**IN VITRO STUDY ON THE IMMUNOLOGICAL EFFECT OF BROMELAIN AND TRYPsin ON MONONUCLEAR CELLS FROM HUMANS**

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**Abstract**

**Background:** Proteolytic enzymes such as bromelain and trypsin are used as an adjuvant therapeutic approach in the treatment of chronic inflammatory, malignant and autoimmune diseases. In vitro studies have shown that proteolytic enzymes modulate surface adhesion molecules on T-cells. In this study we analysed the influence of bromelain and trypsin on the cytokine production and proliferation of peripheral blood mononuclear cells (PBMC) from patients with a classical cellular mediated autoimmune disorder, namely encephalomyelitis disseminata (ED) as well as from healthy controls.

**Methods:** PBMC from patients with ED (n = 12) and healthy controls (n = 12) were cultured for seven days at 37 °C under three different conditions: without antigen, with bromelain and with trypsin (final concentrations 10-1000µg/ml). Proliferation was determined by 3H-thymidine incorporation. Secretion of cytokines into the supernatant was measured by a double sandwich ELISA. Intracellular cytokines were determined by flow cytometric analysis.

**Results:** PBMC from patients with ED and healthy controls showed a significantly increased proliferative response to bromelain (ED: 14053 ± 7585 cpm with bromelain vs. spontaneous proliferation of 430 ± 255 cpm; healthy controls: 10689 ± 4607 cpm vs. 327 ± 193) but not to trypsin. Bromelain induced in all 24 individuals a significant increase of the macrophage/monocyte associated cytokines interleukin (IL)-6, granulocyte-macrophage-colony stimulating factor (GM-CSF), tumour necrosis factor alpha (TNFα) (p<0.01) as well as of the type 1 cytokine gamma-interferon (IFNγ). In contrast, the type 2 cytokines IL-4 and IL-5 were not induced. Flow cytometric analysis revealed a significant increase of IFNγ-producing CD4+ T-cells. There were no differences in cytokine production between ED patients and healthy controls.

**Conclusion:** These results indicate that bromelain – but not trypsin – activates macrophages/monocytes and type 1 cells independently from the underlying disease and may stimulate, therefore, the innate as well as the adaptive immune system.

**Key words:** proteolytic enzymes, bromelain, trypsin, T-cells, macrophages/monocytes, cytokines

**INTRODUCTION**

Proteolytic enzymes such as bromelain and trypsin have been recommended as adjuvant therapeutic approaches in the treatment of viral and malignant diseases [1, 2]. Furthermore, there is evidence that in patients with rheumatic and other inflammatory diseases oral therapy with proteolytic enzymes produces certain analgesic and antiinflammatory effects [3-7]. However, the results obtained in different studies are often inconsistent [3]. Nevertheless, the immunomodulatory potency of proteolytic enzymes at least in vitro is nowadays without doubt. Thus, it was shown that they modulate surface adhesion molecules on T-cells, macrophages and natural killer (NK) cells and induce the secretion of IL-1β, IL-6 and TNFα by peripheral blood mononuclear cells (PBMC) [8-14]. Bromelain can also inhibit the T-cell signal transduction by blocking the Raf-1/extracellular regulated kinase (ERK)-2 pathway [15]. Beneficial effects of orally administered proteolytic enzymes in experimental allergic encephalomyelitis (EAE) suggested the application of these substances in the therapy of T-cell mediated autoimmune diseases [16], and, indeed, in patients with diabetes type 1 an inhibition of T-helper type 1 cells has been described during intake of bromelain [17]. Type 1 and type 2 TH cells are defined by their cytokine production and exhibit distinct functional properties. Thus, type 1 TH cells secrete interferon-gamma (IFNγ) and tumour necrosis factor-beta (TNFβ) and are involved in macrophage activation and delayed-type hypersensitivity (DTH) reactions. In contrast, type 2 TH cells produce interleukin (IL)-4, IL-5 and IL-13 and mediate antibody responses, including IgE production [18]. These T helper cell subsets represent polarized forms of the specific immune response and counteract each other. They are closely associated with resistance or susceptibility to bacterial infections but also appear to be involved in the induction and regulation of autoimmunity [19, 20]. One could, therefore, speculate that the beneficial effect of oral enzymes in the EAE model or diabetes mellitus type 1 may be due to their immunomodulatory properties on the different TH subsets.

Aim of our study was, therefore, to analyse *in vitro* the influence of the proteolytic enzymes bromelain
and trypsin on proliferation and cytokine production by PBMC from healthy individuals and patients with the type 1 TH cell mediated autoimmune disease encephalomyelitis disseminata (ED). It became evident that bromelain, but not trypsin, exhibited an immunomodulatory effect resulting in enhanced cell proliferation activity and secretion of TH type 1 related cytokines by PBMC.

PATIENTS AND METHODS

SUBJECTS

Twelve healthy blood donors (2 female, 10 male; mean age: 41 years, range: 26-60 years) and twelve patients with encephalomyelitis disseminata (ED) exhibiting a relapsing-remitting course were included into the study (9 female, 3 male; mean age: 32 years, range 27-47 years). ED patients were diagnosed by established diagnostic criteria [21] and neurological examinations were carried out by one of the authors (A. Guseo). The patients had an EDSS (Expanded Disability Status Scale) ranging from 1 to 3. None of the subjects has received polyenzyme preparations or had any prophylactic medical treatment.

All patients and healthy blood donors had given their informed consent to participate in the study, according to the 1975 Helsinki Committee ethical guidelines.

ANTIBODIES

Purified anti-human IL-2, IL-4, IL-5, IL-6, GM-CSF, IFN-γ, and TNF-α monoclonal antibodies (mAb) and the respective biotinylated anti-human mAb as well as R-phycocerythrin (PE)-conjugated anti-human IL-4, IL-5, IFN-γ were purchased from Pharmingen (San Diego, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 and CD3 mAb, R-Phycoerythrin (PE)-conjugated anti-human CD8 and CD19 mAb were obtained from Immunotech (Mar- seille, France).

ANTIGENS

Bromelain and trypsin were kindly provided by MUCOS, Geretsried, Germany (Ch. numbers 968301 and 0970298, respectively). Identity and purity had been proven by electrophoresis, isoelectric fociation, and measuring of the absorption spectrum. Analysis of microbiological purity revealed no contamination with E. coli, Salmonellae, Pseudomonas aeruginosa, Staphylococcus aureus, an amount of candida less than 100kBE/g, and total germ number of less than 10.000kBE/g. Endotoxins could not be detected.

CELL CULTURE

PBMC were isolated from heparinized blood by centrifugation through Ficoll-Hyphaque. The cells were washed two times in Hanks’ salt solution and resuspended in 1 million cells/ml in RPMI 1640 medium supplemented with gentamycin and 25% autologous serum. PBMC were cultured without antigen, with bromelain as well as with trypsin in four concentrations ranging from 10 to 1000µg/ml for 7 days at 37°C, 5% CO2 in a humidified atmosphere.

LYMPHOCYTE PROLIFERATION ASSAY

For proliferation assays PBMC (1x10^6/well) were seeded into 96-well cell culture plates and stimulated with bromelain and trypsin in parallel. During the last 18h, the cultures were pulsed with 1H-thymidine (0.74 Mbp/ml, 20µl/well) and then harvested onto glassfiber filters. The incorporated radioactivity was measured by liquid scintillation spectroscopy using a β-counter and given as counts per minute (cpm). All cultures were performed in fourfold.

MEASUREMENT OF CYTOKINES BY ELISA

For cytokine production 5x10^5 PBMC/ml were cultured with bromelain and trypsin in parallel in duplicates (concentrations ranging from 1000µg/ml) in 24-well plates and were maintained as indicated above. Culture supernatants were collected at day 7 and kept frozen at –20°C until quantitative cytokine determination [22].

Briefly, wells of a 96-well microtiter plate were coated overnight at 4°C with 1.75µg/ml anti-human cytokine monoclonal antibody (IL-2, IL-4, IL-5, IL-6, GM-CSF, IFN-γ, TNFα) in hydrogen bicarbonate buffer, pH 9.6, and blocked for 1h at room temperature with phosphate buffered saline (PBS 60mM, pH 4.7) containing 0.5% bovine serum albumin (BSA). Culture supernatants were used undiluted and incubated for 2h at 37°C; 1.25µg/ml biotinylated anti-human cytokine antibodies (IL-2, IL-4, IL-5, IL-6, GM-CSF, IFN-γ, TNFα) were added for 2h at 37°C. After another incubation for 1h at room temperature with avidin-peroxidase (2.5µg/ml), substrate solution (0.5 mg ortho-phenylene-diamine/ml citrate buffer [pH 5.0] and 0.01% H2O2) was added. The reaction was stopped with 25% sulphuric acid and optical density was measured at 450nm in a microtiter plate reader. Results were related to a standard curve obtained with cytokine standards (Pharmingen, San Diego, CA, USA) in different concentrations for each cytokine.

FLOW CYTOMETRY AND INTRACELLULAR CYTOKINE ANALYSIS

For cell staining and intracellular cytokine analysis, PBMC were isolated and cultured for seven days as described above. PBMC were incubated with 0.25µg FITC anti-human CD3 and 0.25µg PE anti-human CD19 or 0.25µg FITC anti-human CD4 and 0.25µg PE anti-human CD8 for 30 min at 4°C. Cells were washed twice with cold phosphate-buffered saline (PBS; centrifugation at 500g for 10min, 4°C) and resuspended in 400µl PBS for final flow cytometric analysis.

For intracellular cytokine analysis, on day seven cells were restimulated with PMA (50ng/ml) and ionomycin (1 µg/ml) in the presence of Brefeldin A (10µg/ml) for 6 hours [22]. Cells were washed twice in
PBS containing 2% fetal calf serum (FCS) and stained with 0.25µg PE anti-human CD3 for 30 minutes. Cells were then washed with PBS/2% FCS and fixed in PBS containing 4% paraformaldehyde for 20 minutes. After two additional washes in PBS/2% FCS containing 0.2% saponin (permeabilization buffer) cells were resuspended in 100µl permeabilization buffer and stained with 0.2 µg PE anti-human IFN-γ, 0.1 µg PE anti-human IL-4 or 0.1 µg PE anti-human IL-5 antibodies. After 30 minutes cells were washed two times in permeabilization buffer and resuspended in PBS/2% FCS for analysis.

Cells were analysed by two-colour flow cytometry using a Becton Dickinson FACScan flow cytometer. The lymphocyte population was gated using forward and side scatter parameters to exclude debris and dead cells. The data were analysed using Lysis II software (Becton Dickinson). Quadrants were set based upon isotype controls for each antibody.

STATISTICS

The Mann-Whitney-test was used to compare data between groups. Differences were considered significant for p < 0.05.

RESULTS

PROLIFERATIVE ACTIVITY OF PBMC AFTER STIMULATION WITH BROMELAIN OR TRYPsin

Incubation of PBMC with bromelain lead to a significantly increased proliferative response when compared to the unstimulated cell cultures (p<0.001). The proliferation activity was dose dependent and strongest proliferation was obtained at bromelain concentrations of 10 and 100µg/ml. There was no significant difference in proliferation activity between healthy controls and ED patients (Fig. 1a).

In contrast, trypsin was not able to induce a marked proliferation of PBMC in both study groups (Fig. 1b).

CYTOKINE SECRETION BY PBMC AFTER STIMULATION WITH BROMELAIN OR TRYPsin

Bromelain at concentrations of 10 or 100µg/ml induced a significant secretion of the macrophage/monocyte-associated cytokines IL-6, GM-CSF and TNFα as well as the production of the type 1 related cytokine IFNγ by PBMC from healthy controls and ED patients (p<0.01). The production of IL-2 and the type 2 cytokines IL-4 and IL-5 was not markedly enhanced by bromelain. Differences in cytokine levels between healthy controls and ED patients were not observed (Fig. 2).

There were no significant differences in cytokine production when PBMC were incubated with trypsin as compared to the unstimulated cultures (data not shown).

EXPRESSION OF CD3/CD19 AND CD4/CD8 ON NON-STIMULATED AND BROMELAIN-STIMULATED PBMC AND DETERMINATION OF INTRACELLULAR CYTOKINES IN CD3 POSITIVE T CELLS.

In order to further analyse the immunomodulatory properties of bromelain we investigated the expression of the surface molecules CD3/CD19 and CD4/CD8 on PBMC from three healthy controls in response to 10 or 100µg bromelain/ml. Bromelain lead to an increased expression of CD3 (mean of the three controls: 77 ± 2.7%) when compared to the unstimulated cultures (70 ± 3.9%). Analyses of CD4 and CD8 revealed an enhanced expression for the CD4 marker (CD4: 51 ± 3.6% vs. unstimulated cultures: 38 ± 5.7%; CD8: 25 ± 2.8% vs. unstimulated cultures: 28 ± 2%). In Figure 3 an example for one healthy controls is shown.

Measurement of intracellular cytokines produced by CD3 positive T cells of the three healthy individuals after incubation of PBMC with bromelain and restimulation with PMA and ionomycin revealed an enhanced production for the type 1 cytokine IFNγ when
Fig. 2. Cytokine levels in supernatants of PBMC from healthy individuals (n = 12) and patients with encephalomyelitis disseminata (= multiple sclerosis, MS) (n = 12) stimulated with bromelain (10 or 100 µg/ml; □) as compared to non-stimulated cultures (□). Results are expressed as pg/ml. Mean (bars) and standard deviation are given.

Fig. 3. Two-color flow cytometric analysis of non-stimulated PBMC and PBMC stimulated with bromelain (100µg/ml). Data are given for one healthy control as an example. The percentages of CD3, CD19, CD4, and CD8 positive cells were determined. Quadrants were set based upon isotype controls for each antibody.
compared to the cell cultures without bromelain (mean of the three controls: 19.5 ± 3.5% vs. 9.7 ± 2.1%). The production of the type 2 cytokines IL-4 and IL-5 was not influenced. In Figure 4 data are given for one healthy control as an example. Again, trypsin had no effect in these experiments.

**DISCUSSION**

In this study it is shown that bromelain – but not trypsin – induced the secretion of the macrophage/monocyte derived cytokines IL-6, GM-CSF, TNFα and IFN-γ – the latter being produced also by T helper type 1 cells –, while the production of the type 2 cytokines IL-4 and IL-5 was not affected, indicating an involvement of cells of the innate immune system. The enhancement of CD3 and CD4 positive T-cells after incubation with bromelain and the increase of CD3 positive T cells producing the type 1 cytokine IFN-γ by FACS analysis is, however, a strong argument that also type 1 T cells are activated. The immunomodulatory effect of bromelain was similar in patients with ED and healthy controls indicating that it had no specific stimulatory effect on autoreactive T-cells involved in the immunopathogenesis of ED.

There are several reports analysing the influence of proteolytic enzymes on immunocompetent cells in the literature. Brakebusch et al. [23] analysed their effect on granulocyte and macrophage function in patients with a disorder of the humoral immunosystem X-linked agammaglobulinemia. They found an acceleration of phagocytosis, respiratory burst and killing of Candida albicans which was – similarly to our experiments on the T-cell level – related to bromelain but not trypsin. Hale et al. [8] showed that treatment of T cells with bromelain for one hour selectively removed CD44, CD45RA, E2/MIC2, CD6, CD7, CD8, and Leu 8/LAM1 but left intact CD2, CD3, and CD28 T-cell surface molecules. They also found that bromelain markedly enhanced CD2-mediated T cell activation. It is, however, still unclear whether T cell activation by bromelain occurs directly via the cleavage of surface molecules or indirectly via antigen presenting cells. In this respect the observation by Mynott et al. [15] is of interest showing that an inhibitory action of bromelain is not simply caused by degradation of certain surface molecules. Thus, T-cell activation by PMA plus ionophore, two membrane-permeable agonists whose action is independent from extracellular receptors, was blocked by bromelain. Furthermore, bromelain inhibited cytokine mRNA accumulation induced by PMA plus ionophore but not that of T cell receptor induced cytokine production. In the last years the biological actions of proteases could be attributed to the activation of a new sub-family of G protein-coupled receptors, termed proteinase-activated receptors (PARs) [24]. After stimulation, PARs couple to various G proteins and activate signal transduction pathways resulting in the rapid transcription of genes that are involved in inflammation. PARs can, therefore, modulate the secretion of cytokines, inflammatory mediators or neuropeptides. Modulation of PAR function could be, therefore, a new aspect for the treatment of inflammatory or immune diseases, and bromelain could be one candidate as such a therapeutic substance.

The activation of TH1-cells and macrophages by bromelain as presented in this *in vitro* study is somewhat contrary to the observation that it may be *in vivo* helpful in TH1/type1 mediated autoimmune diseases such as EAE or diabetes by shifting the pathogenic type 1 pattern to the protective type 2 reaction [16, 17]. However, it has been recently shown that also a TH type 1 response can activate control mechanisms required to suppress autoimmunity, and the recent success of pilot studies using the bacterial vaccine BCG in therapy of ED patients supports this hypothe-
in vivo effects of proteolytic enzymes, the until now presented intestinal tract [31].

Considering the well tolerability and lack of side effects of proteolytic enzymes, the until now presented in vivo and in vitro effects of proteolytic enzymes on immunocompetent cells justify, therefore, further in vivo studies analysing immunological reactions during enzyme treatment in relation to clinical effects.

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