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Modulation of autoimmunity to beta-cell antigens by proteases

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Abstract

Aims/hypothesis. Proteases are used in therapy for autoimmune diseases yet the mechanism of their action remains to be determined. We studied the immunological basis of protease therapy in the context of Type I (insulin-dependent) diabetes mellitus.
Methods. We studied the effects of proteases (trypsin, papain, chymotrypsin, bromelain) on immune reactivity of a series of autoreactive T-cell clones from prediabetic subjects and patients with a recent onset of Type I diabetes and specific to the autoantigens GAD65, IA-2 and insulin-secretory granule protein.
Results. Cell surface expression of adhesion, co-stimulatory and homing molecules on both antigen-presenting cells and T cells was changed after protease treatment. Cytokine analyses showed a selective inhibition of proinflammatory (Th-1) but not Th-2 cytokine production. Autoreactive T-cell proliferation was inhibited at pharmacological serum concentrations, whereas non-specific proliferation to phytohaemagglutinin was not affected at these concentrations. Preincubation experiments on T cells and antigen-presenting cells separately showed that this effect was mediated by APCs, but not T-cells.
Conclusion/interpretation. Proteases have pleiotropic immunological effects supporting an immunomodulatory potential for the intervention of chronic inflammatory diseases and Th-1 mediated oedema formation. [Diabetologia (2002) 45:686–692]

Keywords Immunotherapy, Th-1 lymphocytes, Th-2 lymphocytes, cytokines, autoimmune.

In Western Europe proteases are widely prescribed drugs for treating immune-mediated inflammatory conditions, tissue damage from radiotherapy and sports injuries of athletes [1, 2, 3, 4]. Clinical trials currently test the efficacy of protease to ameliorate immune-mediated diseases like multiple sclerosis and rheumatoid arthritis. Orally administered proteases such as bromelain are absorbed in a non-degraded bioactive form, resulting in increased proteolytic serum activity [2]. Prevention of murine experimental allergic encephalomyelitis (EAE) and autoimmune diabetes by oral hydrolytic enzyme treatment has been shown [5, 6]. Pilot trials in human diseases suggested that proteases have ameliorating effects for multiple sclerosis and rheumatoid arthritis without any side-effects [7]. However, the mechanism of their action is not clear, whilst no molecular basis exists to use mixtures of different proteases.

Type I (insulin-dependent) diabetes mellitus results from a T-cell-dependent immune-mediated destruction of the insulin producing pancreatic beta cells. In first degree relatives the development of the disease can be predicted by the detection of islet-reactive autoantibodies to islets of Langerhans, insulin, GAD and IA-2

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Abbreviations: APC, Antigen-presenting cell; EBV, Epstein-Barr virus; DC, dendritic cell; EAE, experimental allergic encephalomyelitis; GM-CSF, granulocyte-macrophage colony stimulating factor; ICAM-1, intercellular adhesion molecule-1; MACS, magnetic cell sorting; PBMC, peripheral blood mononuclear cell; PHA, phytohaemagglutinin; DMA, phorbol 12-myristate 13-accetate

[8, 9]. Pharmacological intervention therapy requires that the side-effects are acceptably low, because prediabetic subjects are healthy persons except for their subclinical autoimmunity to pancreatic islets.

We present evidence that proteases regulate T-cell activity by miscellaneous interferences in immune responsiveness, including the modulation in the expression of cell surface molecules and the deviation of cytokine production to a non-inflammatory profile.

Materials and methods

T-cell reagents. Three independent T-cell clones were previously isolated from two newly diagnosed Type I diabetic patients and a prediabetic subject 4 years before the clinical manifestation of the disease [10, 11, 12]. Clone 1C6 is directed against beta-cell membrane protein, clone PM1-11 is directed against GAD65 (Diamyd, Stockholm, Sweden) and clone KD9665 is directed against IA-2 (kindly provided by Dr M.R. Christie, King's College, London, UK).

Generation of immature dendritic cells. Monocytes were obtained via positive selection on a MACS column after staining PBMC with CD14-MicroBeads according to the supplier's protocol (Miltenyi Biotec via CLB, Amsterdam, The Netherlands). Isolated monocytes (0.5×10^6/ml, >90% pure) were subsequently cultured for 6 days in RPMI 1640 medium (Gibco Life Technologies, Breda, The Netherlands) supplemented with 10% heat inactivated fetal calf serum, 2 mmol/l glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 1000 U/ml recombinant human IL-4 (Strathmann Biotec AG, Hannover, Germany) and 800 U/ml recombinant human GM-CSF (Leucomax, Novartis Pharma, Arnhem, The Netherlands) in 24-well tissue culture plates (Costar, Cambridge, Mass., USA). Medium and cytokines were refreshed every 3 days. At day 6 the dendritic cells (DC) had obtained an immature phenotype. Dendritic cell maturation was induced by adding 100 ng/ml *E. coli* derived LPS (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands), and 800 U/ml GM-CSF. Dendritic cells (DC) were harvested after 48 h for further analysis.

FACS-Analysis. T-lymphocytes, EBV-transformed B-lymphocytes and monocyte-derived immature DC were co-cultured for 14 h (T-lymphocytes and DC) and 2 h (B-lymphocytes) (optimal viability and recovery), with the proteases trypsin, bromelain, papain, chymotrypsin (Mucos Pharma, Geretsried, Germany), and 50 µg/ml mixtures there of (protease mixture 1: trypsin, bromelain, chymotrypsin and papain in the ratio 24:45:1:60; protease mixture 2: trypsin and bromelain 1:2). Heat-inactivated enzymes were tested as control for the contribution of enzyme activity on phenotype and function of the leukocytes. Cells were washed three times in PBS and incubated with monoclonal antibodies directed to cell surface molecules CD1a, CD3, CD11a, CD20, CD25, CD40, CD44, CD54, CD58, CD80 and CD86, and stained with FITC-labelled anti-murine IgG as described previously (all antibodies were purchased from Becton and Dickinson, San Jose, Calif., USA) [13]. Analyses were done on the FACSCalibur fluorescence-activated cell sorter (Becton and Dickinson) on three separate occasions.

ELISpot analysis. ELISpot analyses were carried out to measure production of IL-4, IL-10 and IFN-γ, using commercial kits and according to the manufacturers' instructions (U-CyTech, Utrecht, The Netherlands). T-cells (n=20 000) of clone PM1-11 and 50 000 irradiated HLA-matched antigen-presenting cells were preincubated with medium alone, PMA and ionomycin or GAD65 autoantigen for 48 h with and without 25 µg/ml of the protease mixtures 1 and 2. Then, non-adherent cells were washed and 3500 cells transferred to wells of ELISA-plates precoated with high-affinity monoclonal antibodies against IL-4, IL-10 or IFN-γ, to which the cytokine produced during incubation will bind. After incubation for 4 h (IL-10 and IFN-γ) or overnight (IL-4), cells were lysed and debris was washed away. Areas to which the cytokine was bound were detected with a combination of biotinylated anti-cytokine antibody and gold-labelled anti-biotin antibody. Finally, a silver enhancement reagent was added yielding black zones ('spots') which show the sites of cytokine secretion. Numbers of spots and surface area of triplicate experiments were calculated automatically using Olympus Micro-Imager software.

T-cell proliferation. T-cells were co-cultured with irradiated autologous or HLA-DR-matched PBMC in culture medium (RPMI 1640, 10% human pooled serum) or stimulated with IL-2, PHA or sub-maximal concentrations of specific autoantigens and synthetic peptide epitopes thereof [10, 11, 12]. Proteases were added in a dose range from 3.1–100 µg/ml. After incubation for 3 days, ^{3}H-thymidine in RPMI 1640 medium was added to each well and cultures were harvested for counting radioactivity. Preincubation experiments were done with 100 µg/ml of proteases for 18 h. Heat-inactivated enzymes were tested as control for the contribution of enzyme activity on phenotype and function of the leukocytes. Thereafter, cells were washed and stimulated as described above. Data represent three separate experiments and are expressed as means of triplicate experiments ± standard deviation. In Student's t tests or Mann-Whitney U tests p values lower than 0.05 were considered significant.

Results

Cell surface analysis. The proteolytic activity of the various proteases and mixes was tested on cell surface molecules by FACS analysis, using a series of monoclonal antibodies reactive to CD3, CD11a, CD25, CD44, CD54 and CD58 on T-cells, HLA-DR, CD20, CD40, CD44, CD54, CD58, CD80 and CD86 on antigen-presenting B-cells and CD1a, HLA-DR, CD40, CD54, CD58, CD80 and CD86 separately. On T-cells, expression of CD3 and CD44 was decreased by all proteases, CD11a expression was increased and CD54 and CD58 were not affected (Table 1). Expression of HLA-DR, CD44, CD54, CD58 and CD80 on B-cells was decreased by protease treatment, whereas expression of CD20, CD40 and CD86 was not affected (Table 2). The phenotype of immature dendritic cells also changed considerably, though differently than that of B-cells, in the sense that most of the markers tested were increased (Table 3). Incubation with heat-inactivated enzymes did not alter the phenotype. Viability was nearly complete. These results indicate that TCR expression and homing receptors, as well as receptors for peptide presentation and costimulation by antigen-presenting cells are affected by protease treatment.

Table 1. Effect of protease treatment on expression of cell surface molecules: FACS analysis – T-cells (1C6; 14 h incubation with proteases)

	CD3	CD11a	CD25	CD44	CD54	CD58
Trypsin	↓	↑	=	↓↓	=	=
Bromelain	↓	↑	=	↓↓	=	=
Papain	↓	↑	=	↓↓	=	=
Chymotrypsin	↓	↑	↓↓	↓↓	↓	↓↓
Mix 1	↓	=	=	↓↓	=	=
Mix 2	↓	↑	=	↓	=	=

CD3: T-cell receptor-associated molecular complex; CD11a: (LFA1): adhesion molecule; costimulation and extravasation; CD25: IL-2 receptor (activated T-cells, B-cells, Mϕ); CD44: adhesion molecule; homing receptor on leukocytes; CD54 (ICAM-1): costimulation, extravasation; CD58 (LFA-3): costimulation; Changes in mean fluorescence intensity were grouped arbitrarily into 'moderate' [0–25% change; (↓)/(↑)], 'intermediate' (25–75%; ↓/↑) and 'severe' (>75%; ↓↓/↑↑)

Table 2. Effect of protease treatment on expression of cell surface molecules: FACS analysis – B-cells (EBV-BLCL; 2-h incubation with proteases)

	DR	CD20	CD40	CD44	CD54	CD58	CD80	CD86
Trypsin	↓	=	=	↓	=	↓	(↓)	=
Bromelain	↓	=	=	↓	↓	↓	↓	=
Papain	↓↓	=	=	↓	↓	↓	(↓)	=
Chymotrypsin	(↓)	=	=	↓	(↓)	(↓)	=	=
Mix 1	↓	=	=	↓	(↓)	↓	(↓)	=
Mix 2	↓	=	=	↓	=	=	n.t.	=

CD20: B-cell marker; CD40: costimulatory molecule; CD44: adhesion molecule; homing receptor on leukocytes; CD54 (ICAM-1): costimulation, extravasation; CD58 (LFA-3): costimulation; CD80 (B7.1): costimulation; CD86 (B7.2): costimulation. Changes in mean fluorescence intensity were grouped arbitrarily into 'moderate' [0–25% change; (↓)/(↑)], 'intermediate' (25–75%; ↓/↑) and 'severe' (>75%; ↓↓/↑↑)

Table 3. Effect of protease treatment on expression of cell surface molecules: FACS analysis – immature dendritic cells (14 h incubation with proteases)

	DR	CD1a	CD40	CD54	CD58	CD80	CD86
Trypsin	=	=	=	=	=	=	(↓)
Bromelain	↑	↑	(↑)	=	↑	↑	↑
Papain	↑	=	=	↑	=	(↑)	↑
Chymotrypsin	↑	=	=	↓	↓	=	=
Mix 1	↑	(↑)	=	=	(↑)	↑	↑
Mix 2	↑	(↑)	=	=	↑	(↑)	(↑)

CD1a: DC marker; CD40: costimulatory molecule; CD54 (ICAM-1): costimulation, extravasation; CD58 (LFA-3): costimulation; CD80 (B7.1): costimulation; CD86 (B7.2): costimulation. Changes in mean fluorescence intensity were grouped arbitrarily into 'moderate' [0–25% change; (↓)/(↑)], 'intermediate' (25–75%; ↓/↑) and 'severe' (>75%; ↓↓/↑↑)

Cytokine production. The effect of proteases on cytokine production profile of autoreactive T cells was studied on the GAD65 specific T-cell clones because this clone showed a Th-0/Th-2 cytokine profile in response to recognising GAD65 [11]. Non-specifically induced interferon-gamma (IFN-γ) production in response to PMA/ionomycin was not affected by protease treatment (Fig. 1). In contrast, IL-4 release increased two fold, while IL-10 production decreased twofold in the presence of protease mixes. However, autoantigen specific release of IFN-γ was completely inhibited, whereas IL-4 and IL-10 responses were not affected. This showed the selective action of the proteases on autoantigen-specific reactivity.

Proliferation experiments. Proliferative responses of a series of autoreactive T-cell clones with different specificity were analysed (Figs. 2, 3). Viability counting was done after preincubation before the functional assay; viability was near complete. A T-cell responsive to insulin-secretory granule membrane protein was specifically inhibited in proliferation against beta-cell membrane homogenate by all proteases tested. Bromelain was the least effective but still capable of blocking the proliferation for more than 80% at 25 µg/ml (Fig. 2A). The proliferation in response to IL-2 was much less affected. Papain was not able to interfere at all, while chymotrypsin as well as mixes of proteases most effectively inhibited the prolifera-

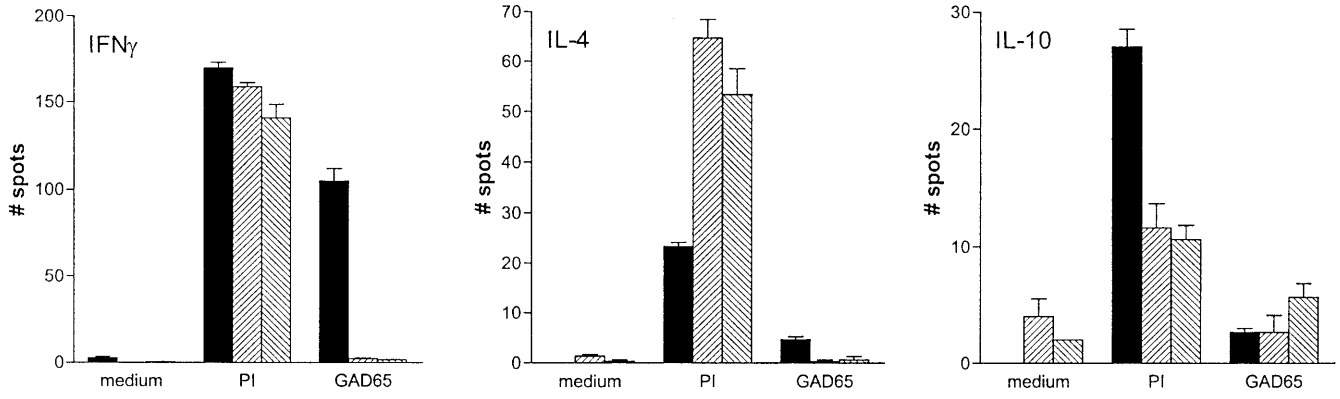

Fig. 1. Cytokine analysis of autoreactive T-cell clone reactive to GAD65 by ELISpot. The autoreactive T-cell clone PM1-11 was tested unstimulated (medium), and stimulated aspecifically (PMA/ionomycin, PI) and specifically (autoantigen GAD65) for production of IFNγ, IL-4 and IL-10 in the absence (left column) and presence of protease mix 1 (middle column) and mix 2 (right column)

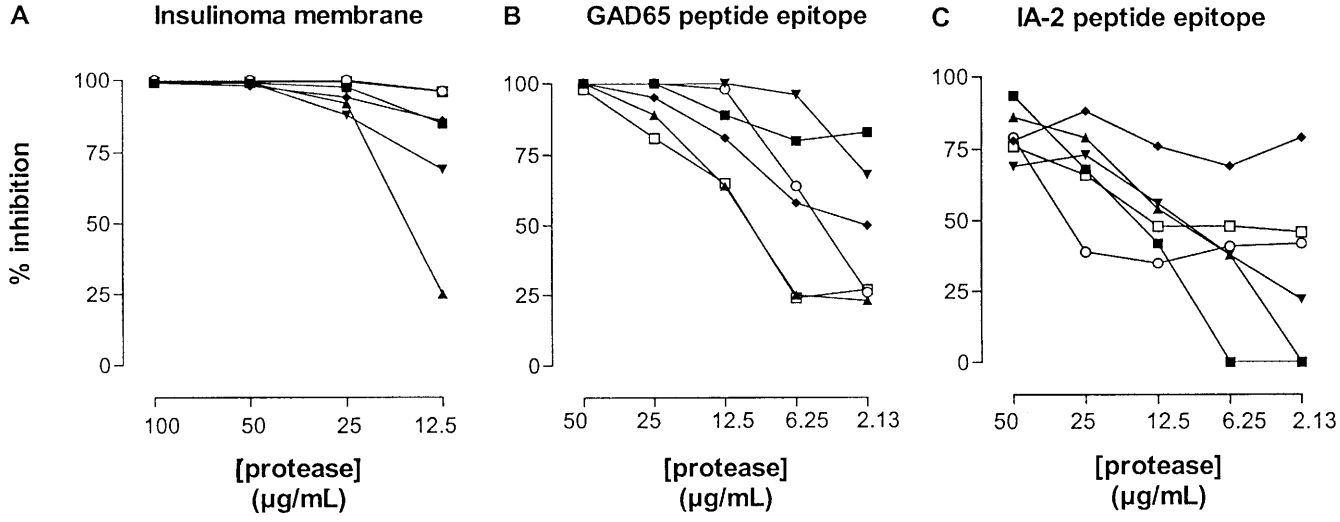

Fig. 2A–C. Effect of protease treatment on antigen-specific T-cell proliferation. Indicated is the percentage of inhibition in the presence of protease compared with proliferation in the absence of protease. The maximum concentration was reduced for T-cell clones against GAD65 and IA-2 following the plateau activities reached with this clone. GAD65 epitope: amino acid positions 335–352 (TVYGAFDPLLAVAD); IA-2 epitope: amino acid positions 831–850 (LYHVYEVNLVSEHIWCEDFL). – ■ – trypsin, – ▲ – bromelain, –▼– chymotrypsin, – ● – papain, – ○ – mix 1, – □ – mix 2

tive response (Fig. 3A). Stimulation by PHA was only inhibited with protease concentrations higher than 50 µg/ml (Fig. 3B). The inhibitory effect could not be explained by proteolysis of the antigen as the GAD65 specific T-cell clone and IA-2 specific T-cell clone could be inhibited in their response to peptide epitopes with varying degrees of efficacy (Fig. 2C). The autoantigens and peptides did not show any effect on immunogenicity after pre-treatment with proteases followed by heat-inactivation of the enzymes before adding the cell cultures (data not shown).

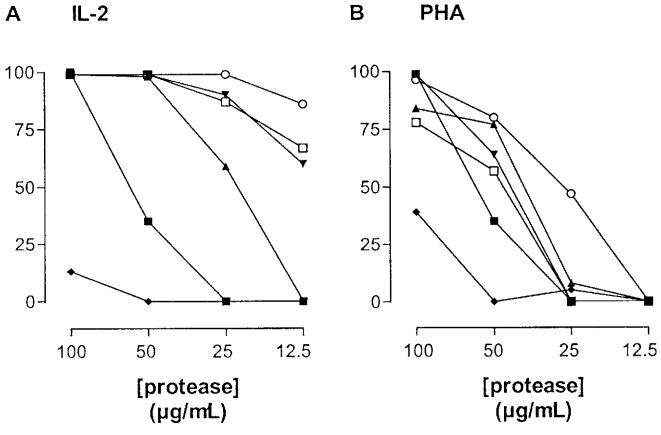

Fig. 3A, B. Effect of protease treatment on non-specific stimulation of T-cell clone 1C6. Indicated is the percentage of inhibition in the presence of protease compared with proliferation in the absence of protease. – ■ – trypsin, – ▲ – bromelain, –▼– chymotrypsin, – ● – papain, – ○ – mix 1, – □ – mix 2

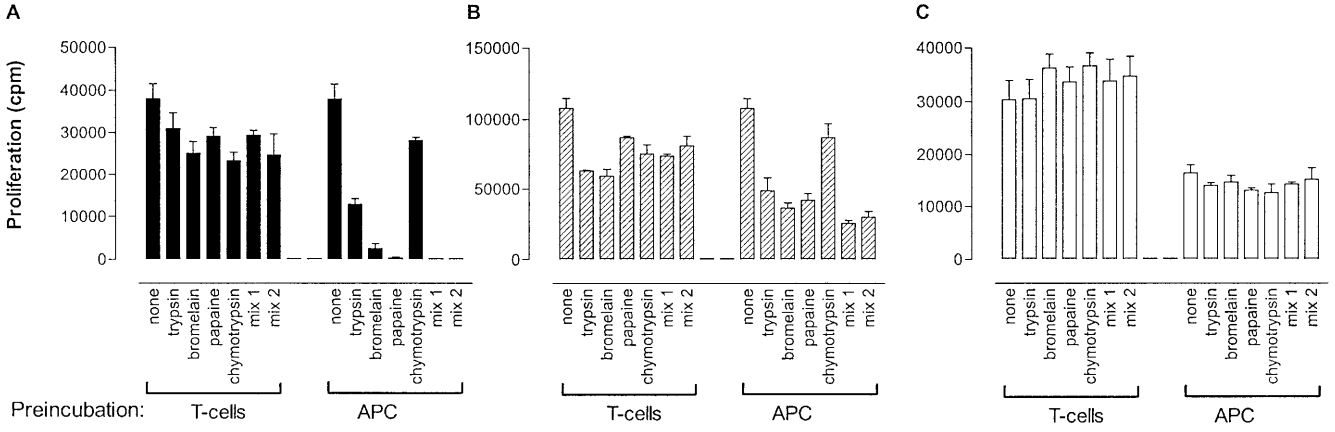

Fig. 4A–C. Effect of preincubation with proteases on proliferation of T-cell clone 1C6. Indicated is proliferative response after preincubation of either the T-cell clones or the antigen-presenting B-cell line. The protease concentration during preincubation was 50 µg/ml. **A** Proliferative response to complete antigen processed by antigen-presenting cells, **B** proliferative response to a synthetic peptide epitope directly presented by antigen-presenting cells, **C** proliferation to IL-2 after preincubation of T-cells, or PHA after preincubation of antigen-presenting cells

Preincubation studies on T cells and antigen-presenting cells separately showed that the inhibitory effect was elicited predominantly on APC (Fig. 4A). Papain and the protease mixes were most effective, regardless of the need for processing by the APC because similar effects were measured using the synthetic peptide epitope (Fig. 4B). Viability of either the APC or T cells appeared unaffected, because the response to IL-2 was not affected by pretreatment with proteases (Fig. 4C).

Discussion

Chronic inflammatory diseases are treated frequently with oral proteases. However, no molecular concept of the mechanism of the therapeutic action has been provided. We found that proteases can modulate T-cell activation by pleiotropic interference in immune responsiveness. Protease treatment diminished cell surface expression of homing receptor, co-stimulatory molecules and receptors involved in epitope presentation and recognition. The cytokine production profile of a T-cell clone stimulated by the specific autoantigen was affected by proteases through the selective inhibition of IFN-γ. Finally, the antigen-specific proliferative responses of a series of Type I diabetes-associated autoreactive T-cell clones were inhibited by protease pre-treatment of antigen-presenting cells at a pharmacotherapeutic serum concentration.

Immune activation of our series of human T-cell clones showed diverse sensitivity to the different proteases. The use of protease mixtures resembling the composition of commercially available drugs diminished the variability of the results of the different T-cell clones at a greater efficacy than the single proteases. Since different T-cells are activated in chronic inflammation and antigen spreading occurs during the progression of the disease, our observations favour the use of protease mixtures for clinical practice [14, 15].

Protease treatment reduced expression of cell surface receptors on T cells and antigen-presenting cells. As the effect of protease activity on T-cell stimulation and activity was primarily mediated by antigen-presenting cells in the preincubation study, the reduction of the homing receptor CD44 expression by T-cells adds to the potential efficacy of protease therapy in vivo [3, 5]. This is supported by the reported reduction of CD44 expression on lymphocytes of patients with multiple sclerosis during protease therapy [3, 7, 16].

We have shown that autoantigen T-cell proliferation can be inhibited by soluble forms of the intercellular adhesion molecule-1 (ICAM-1) in vitro and in vivo [17, 18]. The generation of soluble forms of adhesion molecules by proteolytic cleavage might act as an additional beneficial immunomodulatory function of protease treatment. Indeed, incubation of recombinant forms of ICAM-1 with proteases in vitro lead to incomplete protein degradation resulting in fragments of 30 000–60 000 M_r molecular weight which are compatible with the functionally active recombinant soluble forms studied previously. Reduction in co-stimulatory molecules and low-avidity recognition during T-cell activation favours the development of Th-2 T cells [19, 20].

The quality of immune activation plays an important role during chronic autoimmunity. The ratio of Th-1 and Th-2 cytokine expression regulates disease activity [21, 22, 23, 24, 25]. Th-1 immunity has been shown to be associated with inducing the progression of the disease in multiple sclerosis [26, 27, 28, 29, 30], rheumatoid arthritis [31, 32, 33] and Type I diabetes [24, 34, 35]. Unexpectedly, protease incubation specifically inhibited antigen-specific Th-1 lymphocyte function while Th-2-like cytokines were not affected. A similar observation has been reported in the experimental allergic encephalomyelitis, an animal

model for multiple sclerosis [5]. Oral administration of proteases to mice led to the reduction in Th-1 lymphocytes and completely diminished symptoms of the disease. Cytokine synthesis in human peripheral blood mononuclear cells has also been shown to be affected by oral enzyme treatment [4]. In animal models for rheumatoid arthritis and Type I diabetes protease treatment prevented or delayed the onset of the disease [6, 36, 37]. A randomized placebo-controlled clinical trial in first degree relatives of patients with Type I diabetes with a high risk of developing this disease has been initiated in Germany and Austria to test the immunotherapeutic effect of protease mixtures (ProDiabStudy). Our results also provide a rationale for the current protease therapy in sports. Th-1 cytokines cause increased vascular permeability and oedema [38, 39], whereas IL-10 inhibits cytokine production, vascular leakage and swelling during Th-1 induced delayed-type hypersensitivity [39]. Our findings showing that proteases shut off Th-1 cytokine production without affecting Th-2 type cytokines implies that oedema formation upon physical injury is affected by oral treatment with proteases.

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