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Prevention of Murine EAE by Oral Hydrolytic Enzyme Treatment

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Clinical trials that test the efficacy of Phlogenzym (consisting of the hydrolytic enzymes bromelain and trypsin and the anti-oxidant rutosid) as a treatment for T cell-mediated autoimmune diseases including multiple sclerosis (MS), type 1 diabetes and rheumatoid arthritis are presently ongoing. We tested the effects of Phlogenzym treatment in the murine model for MS, experimental allergic encephalomyelitis (EAE), a disease induced in SJL mice by immunization with proteolipid protein (PLP) peptide 139–151. Oral administration of Phlogenzym resulted in complete protection from EAE. In Phlogenzym-treated mice, the dose response curve of the PLP:139–151-specific T cell response was shifted to the right, that is, the primed T cells required higher peptide concentrations to become activated. Additionally, the T cell response to this peptide was shifted towards the T helper 2 cytokine profile. Both effects are consistent with an increased T cell activation threshold. In support of this interpretation, we found that the accessory molecules CD4, CD44, and B7–1 (all of which are involved in T cell co-stimulation) were cleaved by Phlogenzym, while CD3 and MHC class II molecules (which are involved in the recognition of antigens by T cells) and LFA-1 were unaffected. These data show the efficacy of oral Phlogenzym treatment in an animal model of T cell-mediated autoimmune disease and suggest that the protective effect might be the result of an increase in the activation threshold of the autoreactive T lymphocytes brought about by the cleavage of accessory molecules involved in the interaction of T cells and antigen presenting cells. © 1999 Academic Press

Introduction

The mucosal surface is not an unbreachable barrier between macromolecules present in the external world and the body: after oral uptake, several percent of ingested proteins can be recovered from the blood as native antigens [1–5]. Specialized cells along the gastrointestinal tract, so-called M cells, actively import antigens from the gut lumen [6]. After feeding high dose of protein, such antigen uptake usually results in oral tolerance [7], ensuring that the immune system does not respond to ubiquitous environmental antigens. We addressed the issue of whether this tolerogenic route of protein uptake can be exploited for the therapeutic delivery of bioactive macromolecules.

Various orally administered cytokines can exert systemic effects [8]. For example, the feeding of interferon-α (IFN-α) confers protection from collagen-induced arthritis and reduces the collagen-specific delayed-type hypersensitivity response [9]. Oral

administration of IFN-α also ameliorates infectious diseases caused by vesiculo stomatitis virus [10], feline leukemia virus [11] and *Theileria* infection [12]. Oral, but not parenteral interleukin-12 (IL-12) was effective in redirecting the T helper 2 (Th2) responses to tetanus toxoid towards a Th1-type response [13], and oral administration of IL-6 was shown to enhance the systemic immune response against *Campylobacter* infection and, upon rechallenge, protection from it [14]. The systemic effects seen after the feeding of these cytokines suggest that these proteins were not inactivated by acid and protease digestion but passed through the gut in their biologically active form, exerting systemic effects thereafter. The same applies for some gastrointestinal hydrolases, including trypsin, that undergo enteropancreatic recirculation [15–18]; these enzymes are actively reabsorbed in the gut and, after passing through the blood, are re-secreted by the pancreatic gland. Orally administered trypsin and bromelain, the second hydrolase contained in Phlogenzym, are also absorbed in a bioactive form, resulting in an increase in specific hydrolytic activity in the serum [19–21]. Immune complexes, being sensitive to digestion by these enzymes, can be degraded in the kidney after systemic [22] and oral

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application of these enzymes [23]. Phlogenzym has been widely prescribed in Western Europe to treat immune-mediated inflammatory conditions. Orally administered Phlogenzym proved to be as effective for the treatment of cutaneous *Herpes zoster* (caused by *Varicella* virus) as the reference drug, the anti-metabolite acyclovir [24–26]. Initial trials suggested that Phlogenzym might have ameliorating effects for autoimmune diseases including multiple sclerosis [27] and rheumatoid arthritis [28]. To follow-up on these reports, large scale, double-blind clinical trials are presently being conducted in Germany to test the effects of hydrolytic enzyme treatment on MS, type 1 diabetes and rheumatoid arthritis.

The experiments reported herein were performed to test the efficacy of Phlogenzym on the animal model of MS, experimental allergic encephalomyelitis (EAE). Oral Phlogenzym application prevented EAE development in mice. Because this disease is mediated by autoreactive T lymphocytes, we chose to characterize the underlying T cell response. We observed that, in the Phlogenzym-treated mice, the dose-response curve of the autoimmune T cell response was shifted to the right, and the cytokine profile was shifted from a T helper 1 (Th1)-type cytokine profile, with IFN-γ predominantly produced by the antigen-specific T cells, to a Th2-type profile, with the prevalence of IL-4 and IL-5 production. Both changes can explain the disease-attenuating effects seen.

Materials and Methods

Mice, antigens, and treatments

SJL mice were purchased from The Jackson Laboratories, Bar Harbor, ME, USA and bred at Case Western Reserve University, Cleveland OH, USA under specific pathogen-free conditions. Female mice were used at 6–10 weeks of age. Hen egg white lysozyme (HEL) was purchased from Sigma, St. Louis, MO, USA. PLP peptide 139–151 (H-KISQAVHAAHAE-OH), was purchased from Princeton Biomolecules, Columbus OH, USA. IFA was from Gibco BRL, Grand Island, NY, USA and CFA was made by mixing *M. tuberculosis* H37RA (Difco Laboratories, Detroit, MI, USA) at 2.5 mg/ml into IFA. Phlogenzym, a gift of Mucos Ltd, Geretsried, Bav, Germany, or HEL, was freshly dissolved in tap water, and 0.5 ml was given orally, by gavage. Except where specified otherwise in the text, the dose given was 4.1 mg/mouse, given at a 12 h interval, starting simultaneously with the immunization, throughout the duration of the experiment.

Induction and assessment of EAE

PLP peptide 139–151 was mixed with CFA to yield a 1 mg/ml emulsion, of which 50 μl (50 μg/mouse) was injected once, subcutaneously (sc). Pertussis toxin (List Biological Laboratories Inc., Campbell, CA, USA)

was given intraperitoneally (ip) twice on days 0 and 1 at a dose of 0.2 ng/mouse. Starting from day 8 after immunization, mice were assessed for the development of paralytic symptoms, and the severity of disease was recorded according to the standard scale: grade 1: floppy tail; grade 2: hind leg weakness; grade 3: full hind leg paralysis; grade 4: quadriplegia; grade 5: death. To ensure the nourishment of paralysed mice, elongated water tubes were used and food was placed in the bedding.

Flow cytometry

All antibodies were obtained from PharMingen, San Diego, CA, USA. They were used to stain 1,000,000 cells in single cell suspension. Stained cells were analysed with a FACScan and Cell Quest software (Becton Dickinson, Mountain View, CA, USA). Data are presented on a log scale as histograms.

Proliferation assays

Proliferation assays were performed as previously described [29]. Briefly, single cell suspensions were prepared from the spleen, of which 1×10^6 cells were plated per well in flat-bottomed 96-well microtiter plates in serum-free HL-1 medium (BioWhittaker, Walkersville, MD, USA) supplemented with 1 mM L-glutamine. Antigens or peptides were added at a final concentration of 100 μg/ml or titrated as indicated. During the last 18 h of a 4-day culture, ^{3}H-thymidine was added (1 μCi/well); incorporation of label was measured by counting liquid scintillation.

Cytokine measurements by ELISA spot

These assays were performed as previously described [30]. Briefly, ImmunoSpot plates (Resolution Technology, Columbus OH, USA) were coated overnight with IFN-γ-, IL-4- or IL-5-specific capture antibody, R46A2 (4 μg/ml), 11B11 (2 μg/ml) or TRFK-5 (5 μg/ml) in PBS, respectively. The plates were blocked with 1% BSA in PBS for 1 h at room temperature and washed 4 times with PBS. Spleen cells were plated at 10^6 cells/well alone, in HL-1 medium alone or with peptides at the specified concentrations and were cultured for 24 h for IFN-γ and 48 h for IL-5 and IL-4. Subsequently, the cells were removed by washing and the detection antibody (XMG1.2-HRP at 1 μg/ml for IFN-γ or TRFK-4 at 4 μg/ml and BVD4–24G2-biotin at 2.5 μg/ml for IL-5 and IL-4, respectively) was added and incubated overnight. The hybrids producing these mAbs were obtained from ATCC, and the antibodies were grown and labeled in our laboratory. For IL-5, anti-IgG2a-HRP (Zymed, San Francisco, CA, USA) was added and incubated for 2 h. The plate-bound final antibody was then visualized by adding AEC. To evaluate the results, we used a Series 1 ImmunoSpot Image Analyzer (Resolution Technology).
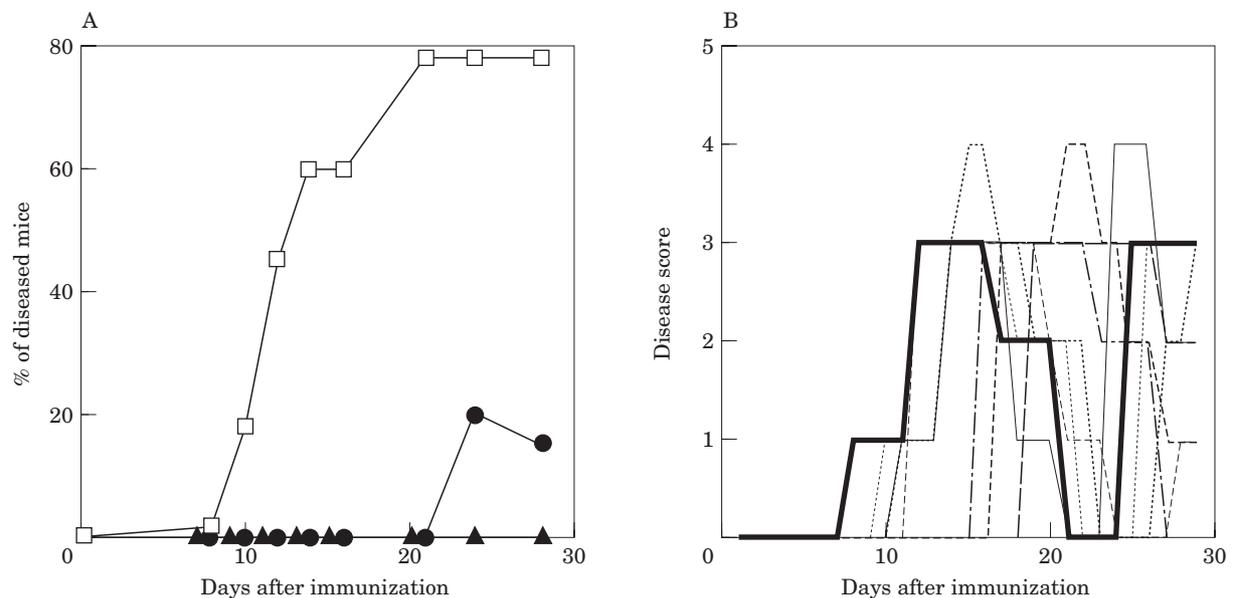
Figure 1. Development of EAE in mice treated orally with Phlogenzym and HEL. (A) Active EAE was induced in groups of 10 SJL mice per experiment by PLP:139–151 peptide immunization, while the mice were being treated twice daily by gavage with either 0.82 mg (●, 20 mice) or 4.1 mg (▲, 30 mice) of Phlogenzym, starting on the day of immunization and continuing throughout the experiment. Control mice were fed the equivalent dose of HEL (□; because there were no differences between the 0.82 or 4.1 mg HEL-fed groups, the data for all 50 control mice are pooled). The severity and course of disease for 10 control-treated mice is shown in panel B (see below). In mice fed with 0.82 mg Phlogenzym, where four of the 20 mice developed EAE, the maximal disease score was 2. (B) The actual course of the disease in the control group fed 4.1 mg HEL, showing the disease score for 10 individual mice, over time, after immunization, as representative for all groups of control-treated mice. The profile of one typical mouse is highlighted. We did not include the 4.1 mg Phlogenzym-fed mice in this panel because they did not show detectable disease at any time (see panel A).

Results

Oral administration of hydrolytic enzymes prevents the development of EAE

We first administered 0.82 mg Phlogenzym per mouse twice daily, orally, by gavage, which, corrected for weight, is the human equivalent dose. Control mice were fed the same dose of the enzyme hen egg white lysozyme (HEL). Enzyme feeding started simultaneously with the disease-inducing immunization with PLP peptide 139–151 and was maintained over the entire duration of the experiment. Mice were observed daily for the development of paralytic symptoms characteristic of EAE. While 39 of the 50 control mice (78%) developed EAE with a mean disease score of 3.7, only four of the 20 0.82 mg Phlogenzym-treated mice developed EAE (20%), with a maximum score of 2 (Figure 1). Since mice have a faster metabolism than humans, we increased the dose of Phlogenzym to test whether this would result in a more profound effect. We found that a five-fold higher dose (4.1 mg/mouse, twice a day) completely prevented the development of EAE in 30 mice tested in three separate experiments (Figure 1). Under continued treatment, none of the mice developed EAE during the 1 month observation period; thus, the oral feeding of Phlogenzym had a strong disease-suppressing effect. The protection afforded by Phlogenzym might not be durable, however, since several

of the mice developed mild but definitive EAE after the treatment was stopped.

Selective cleavage of accessory molecules by Phlogenzym

Because Phlogenzym consists of hydrolytic enzymes that might cleave cell surface molecules involved in the interaction of T cells and antigen presenting cells (APC) accounting for the observed disease-ameliorating effect, we tested which of the cell surface molecules involved in this interaction are susceptible to cleavage by Phlogenzym. T cell hybridoma and B cell tumor lines were incubated with 50 μg/ml of Phlogenzym for 2 h *in vitro*, after which the expression of cell surface molecules was assessed by FACS analysis. Staining for CD4, CD44, and B7–1 was dramatically reduced after the enzyme treatment, while the anti-CD3 staining was unaffected (Figure 2). Also unaffected by the enzyme treatment was cell surface expression of MHC class I, MHC class II, and LFA-1 molecules; moderate reductions in staining for ICAM-1 and B7–2 were seen (data not shown). Identical results were obtained in two-color FACS analysis of freshly isolated spleen cells treated with Phlogenzym after gating on cells positive for CD3 and MHC class II molecules to identify T cells and APC, respectively (data not shown). These data show that, despite their ubiquitous cleavage motifs, and even at high concentrations,
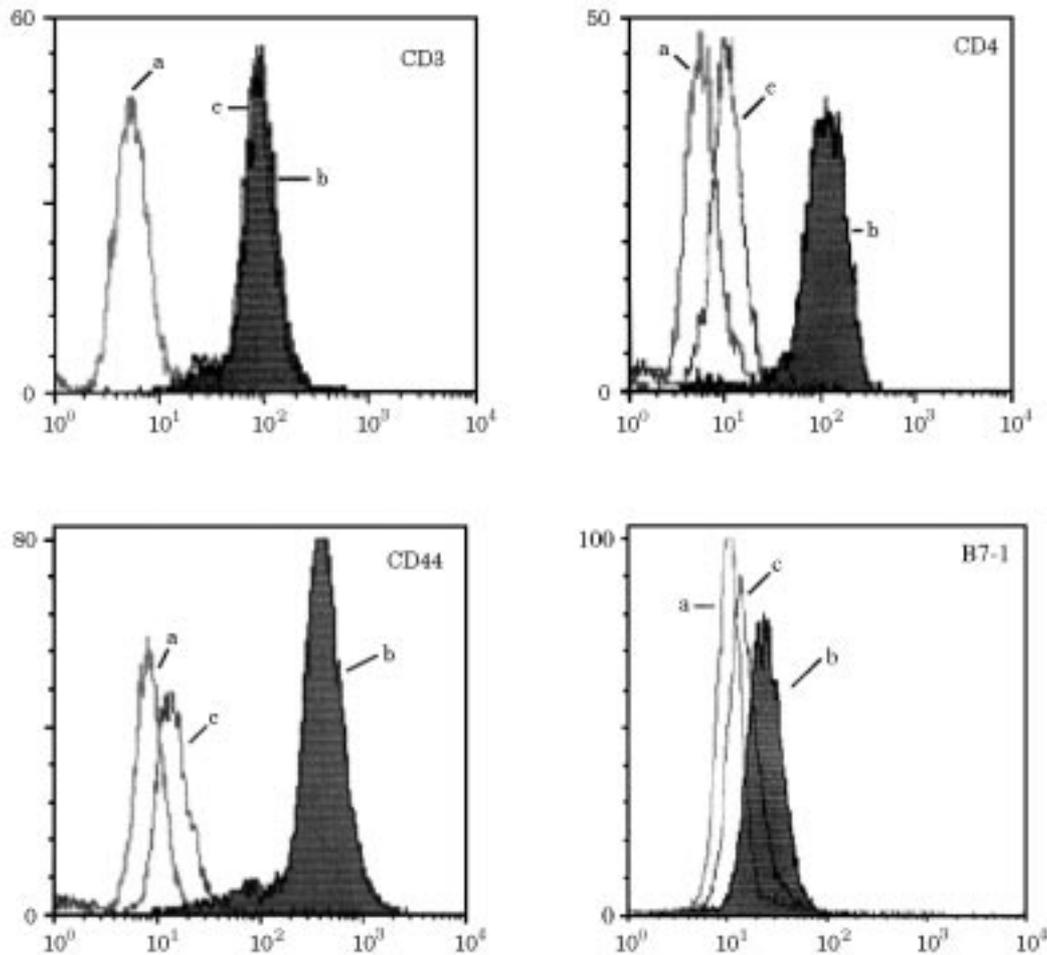
Figure 2. Expression of cell surface molecules after Phlogenzym treatment *in vitro*. A T cell hybrid clone, F.10.9, and a B cell tumor clone B220 were incubated with 50 µg/ml of Phlogenzym at 37°C and subsequently stained. The results in the top two panels are from the T cell hybrid, in the lower two panels from the B cell line (a=isotype control; b=staining of HEL-control-treated cells (which was identical to untreated cells): c=staining after Phlogenzym treatment). The loss of the CD44 signal was also seen on the F10.9 cells. The data are representative of these and two other T and B cell lines tested in two experiments.

these enzymes caused highly selective changes in cell surface molecule expression affecting some, but not all, cell surface molecules involved in the T cell/APC interaction.

Shifting of the dose response curve for T cell activation in Phlogenzym-fed mice

If Phlogenzym cleaves CD4, CD44, and B7–1 molecules *in vivo*, it should shift the dose-response curve for T cell activation to the right [35] since the T cell activation threshold depends on the level of accessory molecule expression [31–36]. We therefore tested the proliferative recall response to PLP:139–151 peptide in freshly isolated spleen cells of mice that were immunized with this peptide and fed with Phlogenzym or HEL (as in Figure 1, administering by gavage 4.1 mg enzyme/mouse, twice a day). Representative results are shown in Figure 3. The magnitude of the response in the Phlogenzym-treated mice was 10–40% of that in the control mice. Additionally, at submaxi-

mal peptide concentrations, the dose-response curve was invariably shifted to the right in the Phlogenzym-treated group; therefore, *in vivo*, Phlogenzym treatment affected T cell function in a way that is consistent with an increased T cell activation threshold caused by the cleavage of accessory molecules involved in T cell/APC interaction.

Th2-switching of the autoantigen-specific T cell response in Phlogenzym-treated mice

Weak, low avidity T cell stimulation tends to favor induction of Th2 responses [37, 38]. Also, because B7–1 molecules favor Th1 development [39], their cleavage (Figure 2) might cause Th2-switching of the autoimmune T cell response. To test for this possibility, we immunized mice with peptide PLP:139–151 and examined the cytokine profile of the recall response to this peptide. Groups of mice were fed with the 2×4.1 mg/mouse/day dose of Phlogenzym or HEL and tested 21 days after immunization, at
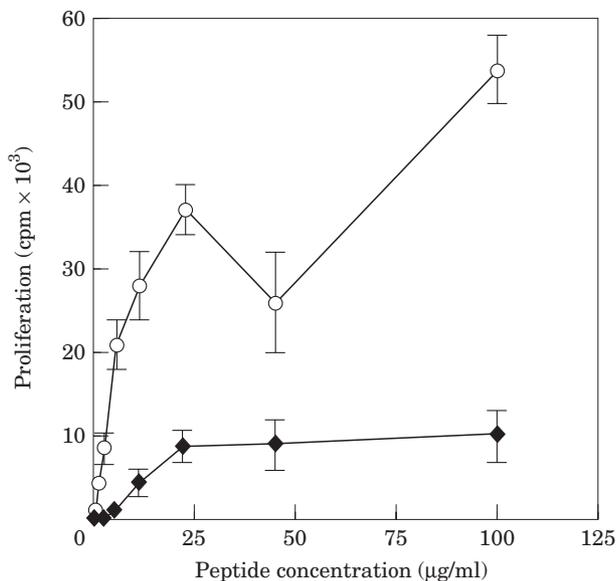
Figure 3. The PLP:139–151 peptide-specific proliferative recall response in mice fed with Phlogenzym (◆) vs. HEL (○). Mice were immunized with PLP:139–151 and treated with the 4.1 mg dose of Phlogenzym or HEL as specified in Figure 1. On day 21, the spleen cells were tested for their proliferative recall response in the presence of the PLP peptide concentrations shown. The data are expressed as the mean and SD of triplicate cultures of one mouse in each group, being fully representative for five mice tested in each group in this experiment. The results were reproduced in two experiments.

which time most of the HEL-fed control mice but none of the Phlogenzym-treated mice had developed EAE. Spleen cells from diseased, HEL-fed mice showed no significant IL-4 or IL-5 production in response to the PLP:139–151 peptide over the medium control but gave a vigorous IFN-γ recall response to this peptide (data not shown); the Phlogenzym-fed mice, which were not diseased, exhibited vigorous PLP:139–151-specific IL-4 and IL-5 production (Figure 4). The IFN-γ-recall response was not significantly different between the two groups (data not shown); therefore, there was a profound Th2 bias in the Phlogenzym-treated mice. In accordance with previous observations [40], this Th2-type cytokine recall response was also seen in the control mice 2 months after the immunization, by which time these mice had recovered from EAE (Figure 4).

Discussion

The data presented here show that oral administration of the hydrolytic enzyme preparation Phlogenzym can prevent the development of EAE in susceptible mice. This report confirms and extends upon previous reports in which this enzyme treatment has been shown to inhibit another T cell-mediated process, the rejection of rat aorta transplants across minor histocompatibility barriers [41].

An intriguing aspect of these findings is that the therapeutic effect was seen after oral administration of

macromolecular proteins. In addition to certain cytokines that have been shown to exert systemic effects after oral application [8–14], the feeding of enzymes now seems to be suited to the induction of systemic effects. Trypsin, a constituent of Phlogenzym, is actively imported from the gut [15–18]. Bromelain, the second protease constituent of Phlogenzym, is also absorbed in its active form [19–21]. Apparently, these proteins are resistant to acid inactivation and degradation by the digestive enzymes present in the gastrointestinal tract, and, when fed in high doses, can reach serum concentrations that exert pharmacological effects. Because the oral route of protein entry favors the induction of immunological tolerance, particularly after high-dose feeding [7], the ability to deliver therapeutic proteins by feeding might be an attractive alternative to injecting them, which is more likely to induce a neutralizing antibody response, as frequently seen after injections of monoclonal antibodies or β-interferon. The need to feed a higher dose might be offset by the reduced immunogenicity.

Despite the large number of cleavage motifs for bromelain and trypsin, these enzymes cleaved some (CD4, CD44, and B7–1) but not other (CD3, MHC class I and class II, and LFA-1) cell surface molecules. The latter proteins might be protected either because of their glycosylation or because their cleavage motif is buried within the core of the folded, native protein: in either case, the enzyme has no access to the substrate site. Since the serum contains an array of proteases, including trypsin [15–17], most cell surface proteins must have evolved to be resistant to such hydrolytic enzymes. Only when acting in the special microenvironment of the digestive tract, where proteins are denatured and, hence, unfolded by the low pH and stripped of their sugar side chains, are enzymes like trypsin able to exert broad substrate specificity. The selective cleavage of certain cell surface molecules, but not of others, should, however, have specific effects on cell functions. During inflammatory reactions, macrophages and neutrophils release an array of proteases [42], a reaction that may serve more specific functions in regulating the immune response than presently anticipated. Because they specifically cleave certain cell surface molecules, the use of hydrolytic enzymes may be an intriguing alternative to the use of blocking antibodies, or of recombinant ligands, to target cell surface molecules selectively.

We could not detect downmodulation of CD4, CD44, and B7–1 *in vivo* after feeding the enzymes (data not shown). Since the enzymatic activity of both bromelain and trypsin is optimal in an acidic environment, it is likely that these enzymes act primarily at inflamed sites, where the pH is low. We did, however, detect functional changes in the T cell response that were consistent with the cleavage of these accessory molecules during T cell priming. The right-shifted dose-response curve of the T cells primed in enzyme-treated animals (Figure 3) is consistent with an increased T cell activation threshold caused by reduced accessory molecule expression. For instance, peptide concentrations up to 100 times higher are
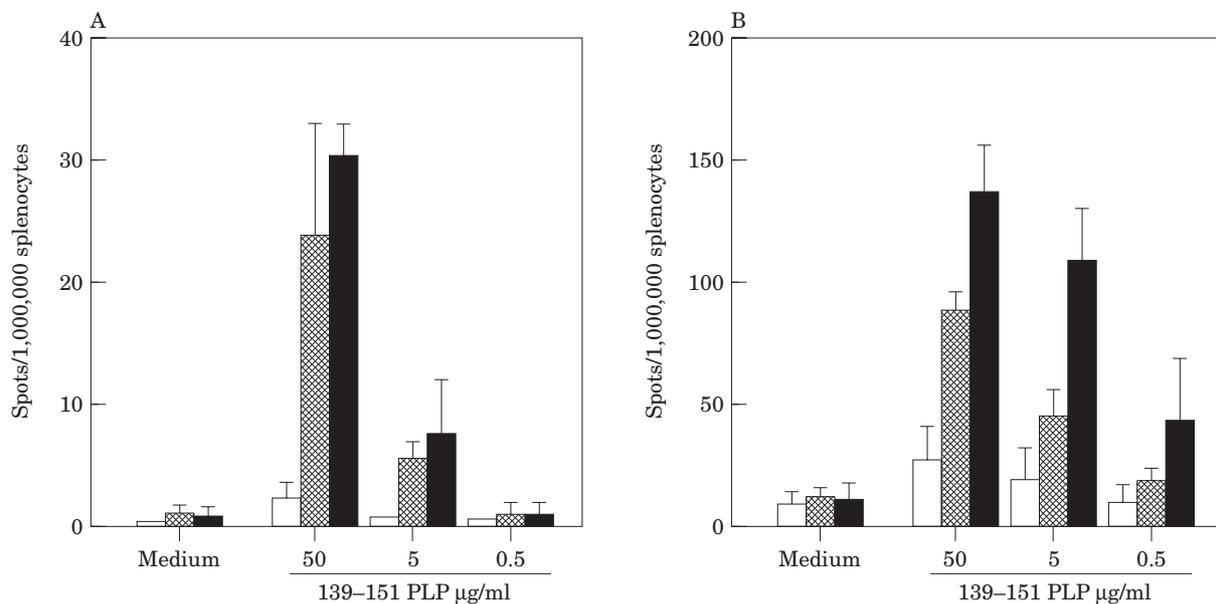
Figure 4. The cytokine recall response in mice treated with Phlogenzym and HEL. Five mice/group were immunized and treated as specified in Figure 3. Spleen cells were tested in ELISA spot assays of IL-5 (A) or IL-4 (B) production in the presence of medium alone or of PLP:139–151 peptide at the concentrations indicated. Diseased control mouse fed the 4.1 mg dose of HEL and tested on day 21 (open bars); non-diseased mouse fed the same dose of Phlogenzym and tested on day 21 (hatched bars); HEL-control mouse tested on day 60, after recovering from EAE (solid bars). The data are expressed as the mean+SD of triplicate cultures of one mouse in each group and are fully representative of five mice tested in each group in this experiment. The results were reproduced in three experiments.

required to activate T cells in LFA-1 knockout mice [31] (LFA-1 is the ligand for ICAM-1) whereas increased ICAM-1 expression lowers the T cell activation threshold [32, 33]. Also CD44, CD4, and B7–1 have co-stimulatory effects on murine T cell activation [32, 34]. Reduced density of these molecules on the surface of T cells and/or antigen presenting cells therefore renders T cells less activatable, i.e., increases their activation threshold [37]. For individual clones, such treatments cause a shift in their functional avidity for the antigen [36], converting high avidity recognition into low avidity recognition. Low avidity T cell responses favor the development of Th2 cells [37, 38]; therefore, both of the changes that Phlogenzym treatment induced in the autoantigen (PLP: 139–151)-reactive T cell population, the right shift in the dose-response curve and the Th2 shift, are anticipated consequences of reduced densities of co-stimulatory molecules that are cleaved by the hydrolases contained in Phlogenzym.

While the decreased expression of co-stimulatory molecules should affect the recognition of antigen by all T cells irrespective of their antigen specificity, such treatment may inhibit autoreactive T cells more than it does T cells responding to foreign antigens. While the autoreactive T cell repertoire has the tendency to consist of low affinity clones that escaped negative selection [36, 43], the T cell repertoire for foreign antigens has also not been negatively selected and encompasses high affinity clones. It should, therefore, be possible to increase the T cell activation threshold by reducing co-stimulation to the extent that the autoreactive clones are silenced but the foreign antigen-reactive clones remain largely

unaffected [44]. Accordingly, targeting the accessory molecules involved in T cell activation thresholds might be an immunomodulatory therapy selective for autoimmune disease because it does not result in generalized immunosuppression. Antibodies that block accessory molecules have been widely used to interfere with the development of autoimmune diseases including EAE [45]; our data suggest that similar results can be obtained by oral Phlogenzym therapy.

While the changes that we observed within the autoreactive T cell response itself may suffice to account for the disease protection seen, additional effects cannot be ruled out and are even likely. For example, because CD44 and ICAM-1 molecules are involved in cell migration [46], their cleavage may interfere with recruitment of T cells or macrophages. Also, the enzymes may cleave molecules that we have not measured, causing additional effects. Yet, our data clearly show that the enzyme treatment prevents the development of EAE and attenuates the autoimmune T cell response towards a low affinity, Th2 pathway. Furthermore, the data presented suggest that oral delivery of bioactive proteins can be exploited for therapeutic purposes, and that these hydrolytic enzymes can be used as a novel class of immune modulators.

Acknowledgements

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