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The role of T_G lymphocytes in cell-mediated immunity in patients with periodontal disease

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SUMMARY

Blood mononuclear cell suspensions from patients with a severe form of periodontal disease failed to respond by *in vitro* stimulation to a sonicate from the oral bacterium, *Veillonella alcalescens*. The proliferative response could be restored by the depletion of T_G cells by rosetting with IgG-coated ox erythrocytes and by reconstitution of the cell suspension with 10% plastic-adherent monocytes. Small but statistically significant restoration of the Veillonella response was also achieved by the addition of indomethacin or mefenamic acid to unfractionated cell cultures, indicating only a minor role of prostaglandin (PG) synthesis in the expression of suppressor cells. Since the *in vitro* response to an unrelated antigen PPD had been found unimpaired, the described T_G-cell-mediated suppression of the Veillonella response is apparently antigen-specific.

INTRODUCTION

Peripheral blood lymphocytes from patients with gingivitis and mild periodonitis cultured in autologous sera are stimulated by antigens from oral bacteria (such as *Veillonella alcalescens*), whereas no significant response is observed in cultures from patients with severe periodonitis (Ivanyi & Lehner, 1970; Ivanyi, Challacombe & Lehner, 1973; Baker *et al.*, 1978). The lack of lymphocyte stimulation in the advanced stage of the disease was ascribed to IgG serum antibodies which suppressed specifically the response of Veillonella-reactive T cells. Conversely, lymphocyte responses could be restored by anti-Veillonella antibodies belonging predominantly to the IgM class (Ivanyi *et al.*, 1973).

Moretta *et al.* (1977) have recently shown that human peripheral blood T cells (as defined by rosetting with sheep erythrocytes), are separable into distinct subpopulations with cell surface receptors for either IgM or IgG. Those with receptors for IgM (T_M) provided help, whereas T cells with receptors for IgG (T_G) suppressed the differentiation of B lymphocytes induced by pokeweed mitogen in the presence of helper cells. The suppressor activity of T_G cells required prior interaction of these cells with immune complexes. In accord with this concept, the aims of this study were to ascertain the possibility that the suppression of the *in vitro* response to Veillonella in the advanced stage of periodontal disease was mediated by T_G cells.

MATERIALS AND METHODS

Patients. Eleven patients (five males and six females) ranging in age from 28 to 50 years with severe periodontitis (SP) were investigated. Russell's periodontal index (PI) was used to determine

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the degree of severity of periodontal disease. Pocket depth and bone loss were also measured. Each patient had PI > 4·0, pocket depth over 5 mm and bone resorption over half of the root.

The control group consisted of five patients with gingivitis or mild to moderate periodontitis (GMP; PI < 4·0, pocket depth up to 5 mm, bone resorption up to half of the root). The controls were matched for sex and age.

Preparation of lymphocyte subpopulations. Cell fractionation was performed using the methods described in detail by Moretta *et al.* (1976). Briefly, mononuclear cells (MNC) were obtained from peripheral blood by separation on Ficoll—Triosil and washed three times in medium TC199. Mononuclear cells were first depleted of adherent cells by incubation on plastic (Falcon 3013) at 37°C for 45 min in medium 199 supplemented with 10% fetal calf serum. The adherent cells (AM) were removed with a rubber policeman, washed twice and used to reconstitute the lymphocyte cultures at a concentration of 1×10^5 cells per ml of culture. Of the adherent cells, 85–90% were identified as monocytes by α-naphthyl acid esterase (ANAE) staining (Kullenkamph, Janossy & Greaves, 1977). The non-adherent cells were mixed with neuraminidase-treated sheep erythrocytes (Weiner, Bianco & Nussenzweig, 1973), centrifuged (4°C, 5 min, 150 *g*) and kept on ice for 60 min. After gentle resuspension, the cells were centrifuged on Ficoll–Triosil, the pellet was recovered and erythrocytes were lysed with ammonium chloride (Boyle, 1968). The lymphocytes were washed twice in medium 199 and these are referred to as T cells.

IgG-coated ox erythrocytes (Hayward *et al.*, 1978) were added to T cells and the suspension centrifuged as above. The rosettes were separated on a Ficoll–Triosil gradient and the cells remaining at the interface were aspirated and washed. This cell population is referred to as T_M cells. The pellet which included rosetted cells was recovered and, after erythrocyte lysis, the lymphocytes were washed. These cells are referred to as T_G-enriched cells.

As shown in Table 1, 80% of the cells from T_M fraction made IgM (Fc) rosettes and 1% made IgG (Fc) rosettes; 0·5% of the cells were identified as monocytes. On the other hand, T_G cells made 85% IgG (Fc) rosettes and 1% made IgM (Fc) rosettes. This fraction contained 1·5% monocytes.

It has been shown previously that T-cell proliferation induced by Veillonella antigen is monocyte-dependent (Ivanyi & Lehner, 1974). In order to retain some monocytes in lymphocyte cultures, in the next experiments unfractionated mononuclear cells were rosetted with IgG-coated ox erythrocytes, centrifuged and the cells remaining at the interface were collected. This population is referred to as MNC-T_G fraction, and contained $5 \pm 2\%$ monocytes; $79 \pm 3\%$ cells made IgM rosettes and $1 \pm 0{\cdot}5\%$ made IgG rosettes on retesting; $90 \pm 1\%$ cells formed rosettes with sheep erythrocytes.

Cultures. Lymphocytes were cultured at a concentration of 1×10^6 cells per ml in culture medium RPMI 1640 enriched with added L-glutamine (2 mmol/ml), penicillin (100 units/ml), streptomycin (100 μg/ml) and 20% autologous serum. The cultures were maintained for 5 days and then harvested and assessed as described previously (Ivanyi & Lehner, 1970). The results were expressed as counts per minute (c.p.m.) per 1×10^6 viable lymphocytes. All cultures were performed in triplicate.

Stimulants. Veillonella alcalescens, cultured and disintegrated by ultrasonication as described previously (Ivanyi & Lehner, 1970), was used at the optimal concentration of 20 μg of protein per ml of culture (Ivanyi & Lehner, 1978). Preservative-free PPD was used at the previously determined optimal concentration of 10 μg per ml of culture.

In some experiments, indomethacin and mefenamic acid were added to lymphocyte cultures with or without the antigen at an optimal concentration of 1 μg per ml (Fig. 1). The statistical analysis was done using Student's *t*-test for paired data. The degree of enhancement was calculated from the formula:

$$\% \text{ Increase} = \frac{\text{c.p.m. of cultures with PG synthetase inhibitor and Veillonella} - \text{c.p.m. of cultures with Veillonella alone}}{\text{c.p.m. of cultures with Veillonella alone}} \times 100$$

The same formula was used for calculating the percentage increase in T_G-depleted cultures stimulated by Veillonella.

Table 1. Comparison of fractionated T-cell subpopulations by surface markers and by ANAE

	Cell types (percentage)				
Cell fractionation	T_M*	T_G	B‡	Monocytes§	Unidentified
MNC	40±9	25±11	11±3	14±6	10·0±3
T	68±10	19±6	1±0·5	2·0±0·8	10·0±2
T_M	80±8	1±0·5	0	0·5±0·1	18·5±4
T_G	1±0·3	85±4	0	1·5±1	12·5±3

Mean values from two experiments (four patients).
* Cells that formed rosettes with OxE coated with rabbit IgM antibodies.
† Cells that formed rosettes with OxE coated with rabbit IgG antibodies.
‡ Surface-immunoglobulin-positive cells by immunofluorescence.
§ Cells with large and confluent granules by staining for α-naphthyl acid esterase activity.

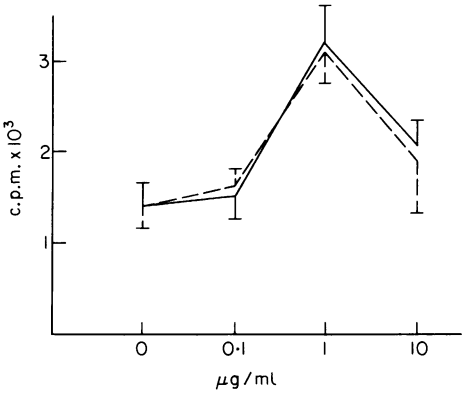

Fig. 1. Indomethacin (——) and mefenamic-acid (- - - -) dose-dependent enhancement of lymphocyte stimulation induced by Veillonella. Mean values from four experiments.

RESULTS

In vitro *stimulation of T-cell subpopulations*

Mononuclear cells and fractionated T-cell subpopulations from six patients with severe periodontitis were cultured in the presence of Veillonella or PPD (Fig. 2). Mononuclear cells and T-cells depleted of monocytes were not stimulated to a significant degree by Veillonella (mean c.p.m. 1,075±185 for MNC and 809±122 for T cells). This lack of response to Veillonella was not reversed by the removal of T_G cells from the total T cells (mean c.p.m. 909±122). In addition, even reconstitution of T-cell cultres with 10% adherent monocytes did not change the level of Veillonella-induced DNA synthesis in lymphocytes (mean c.p.m. 823±110). However, monocyte-reconstituted T_M cultures responded to Veillonella by a significantly enhanced DNA synthesis (mean c.p.m. 8,542±1,736). Unlike the response to Veillonella, mononuclear cells from the same patients gave a vigorous response to the unrelated antigen PPD as shown by DNA synthesis (mean c.p.m. 19,989±4,476). Severe monocyte depletion resulted in a decrease of T-lymphocyte stimulation in both T-cell cultures (mean c.p.m. 4,340±1,078) and T_M cultures (mean c.p.m. 4,682±1,021). Addition of the adherent monocytes restored the level of T-lymphocyte stimulation

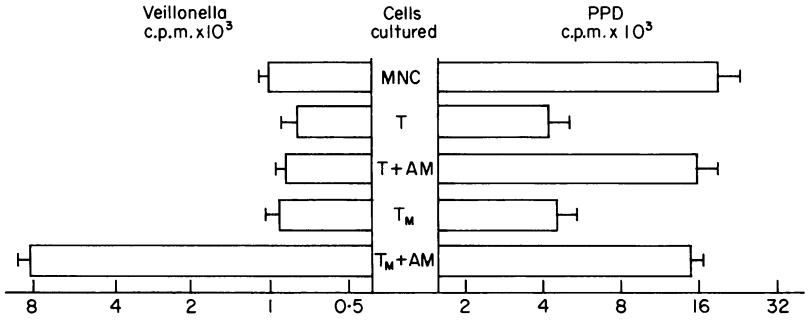

Fig. 2. *In vitro* stimulation of T-cell subpopulations by Veillonella and PPD in patients with severe periodontitis (mean values). The adherent cells were added to lymphocyte cultures at a concentration of 1×10^5 per ml of culture. Counts per minute of control cultures with saline: MNC = 616 ± 63; T = 498 ± 47; T_M = 585 ± 63; T + AM = 676 ± 71; T_M + AM = 632 ± 69.

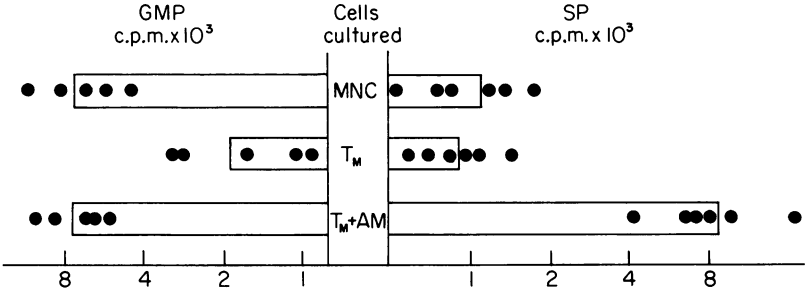

Fig. 3. *In vitro* stimulation of T-cell subpopulations by Veillonella in two groups of patients. GMP = gingivitis and mild periodontitis, SP = severe periodontitis.

induced by PPD irrespective of the presence or absence of T_G cells in cultures (mean c.p.m. 16,221 ± 3,510 and 15,358 ± 2,867 respectively).

In view of the enhanced DNA synthesis in T_G-depleted T lymphocytes from SP patients, we further studied the effect of this depletion on the level of lymphocyte stimulation in five patients with gingivitis or mild periodontitis (GMP) responsive to Veillonella antigen. Fig. 3 compares the results in two groups of patients. Mononuclear cells from patients with GMP were stimulated by Veillonella as shown by enhanced DNA synthesis (mean c.p.m. 7,300 ± 1,225), whilst T_M cells responded to a lesser extent (mean c.p.m. 1,986 ± 464). Addition of the adherent monocytes to these cultures restored T-lymphocyte proliferation to the level observed in unfractionated cell cultures (mean c.p.m. 7,500 ± 884). The results show that the removal of T_G cells did not significantly enhance the Veillonella-induced lymphocyte stimulation in GMP patients, as compared with SP patients.

The effect of indomethacin and mefenamic acid on lymphocyte stimulation induced by Veillonella in patients with severe periodontitis

Since previous studies have shown that lymphocyte responses to both specific antigens and mitogens can be enhanced by *in vitro* blockade of prostaglandin synthesis (Goodwin, Bankhurst & Messner, 1977), we also tested the effect of indomethacin and mefenamic acid on lymphocyte stimulation in five patients with severe periodontitis (Fig. 4).

Mononuclear cells in the absence of the drugs were not stimulated to a significant degree by Veillonella (mean c.p.m. 912 ± 90). Addition of indomethacin and mefenamic acid into these cultures resulted in a significant enhancement of DNA synthesis (mean c.p.m. 1,895 ± 365, $P < 0{\cdot}05$,

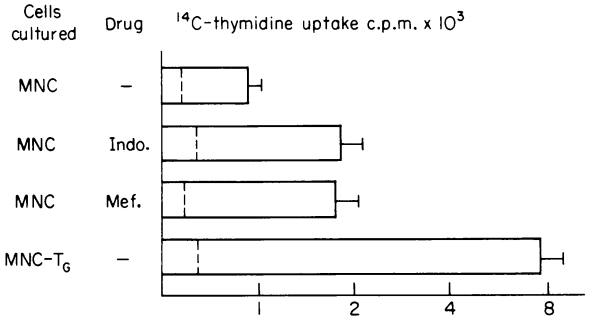

Fig. 4. The effect of indomethacin and mefenamic acid on *in vitro* stimulation of fractionated mononuclear cells by Veillonella. Mean values from five experiments. The dotted line represents mean c.p.m. in control cultures with saline.

% increase = 100 ± 21; and 1,769 ± 337, $P < 0{\cdot}05$, % increase = 95 ± 17 respectively). However, T_G-depleted mononuclear cells, without any drugs added, responded to Veillonella with the highest level of proliferation (mean c.p.m. 7,694 ± 1,306, $P < 0{\cdot}001$; % increase = 784 ± 173).

DISCUSSION

We have confirmed the previous findings that peripheral blood mononuclear cells from patients in an advanced stage of periodontal disease do not respond to a significant degree to a sonicate of the oral Gram-negative bacterium, *Veillonella alcalescens* (Ivanyi & Lehner, 1970; Ivanyi *et al.*, 1973). Very low stimulation indices with Veillonella in patients with advanced periodontal disease (pocket depth of 4 mm or greater) were also reported by Page *et al.* (1978). Similar results showing a lack of response of lymphocytes from patients with severe periodontitis to plaque antigens have been described by Baker *et al.* (1978). These authors have also shown that after therapy (either full extractions or combination of extractions with periodontal surgery), lymphocytes from the same subjects responded significantly to plaque antigens. On the other hand, Patters *et al.* (1976) reported that Veillonella could stimulate lymphocytes from eight patients with 'moderate to severe' stages of periodontitis. This discrepancy may be due to variations in the degree of severity of the disease, as in the study by Baker *et al.* (1978), and in our previous as well as present experiments only patients with severe periodontitis were investigated. These patients had generalized deep periodontal pockets, and many required extractions because of extensive alveolar bone loss.

The inhibition of lymphocyte proliferation in the advanced stage of the disease was previously ascribed to 'IgG blocking antibody', which may be cytophilic for T cells (Ivanyi *et al.*, 1973). The results in this paper showed that the lack of lymphocyte responses to Veillonella in patients with severe periodontitis could be restored by the depletion of T_G cells to a level of DNA synthesis comparble with that observed in patients in mild stages of the disease (Fig. 3). Thus the cell responsible for suppression of response of the unfractionated mononuclear cells probably carries surface Fc receptors for IgG (T_G subpopulation). This suppressor cell seemed to be Veillonella-antigen-specific, as it did not inhibit lymphocyte proliferation induced by the unrelated antigen PPD. However, it has been reported previously that lymphocytes from patients with servere periodontitis failed to respond not only to Veillonella, but also to sonicates from several other Gram-negative bacteria (Ivanyi & Lehner, 1970). As the effect of T_G depletion on lymphocyte responses to sonicates from other Gram-negative organisms was not tested in this study, the antigenic cross-reactivity within Gram-negative species cannot be excluded.

The suppression mediated by T_G cells may involve binding with either free or antibody-complexed Veillonella antigen. The latter explanation would agree with the results of Moretta *et al.* (1977) who have shown that the suppression of the differentiation of B lymphocytes by T_G cells required prior interaction of these cells with immune complexes. An increase in the proportion of

T_G cells has been noted in sarcoidosis (Katz, Haynes & Fauci, 1978) and in children with nephrotic syndrome after steroid therapy (Trompeter, Layward & Hayward, 1978). In inflammatory bowel disease, a positive correlation was found between the number of circulating T_M cells and the level of response to mitogens *in vitro* (Victorino & Hodgson, 1980). However, these studies determined only total numbers of the respective T subsets which could hardly reflect even profound changes in antigen-specific T-cell clones.

It has been shown recently that human T-cell subsets can be defined by specific cell surface antigens recognized by monoclonal antibodies (Reinherz *et al.*, 1980). These authors have suggested that the T_G subset is a heterogeneous population of cells as 50% of cells were shown to have an antigen in common with monocytes. Nevertheless, the T-suppressor-cell population as detected by OKT5 monoclonal antibody was enriched in the T_G fraction at least two-fold when compared with the T_M fraction. Besides, other studies have shown that the monoclonal antibodies to human T cells (OKT3) react with the majority of T_G cells (Perlmann, personal communication). It seems conceivable, therefore, that irrespective of the presence of contaminating cells, the Veillonella-reactive suppressor cells were T cells.

Prostaglandins of the E series have been shown to suppress many *in vitro* immune responses including mitogen- or antigen-induced lymphocyte sitmulation (Goodwin *et al.*, 1977). Significant amounts of prostaglandins are secreted endogenously by cultured monocytes but not by lymphocytes (Bray, Powell & Lydyard, 1981). Conceivably, IgG antibodies from the autologous serum and Veillonella may form immune complexes which stimulate the prostaglandin synthesis of monocytes and in turn these prostaglandins could activate T_G cells via their high-affinity PGE receptors (Goodwin, Kaszubowski & Williams, 1979). To determine whether prostaglandin synthesis is involved in activation of Veillonella suppressor cells, we studied the effect of two prostaglandin synthetase inhibitors on the level of lymphocyte stimulation by Veillonella in patients with severe periodontitis. Both indomethacin and mefenamic acid induced low enhancement of DNA synthesis induced by Veillonella which was significant at the 5% level. However, the percentage enhancement (up to 100) was similar to that observed previously for indomethacin-induced amplification of PHA responses of lymphocytes from healthy subjects (Goodwin *et al.*, 1977) and for indomethacin-induced amplification of Veillonella responses of lymphocytes from patients with gingivitis and mild periodontitis (unpublished results).

In contrast, in patients with Hodgkin's disease where the defect in cell-mediated immunity was attributed to the prostaglandin-producing suppressor macrophages, the mean increase in PHA-induced thymidine incorporation caused by indomethacin was around 300% (Goodwin & Webb, 1980). It seems likely, therefore, that prostaglandin synthesis plays only a minor role in the activation of T_G cells in patients with severe periodontitis. This is in accord with the recent studies of Kemp *et al.* (1980) who observed gross amplification by indomethacin of macrophage-mediated suppression but only marginal increase in suppressor T-cell-controlled activities.

The mechanisms which lead to an enhanced activity of T_G cells in severe stages of the disease are not fully understood. Antigen concentration within the periodontal tissue as well as IgG antibody levels or the type of antigen–antibody complexes may play a role. Association of IgG antibody titres to Veillonella and periodontal inflammatory index was reported previusly (Ostravik & Brandtzaeg, 1977) and the amount of IgG eluted from the periodontal tissues correlated with the severity of periodontal disease (Clagett & Page, 1978). Thus activation of suppressor cells by antigen–antibody complexes may occur in the inflamed periodontal tissue, and these may disseminate via the circulatory system. Indeed, the lack of response to Veillonella was also observed in cultures of mononuclear cells isolated from the periodontal tissue of patients with severe periodontitis (Ivanyi, 1980). Thus Veillonella-induced suppressor T cells may play a role in the mechanisms which regulate the progression of periodontal disease into its severe form.

Finally, not all patients with gingivitis or mild periodontal disease responded to Veillonella, as lymphocytes from 10% of patients with gingivitis and 30% of patients with mild periodontitis showed no significant *in vitro* proliferation (Ivanyi & Lehner, 1970, 1974). This lack of *in vitro* cell-mediated immunity may be interpreted as a warning signal of the progression of the disease to its severe form. This view is supported by results obtained from experimental studies in gnotobiotic rats monoinfected with another Gram-negative bacterium *Eikenella corrodens* in which the increase

in severity of periodontal disease corresponded to a marked decrease in cell-mediated immune responses to this antigen (Listgarten *et al.*, 1978).

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