Inhibitory effect of enzyme therapy and combination therapy with cyclosporin A on collagen-induced arthritis

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Abstract

Objective
There is increasing interest in the use of combination therapy for rheumatoid arthritis and in the possibility of combining the conventional drug approach with newer antirheumatic therapy. The present study investigates the efficacy of long-term prophylactic enzyme therapy and combination therapy with cyclosporin A in rats with collagen-induced arthritis.

Methods
Rats with collagen-induced arthritis were administered the following drugs: cyclosporin A (5 mg/kg/day and 10 mg/kg/day orally); a mixture of enzymes containing pure substances (bromelain, trypsin, rutin) in the same ratio as in Phlogenzym® (PHL, 150 mg/kg, twice daily intrarectally); and a combination of 5 mg/kg/day cyclosporin A plus 300 mg/kg/day PHL for a period of 50 days from the immunization. Levels of serum albumin, serum nitrite/nitrate concentrations, changes in hind paw swelling and bone erosions were measured in the rats as variables of inflammation and destructive arthritis-associated changes.

Results
Treatment with 10 mg/kg cyclosporin A, as well as combination therapy with half dosages of cyclosporin A (5 mg/kg) plus PHL significantly inhibited both inflammation and destructive arthritis-associated changes. Significant differences in favor of combination therapy with 5 mg/kg CsA + 300 mg/kg PHL as compared to 5 mg/kg CsA alone were seen in hind paw swelling. Also, reduction of the radiographic scores was more significant in the combination therapy group. Five mg cyclosporin A or PHL alone reduced the disease markers studied to a lesser extent, and in the case of enzyme therapy this occurred at a later stage of arthritis development.

Conclusion
Our results show the inhibitory effect of enzyme therapy on collagen-induced arthritis in rats, as well as the efficacy of cyclosporin A given in low doses in combination with enzyme therapy, which may be useful in the treatment of rheumatoid arthritis.

Key words
Collagen-induced arthritis, enzyme therapy, cyclosporin.
Introduction
Collagen-induced arthritis (CIA) is an autoimmune model that in many ways resembles rheumatoid arthritis (RA) (1). Immunization of genetically susceptible strains of rodents and primates with type II collagen leads to the development of a severe polyarticular arthritis that is mediated by an autoimmune response. Like RA, synovitis and cartilage and bone erosions are hallmarks of CIA, and susceptibility to both RA and CIA is linked to the expression of specific class II MHC molecules. CIA is a frequently used animal model to study disease pathology and drug efficacy with respect to human rheumatoid arthritis (2,3).
Cyclosporin (CsA), a cyclic and lipophilic fungal peptide, has been investigated widely in terms of both basic and clinical immunology for its unique immunosuppressive activity, i.e., the predominant suppression of T-lymphocyte functions. The drug has been clinically used in the treatment not only of rejection in organ transplantation but also of rheumatoid arthritis, and its potent effect in clinical conditions is recognized (4, 5). Although clinical trials have provided evidence for the clinical efficacy of CsA, its toxicity profile with respect to renal function impairment and gastrointestinal intolerance has led to a search for combination therapies which require lower doses while offering maintained or increased overall therapeutic efficacy (6, 7). In CIA, CsA can prevent the onset of the disease in a dose-dependent manner when initiated at the time of collagen immunization (3, 8). In rat arthritis, CsA has been combined with other therapeutic agents with interesting results. Combination of CsA with methotrexate (6), calcitriol (9) and the potent angiogenesis inhibitor AGM-1470 (10) enhanced the therapeutic effect of CsA without aggravating the associated toxicities.
Enzyme therapy is used in a wide range of inflammatory diseases, including glomerulonephritis, sports-related trauma and arthritis (11-16). Moreover, in addition to their antiinflammatory effects commercial preparations of proteases (Wobenzym®, Phlorgenzym®) have shown significant effects upon several critical immune functions. Improvement of joint inflammation and destruction with enzyme therapy was observed in collagen II induced progressive arthritis in male DBA/1 mice and in rabbits with experimental arthritis induced by intraarticular application of ovalbumin (17, 18).
The aim of the present study was to determine the effects of enzyme therapy, CsA, and the possible therapeutic potential of CsA combination with enzyme treatment on the development and disease severity of type II collagen-induced arthritis in rats.

Materials and methods
Materials
Phlorgenzym® (PHL) containing 90 mg bromelain (450 U), 48 mg trypsin (24 cat) and 100 mg rutin (rutoside.3H2O) per tablet was purchased from MUCOS Pharma GmbH, Gerestreid, Germany. The powder used in this study contained the pure substances in the same ratio as in the tablets. Sandimmun Neoral® oral solution (Sandoz Pharma Ltd, Basel, Switzerland) contained 100 mg/ml CsA plus the antioxidant E-307, 12% ethanol by volume, and excipients.

Immunization process
Bovine cartilage type II collagen, prepared by the method of Miller and Rhodes (19), was dissolved in 0.1 M acetic acid by stirring overnight at 4°C at a concentration of 2 mg/ml and an emulsion was prepared of this collagen solution with an equal volume of Freund’s incomplete adjuvants (DIFCO Laboratories, Michigan, USA). All rats were immunized by intradermal injection of 0.5 ml of the cold emulsion into the base of the tail and into a few sites on the back on day 0. Seven days after the initial injection, a booster injection of 0.2 ml of emulsion was administered intradermally at the base of the tail.

Animals
Male Lewis rats (160 - 180 g) obtained from Charles River (Wiga, Germany) were kept during the experiment in standard animal facilities that complied with the regulations of the Euro-
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Treatment

CsA and PHL were administered in corresponding daily doses from day 0 (at immunization) to day 50. CsA solution was diluted every day with olive oil to yield the desired concentration, and applied orally (p.o.) once a day. The enzyme mixture was administered rectally twice a day, at 8 a.m. and 5 p.m. PHL 300 mg/kg body weight was applied in two daily doses of 150 mg/kg each in 0.1 ml saline solution. Fresh PHL suspension was prepared every day. The untreated groups received vehiculum (olive oil, sterile saline) in the same manner daily for 50 days.

The animals were divided into the following six groups of ten: Group 1 - untreated non-arthritic controls; Group 2 - untreated arthritic controls; Group 3 - arthritic rats treated with CsA 5 mg/kg body weight; Group 4 - arthritic rats receiving 10 mg/kg CsA; Group 5 - arthritic rats given PHL (300 mg/kg); and Group 6 - arthritic rats administered the combination of CsA (5 mg/kg) + PHL (300 mg/kg).

Parameters of effect of treatment

Hind paw swelling. The volume of hind paw swelling was measured plethysmographically on days 14, 21 and 28 in the left and right paws.

Serum albumin levels. Serum albumin levels were assessed on days 15, 24 and 31 in rat serum by a spectrophotometric method, using an SYS 1 kit (BM/Hitachi, Boehringer Mannheim, Germany) on a Hitachi 911 automatic biochemical analyzer (Boehringer Mannheim, Germany).

Serum nitrite/nitrate. The nitrite/nitrate concentration in deproteinized serum was determined by the method of Cor-

Table I. The effect of enzyme therapy and combination therapy with cyclosporin A on the swelling of hind paws (ml) in rats with CIA.

<table>
<thead>
<tr>
<th>Groups of rats</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-arthritic untreated controls</td>
<td>1.38 ± 0.07</td>
<td>1.40 ± 0.00</td>
<td>1.40 ± 0.00</td>
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<tr>
<td>Arthritic untreated controls</td>
<td>2.37 ± 0.21</td>
<td>2.75 ± 0.26</td>
<td>2.58 ± 0.23</td>
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<tr>
<td>Arthritic rats treated with:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mg/kg CsA</td>
<td>1.74 ± 0.22***</td>
<td>2.67 ± 0.54</td>
<td>2.35 ± 0.41</td>
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<tr>
<td>10 mg/kg CsA</td>
<td>1.55 ± 0.13***</td>
<td>1.55 ± 0.42***</td>
<td>1.50 ± 0.28***</td>
</tr>
<tr>
<td>300 mg/kg PHL</td>
<td>2.32 ± 0.24</td>
<td>2.44 ± 0.14**</td>
<td>2.21 ± 0.20**</td>
</tr>
<tr>
<td>5 mg/kg CsA + 300 mg/kg PHL</td>
<td>1.88 ± 0.29**</td>
<td>2.35 ± 0.25**</td>
<td>2.08 ± 0.45**</td>
</tr>
</tbody>
</table>

Data represent mean values and standard deviation (mean ± S.D.) for 10 rats on a given day. Significantly different from arthritic control rats ** p < 0.01, *** p < 0.001. Significance of the difference between arthritic rats treated with 5 mg/kg CsA and with combination therapy /p < 0.05.
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Table II. The effect of enzyme therapy and combination therapy with cyclosporin A on serum albumin concentrations (g/L) in rats with CIA.

<table>
<thead>
<tr>
<th>Groups of rats</th>
<th>Day 15</th>
<th>Day 24</th>
<th>Day 31</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-arthritic untreated controls</td>
<td>33.48 ± 1.04</td>
<td>34.08 ± 0.62</td>
<td>34.03 ± 0.58</td>
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<tr>
<td>Arthritic untreated controls</td>
<td>25.29 ± 1.51</td>
<td>28.93 ± 0.77</td>
<td>30.12 ± 1.07</td>
</tr>
<tr>
<td>Arthritic rats treated with:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mg/kg CsA</td>
<td>29.03 ± 0.55**</td>
<td>30.80 ± 1.76**</td>
<td>32.65 ± 1.16**</td>
</tr>
<tr>
<td>10 mg/kg CsA</td>
<td>29.75 ± 0.89***</td>
<td>31.96 ± 1.35***</td>
<td>33.81 ± 1.12***</td>
</tr>
<tr>
<td>300 mg/kg PHL</td>
<td>27.18 ± 1.12*</td>
<td>30.03 ± 1.28*</td>
<td>31.41 ± 1.37*</td>
</tr>
<tr>
<td>5 mg/kg CsA + 300mg/kg PHL</td>
<td>28.52 ± 0.75**</td>
<td>30.99 ± 1.65**</td>
<td>32.93 ± 1.00**</td>
</tr>
</tbody>
</table>

Data represent mean values and standard deviation (mean ± S.D.) for ten rats on a given day. Significantly different from arthritic control rats *p < 0.05, **p < 0.01, ***p < 0.001.

A reduction in inflammatory activity was observed with 10 mg/kg CsA, p < 0.001 (Table II). However, both the half dose of CsA and CsA + PHL also induced a statistically significant inhibition of albumin decrease compared with untreated arthritic rats (p < 0.01). A weaker but still significant effect was observed when administering PHL alone (p < 0.05). However, the combination of CsA with PHL had no additional effect compared to CsA alone.

Table III. The effect of enzyme therapy and combination therapy with cyclosporin A on serum nitrite/nitrate concentrations (nmol/ml) in rats with CIA.

<table>
<thead>
<tr>
<th>Groups of rats</th>
<th>Day 15</th>
<th>Day 24</th>
<th>Day 31</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-arthritic untreated controls</td>
<td>11.24 ± 2.60</td>
<td>13.06 ± 3.14</td>
<td>13.03 ± 2.24</td>
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<tr>
<td>Arthritic untreated controls</td>
<td>27.23 ± 2.94</td>
<td>27.75 ± 4.74</td>
<td>32.73 ± 2.35</td>
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<tr>
<td>Arthritic rats treated with:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mg/kg CsA</td>
<td>14.43 ± 3.47***</td>
<td>14.76 ± 2.92***</td>
<td>12.07 ± 2.67***</td>
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<tr>
<td>10 mg/kg CsA</td>
<td>12.28 ± 2.97***</td>
<td>10.71 ± 2.07***</td>
<td>14.16 ± 2.56***</td>
</tr>
<tr>
<td>300 mg/kg PHL</td>
<td>17.32 ± 2.26***</td>
<td>19.78 ± 2.62***</td>
<td>14.32 ± 6.31***</td>
</tr>
<tr>
<td>5 mg/kg CsA + 300 mg/kg PHL</td>
<td>11.99 ± 2.78***</td>
<td>14.65 ± 2.50***</td>
<td>12.53 ± 3.68***</td>
</tr>
</tbody>
</table>

Data represent mean values and standard deviation (mean ± S.D.) for ten rats on a given day. Significantly different from arthritic control rats ***p < 0.001.

Serum nitrite/nitrate concentrations
Concentrations of nitrite/nitrate in the serum reflect the NO production in various tissues and the inflammatory response. The clinical onset of CIA was associated with a significant rise in nitrite/nitrate concentrations. Cyclosporin alone (5mg/kg and 10 mg/kg) as well as the combination therapy (CsA + PHL) brought the nitrite/nitrate levels back to normal, similar to those in the healthy controls (Table III). PHL alone also markedly suppressed nitrite/nitrate production in CIA rats. The paradoxical increase in nitrite/nitrate concentrations was observed on day 31 in comparison with the serum level on day 24 in the group of rats treated with 10 mg/kg CsA (p < 0.01).

Evaluation of bone erosions
A statistically significant reduction in the radiographic scores was observed for the group treated with CsA (5 mg/kg and 10 mg/kg) and for the combination of CsA + PHL (5 mg/kg + 300 mg/kg) in comparison with untreated CIA rats (Fig. 1). Although there was no significant difference between combination therapy with CsA + PHL and CsA alone (5 mg/kg), the beneficial effect of PHL manifested itself by a greater reduction in the radiographic scores in favour of the combination therapy (p < 0.001 vs p < 0.01).

Discussion
Our experiments in CIA rats focused on the effects of long-term prophylactic treatment with enzymes, and on the possibility of reducing CsA doses by administering the drug together with enzyme therapy. The efficacy of cyclosporin in CIA, an experimental model of arthritis, has been well documented (3, 22). The results obtained in the present study confirm and extend the previously reported effects of CsA. The effect of CsA on nitrite/nitrate levels in CIA has not been previously studied. In our study, CsA at a dose of 10 mg/kg/day administered from the day of immunization was very efficacious in the treatment of rats developing CIA,
and markedly reduced the clinical signs of arthritis: hind paw swelling, bone erosions and ankylosis. CsA given at a half dose (5 mg/kg) only partially suppressed these arthritis markers. PHL alone significantly decreased the hind paw volume and moderately reduced bone erosions and ankylosis. The combination of CsA at a half dose with enzyme therapy potentiated the CsA effect, resulting in a more significant reduction in hind paw swelling and the radiographic score, similar to what was shown in our previous study of rat adjuvant arthritis (23).

In addition, the effects of these drugs on the acute phase response were studied. Serum albumin acts as a negative acute phase protein in rat arthritis and the decrease of serum albumin reflects changes in the synthesis of this protein in the liver secondary to the activation of hepatic cells by inflammatory cytokines, mainly IL-1 (24,25). CsA markedly reduces the albumin decrease in rat adjuvant arthritis (24, 26). A similar effect was observed on our CIA model. CsA in both doses (5 mg/kg, 10 mg/kg) significantly elevated serum albumin levels. A weaker but still significant effect was observed with enzyme therapy. The combination of CsA with PHL had no additional effect compared to CsA alone. Probably, PHL affects the inflammatory cytokine-induced albumin reduction by a mechanism different from that of CsA (acting on different cytokines or their receptors). The effect of the combination of different cytokines on the production of acute phase reactants in the liver is usually not additive, and is strongly dependent on the actual combination of cytokines and their amounts (27).

Nitric oxide (NO), an unstable free radical produced by the action of the enzyme NO synthase (NOS) on L-arginine, is a mediator of multiple physiological functions, and may also mediate local inflammation and tissue destruction (28). Recent evaluations of NO synthesis in adjuvant arthritis (29-31) as well as streptococcal cell wall-induced arthritis showed an association between arthritis, increased NO production and the induction of NOS (32). Moreover, inhibitors of NOS have been shown to suppress arthritis in several animal models (33,34), and increased NO levels have been found in patients with rheumatoid arthritis (35,36). CIA induces significant expression of inducible NOS (iNOS), to an extent that is lower than the levels observed in adjuvant-induced arthritis (22,37). Recent studies, however, indicate that inhibitors of iNOS and peroxynitrite scavenging inhibit the progression of collagen- induces arthritis (37,38), although iNOS inhibition alone is insufficient to suppress the development of CIA.

In our present study markedly increased serum nitrite/nitrate concentrations were measured in CIA rats. Enzyme therapy significantly decreased nitrite/nitrate levels in arthritic rats throughout the experiment. The inhibition of NO production may be involved in the antiinflammatory effect of enzymes. A more detailed clarification of the inhibitory action of PHL on serum nitrates/nitrates requires direct investigation of its effects on NO-synthase isoenzymes. CsA in both the lower and the higher dose used almost completely inhibited nitrate/nitrate increase. This effect of CsA in CIA arthritis has not been studied previously. Higher CsA doses (25 mg/kg/day) given to spontaneously hypertensive rats are, however, known to inhibit endothelial NO formation, which can result in an increase of arterial pressure (39). On day 31, CsA at 10 mg/kg paradoxically increased the concentrations of nitrites/nitrates compared to their concentrations on day 24. The increased nitrite/nitrate levels in these rats may be a consequence of CsA-induced impairment of renal function. In man, more than half of the serum nitrate is eliminated via the kidneys (40). The effect of combination therapy with PHL + CsA on nitrite/nitrate levels cannot be assessed since as little as 5 mg/kg CsA is able to bring serum nitrite/nitrate levels in arthritic animals to normal levels (Table III).

Chintalacharuvu et al. (17) used enzyme therapy (Phlogenzym®) in the treatment of collagen II induced progressive arthritis in male DBA/1 mice. In their experiments, mice were treated twice daily with either a commercial cocktail of proteases (200 mg/kg/day) or ibuprofen. The improvement in joint inflammation and the reduction of joint destruction, as well as the normalisation of IgG glycosylation and IFN-gamma production achieved were similar in the ibuprofen and the protease treated groups. However, protease treatment protected and preserved articular cartilage, normalized the sialylation of anti-collagen antibody and restored IL-5 synthesis. These immunomodulatory effects of proteases were not seen with ibuprofen. The ability of proteases to delay or forestall erosive and destructive arthritis or ankylosis was also observed in mouse CIA.

In the present experiments the rats received prophylactic treatment, and the enzymes were applied in a form different from that used by Chintalacharuvu et al. (17). Our results nevertheless confirm the beneficial effect of enzyme therapy on collagen-induced arthritis reported by these authors. Initially, proteases were employed in glomerulonephritis and arthritis induced by immune complexes (14, 41, 42). Chintalacharuvu et al. (17) explained the mechanism as being a result of the capacity of proteases to cleave Ig molecules, especially in the tuftsin-like region in the second constant heavy chain (CH2) domain. Such proteolysis of the Ig component(s) of an immune complex would, in principle, solubilize the complex; this effect on immune complexes could indeed be documented both in vivo and in vitro. More recently, additional effects of proteases on other Ig superfamil members have been reported (12, 43).

Proteases also cleave the adhesion molecules critical to leukocyte activation and migration into the site of inflammation (CD54 and CD44) (12, 42, 44). CD44 is a homing receptor for collagen matrix. The immunomodulatory effect of proteases likely derives in part from cleavage of adhesion molecules critical to T-cell activation (CD4, CD80 and CD86) (12, 43, 45). Moreover, it has been suggested in a preliminary study that proteases can also influence the secretion and effect of transforming growth factor-1 (16).
The combination dosing regimens of proteolytic enzymes and CsA in CIA rats produced pharmacologically additive inhibitory activities with respect to some of the clinical signs of arthritis in rats (hind paw swelling, radiographic score) possibly due to their different mechanisms of action. The major effect of CsA is the inhibition of T cell activation and synthesis of cytokines IL-2, IL-3, IL-4, GM-CSF, TNF-α. Recently, the beneficial effect of combining enzyme therapy with methotrexate has been described in rheumatoid arthritis patients (13). Enzyme therapy allowed reduction of the methotrexate dose and consequently a reduction in its side effects. The safety of protease therapy in general is well established and it is widely used in a variety of clinical settings.

In conclusion, our study provides evidence for the beneficial effect of enzyme therapy and CsA + PHL combination therapy in the rat CIA model. Preliminary screening using this model might identify additional drug combinations that could be effective and relatively less toxic. The results of the present investigation could lead to clinical treatment trials for rheumatoid arthritis.

References


