvested onto glass-fibre filters. Proliferation, as correlated with ^{3}H-TdR incorporation, was measured by liquid scintillation spectroscopy. Mean ct/min ± s.e.m. of the mean for triplicate antigen-containing cultures were converted to stimulation indices (SI) in relation to the saline control cultures (background response to emulsified nitrocellulose alone). Analysis of the unstimulated cultures showed that a ratio of two included 3 s.d. above the mean and represented a significant degree of stimulation. In order to confirm the optimal concentration of the antigenic fractions (prepared from gels loaded with 100 μg soluble extract) lymphocytes from one subject were also tested with 20 μl fractions diluted 1/2 in RPMI 1640 (Table 2). The results obtained at the two antigen doses were comparable.

Table 1. Age, sex and clinical status of subjects with severe periodontitis (SP) and controls

Subject groups	Female	Male	Total	Age (years) Mean ± s.e.m.	Range
Control	6	6	12	23 ± 0·6	18–31
SP	7	6	13	22 ± 0·5	18–31

RESULTS

In vitro lymphocyte stimulation by V. parvula *fractions*

Lymphocytes from all 12 controls responded by enhanced DNA synthesis to whole antigenic extract with SI varying from 2·0 to 4·7 (Table 3). The obtained pattern of responses to fractions derived from SDS–PAGE-fractionated *V. parvula* sonicate showed that almost all stimulatory activities could be classified into four regions: 74–66, 52–46, 22–19 and 12 kD. No difference was observed between the immunogenicity of low and high mol. wt fractions. No test fraction caused inhibition of the responses below background levels. Similar results were obtained in four patients with mild periodontitis whose responses to the whole extract varied from SI = 3 to SI = 9 (results not shown).

Out of 13 patients with SP, lymphocytes from five patients responded to low but significant proliferation to whole antigenic extract, while lymphocytes from eight patients were not stimulated by this extract (patients no. 3, 4, 6, 8, 9, 10, 11 and 13; Table 3). However, lymphocytes from seven out of these eight patients

Table 2. Reproducibility of lymphocyte stimulation patterns of two antigenic doses

Antigen (100 μg/gel)	Dilution of each fraction	Whole extract* (ct/min)	Molecular weight (kD) fractions†				
			76–66	52–46	22–19	12	
Veillonella parvula	Nil	2815	3114	2455	1797	1797	
	1/2	2611	2980	2531	1724	1646	
			87–74	35–31	28–25	17–15	12
Bacteroides gingivalis	Nil	4459	2657	4622	2959	1287	2150
	1/2	3987	2266	4176	2692	1311	2284

* Response to unfractionated antigen bound to nitrocellulose and emulsified.
† Expressed as ct/min.

Table 3. Proliferative responses of lymphocytes from controls and patients with severe periodontitis to fractionated extracts from *Veillonella parvula*

Donor no.	Saline* (ct/min)	Whole extract†	Molecular weight (kD) fractions‡ 74–66	52–46	22–19	12
Control						
1	599	4·7	5·2	4·1	3·0	3·0
2	1142	2·0	2·1	2·8	2·2	2·0
3	764	3·9	3·4	2·7	5·0	2·1
4	672	2·9	3·0	2·2	2·4	3·0
5	845	2·0	2·7	2·9	3·0	2·0
6	421	3·1	3·0	3·2	2·6	2·5
7	535	4·3	2·9	3·1	1·9	1·6
8	928	2·6	2·4	1·8	1·8	1·8
9	1421	3·3	1·9	2·6	2·4	3·1
10	1387	2·9	2·0	2·4	3·2	1·9
11	714	2·5	1·8	1·7	2·5	2·2
12	928	3·2	3·1	2·6	2·3	2·4
Patient						
1	481	2·4	1·6	1·8	2·8	2·0
2	1298	2·0	1·9	2·4	1·7	1·7
3	429	1·5	2·1	1·2	1·5	2·3
4	1523	1·1	1·5	2·0	1·6	2·0
5	976	2·0	2·0	2·7	2·4	1·8
6	901	1·2	1·4	3·6	4·3	1·2
7	864	2·2	2·0	2·6	1·4	1·6
8	796	1·9	3·4	1·5	2·8	1·8
9	537	0·8	2·4	1·7	3·2	1·9
10	641	0·9	3·2	2·0	1·6	1·2
11	1176	1·4	1·6	1·2	2·3	2·4
12	628	2·4	1·9	1·9	1·8	2·0
13	843	0·7	1·6	1·4	1·4	1·8

* Background response to emulsified nitrocellulose alone.
† Response to unfractionated antigen bound to nitrocellulose and emulsified.
‡ Expressed as stimulation index (ratio between antigen and saline stimulated cultures).

responded to individual fractions by slightly enhanced DNA synthesis (four patients to 74–66 and 22–19 kD regions and three patients to 52–46 and 12 kD regions). Furthermore, in the presence of two fractions (66–54 and 40–35 kD) ^{3}H-TdR incorporation was below the saline background level (SI 0·4–0·6) in five out of 13 patients with SP (results not shown).

In vitro lymphocyte stimulation by B. gingivalis *fractions*

It was of interest to ascertain whether the lack of response to *Veillonella* extract is extended also to the response to other periodontopathic bacteria. Thus, lymphocyte samples from *Veillonella*-unresponsive SP patients and four controls were examined for proliferative responses to *B. gingivalis* fractions (Table 4). Lymphocytes from seven out of eight patients responded to whole antigenic extract with SI varying from 2·5 to 8·1, while lymphocytes from one patient were not stimulated to a significant degree. The obtained pattern of responses to fractions derived from SDS-PAGE-fractionated *B. gingivalis* sonicate showed that almost all stimulatory activities could be classified into five regions: 84–74, 35–31, 28–25, 17–15 and 12 kD. The 28-25-kD fraction seemed to be immunodominant, as lymphocytes from seven out of eight patients responded to this region by enhanced DNA synthesis. Similar results were obtained in the four tested controls. No test fraction caused inhibition of the responses below background levels.

Table 4. Proliferative responses of lymphocytes from eight patients with severe periodontitis to fractionated extracts from *Bacteroides gingivalis*

Donor no.	Whole extract	Molecular weight (kD) fractions* 84–74	35–31	28–25	17–15	12
Patient						
3	3·1	4·4	1·5	2·4	1·8	2·4
4	4·2	1·6	3·7	2·0	2·3	2·2
6	3·8	1·7	2·9	3·5	3·7	2·3
8	2·9	1·8	3·3	2·9	1·8	2·8
9	2·5	2·4	1·9	1·8	1·9	1·6
10	6·4	6·5	4·8	3·9	2·0	2·0
11	8·1	2·0	3·2	6·8	3·0	1·6
13	1·7	2·9	1·6	2·8	1·7	1·5
Control						
1	5·4	3·8	1·9	6·5	2·0	2·3
2	4·7	4·1	2·1	4·5	2·4	3·2
3	7·9	1·8	6·2	5·8	2·0	2·0
4	6·3	2·0	3·7	6·0	3·2	1·9

* Expressed as stimulation index.

DISCUSSION

The peripheral blood T cell repertoire of patients with SP was analysed using SDS–PAGE-separated and electroblotted *V. parvula* and *B. gingivalis* bacterial extracts. The technique is based on the premise that small particles of nitrocellulose-bound antigens are adequately presented to T cells (Abou-Zeid *et al.*, 1987). However, the separation of bacterial extracts into 20 fractions gives only crude resolution, which represents a certain limitation of the technique. In order to standardize the proliferative assays, the fractions from several replicate SDS–PAGE blots were pooled into batches that provided identical stimulants for lymphocyte cultures throughout the study.

The cellular responses of controls and patients with SP to fractionated *V. parvula* antigens were represented by four main proliferation-inducing fractions of 74–66, 52–46, 22–19 and 12 kD. The individual differences in the response patterns may be attributed either to genetic or environmental factors. It is of particular interest that the proliferative response of lymphocytes was directed to at least one of these fractions in seven out of eight patients with SP who failed to respond to the whole, nitrocellulose-bound and emulsified antigen. It has been reported previously that SDS–PAGE/nitrocellulose/DMSO-processed antigens have up to 80-fold enhanced stimulatory capacity compared with soluble antigens (Abou-Zeid *et al.*, 1987). This finding was not confirmed in the present study, since the stimulation indices in SP patients were comparable with stimulation induced by soluble *Veillonella* antigen in previous

studies (Ivanyi *et al.*, 1981; Ivanyi, 1986). The responsiveness to fractionated antigens in non-responders to whole bacterial extract was also reported by other investigators (Converse *et al.*, 1988; Mendez-Samperio *et al.*, 1989). The interpretation may rest in the abolition of a 'suppressor' constituent by the separation of the whole antigen extract. Alternatively, the denaturing conditions of the technique could have yielded 'neo-epitopes' that may not be representative of *in vivo*-processed antigenic determinants.

Lymphocytes from *Veillonella*-unresponsive SP patients were also examined for proliferative responses to *B. gingivalis* fractions. Lymphocytes from all but one patient responded to whole antigenic extract by enhanced DNA synthesis. The obtained pattern of responses to *B. gingivalis* fractions showed that almost all stimulatory activity could be classified into five regions: 84–74, 35–31, 28–25, 17–15 and 12 kD. These results show the evidence of distinct determinants of *V. parvula* and *B. gingivalis*. However, the 12-kD fraction cross-reacted between the two species. It is possible that polypeptide fragments from larger native molecules partly contributed to this activity. Finally, a comparison with the previous Western blot analysis of the antibody repertoire (Watanabe, Marsh & Ivanyi, 1989) showed that the 27-kD fraction from *B. gingivalis* was stimulatory of both B and T cell responses, whereas responses to the remaining stimulatory fractions from *B. gingivalis* and *V. parvula* were not associated.

Identification of antigens from periodontopathic bacteria, stimulatory for the T cell repertoire provides a starting point for the molecular definitions of immunologically active constituents and of their role in human periodontal disease.

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