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Immunomodulation by proteolytic enzymes

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Immune-mediated pathophysiological mechanisms contribute to the development of many forms of renal disease, including interstitial nephritis, glomerulonephritis, and kidney graft rejection. Much recent evidence suggests that the development of all of these diseases critically depends on the activation and expansion of specific T lymphocytes. Even those renal diseases that are primarily mediated by antibodies (SLE, IgA nephropathy) are critically dependent on T-cell involvement. There are very few exceptions to this rule. Thus, if we learn how to modulate T-cell responses in general, we may learn how to treat many immune-mediated pathological conditions. With respect to this, there may be an important lesson to be learned from Dr Gaciong's and Dr Heidland's report in this issue (p. 987) on the treatment of allograft arteriosclerosis by hydrolytic enzymes

In the rat allograft arteriosclerosis model, the host's T-cell response is directed against an aortic tissue transplant that is derived from a different strain of rat, i.e. the transplant is allogeneic. Triggered by recognition of the allogeneic histocompatibility antigens, alloantigen specific T cells become activated and release cytokines. As with any T-cell-mediated inflammatory reaction, these cytokines then activate cells of the reticuloendothelial system and induce a type IV (delayed hypersensitivity type) inflammatory reaction. In this particular model, arteriosclerosis-like lesions develop. If the same immune reaction occurred in the skin, classical delayed type hypersensitivity lesions would develop; if the T cells recognized an antigen in the brain, the β cells of the pancreas, the joint or the glomerular mesangium, the process may manifest itself as multiple sclerosis, type 1 diabetes, rheumatoid arthritis, or glomerulonephritis respectively. A similar type of T-cell response can also underlie chronic kidney graft rejection. Thus, the Warsaw–Würzburg group's findings may apply to other T-cell-dependent diseases. In support of this generalizability, several other studies showed that the enzymes inhibit a variety of immune-mediated kidney diseases and are also potent immune modulators of a murine model of multiple sclerosis [Refs. 1–5 and 14 in the paper]. While in some of these studies the enzymes were injected and in others they were administered orally, they prevented disease under both conditions

These encouraging animal studies have already been partially corroborated in humans. The enzyme preparation used in these animal studies has been available in Germany for decades (Wobenzym, and more recently Phlogenzym) and has enjoyed considerable popularity. While primarily prescribed for sinusitis and herpes zoster, the enzyme preparation has had the unproven reputation for ameliorating a broad spectrum of immune-mediated inflammatory diseases, including rheumatoid arthritis and multiple sclerosis. Only recently have these incidental observations been followed up systematically and clinical trials been initiated to evaluate the therapeutic potential of the enzymes. While the clinical trials are in an incipient stage, the studies on experimental animals are encouraging. Is this another example of the classical case where popular wisdom gained empirically, rather than by design, has led to a new form of therapy? How do these enzymes exert their immunomodulatory effect?

One of the main constituents of Phlogenzym is trypsin. Trypsin undergoes enteropancreatic recirculation: after being secreted by the exocrine pancreas, it is resorbed from the gut lumen, transported in the blood and resecreted by the pancreas [1–3]. Trypsin is a normal constituent of the serum, usually present there at 300 mg/l [4], most of which is of intestinal origin [2]. Owing to this active transport, $\sim$10% of an orally administered trypsin dose ends up in the blood [reviewed in 3]. Like other serum proteases, serum-trypsin is bound to the antiprotease, alpha 2 macroglobulin (α_2M) [5]. In this complex trypsin is protected from autodigestion and from degradation by other serum proteases, but retains it enzymatic activity [3]. Because of its high molecular weight, the trypsin–α_2M complex is confined to the blood stream, as are most macromolecular serum proteins. At sites of inflammation, however, with vascular permeability locally increased, serum protein exudate enters the interstitial compartment. Thus trypsin, along with the other serum proteases and antiproteases, complement components, and IgM, is delivered and enriched at the site of inflammation, i.e. the site of the immune action. Enrichment of trypsin at sites of inflammation can be readily demonstrated *in vivo*. Therapeutically administered trypsin, when injected or given orally, increases the effective concentration of the protease (as can be

demonstrated with labelled trypsin), thus multiplying its physiological action at sites of inflammation.

How does trypsin affect T cell functions? When T lymphocytes or macrophages are cultured *in vitro* in the presence of trypsin, only three cell surface molecules, of 21 studied, were found to be sensitive to cleavage, while cell viability and the 18 other molecules were completely unaffected, even at very high concentrations of the enzyme [6]. Interestingly, the three molecules cleaved, CD4, CD44, and B7-1, are important regulators of the T-cell response. Apparently, trypsin has a highly selective cleavage profile on cell surface molecules. Superficially this finding may be surprising, since the trypsin cleavage motif is present in most proteins (and hence one might expect all proteins to be sensitive to trypsin cleavage). Indeed, trypsin has a broad cleavage profile when it acts as a digestive enzyme in the gut where the proteins it encounters have been denatured by low pH and stripped of their carbohydrate side-chains by amylases and other digestive enzymes. As opposed to this broad 'gut cleavage profile' of trypsin, strikingly different rules apply when trypsin acts systemically. In an inflamed tissue, proteins on cell surfaces, or in solution, are native molecules whose trypsin sites are masked by glycosylation, or buried within the protein core, and hence are inaccessible (glycosylation of cell surface proteins and of secreted proteins serves primarily as protection of the protein from serum proteases). Thus, the 'systemic cleavage profile' of trypsin appears to be highly restricted, selectively affecting only certain types of molecules, i.e. it has specificity! It is conceivable that trypsin-sensitive molecules can be specifically targeted for therapeutic purposes with trypsin, analogous to their blockade with monoclonal antibodies. Bromelain, another constituent of the enzyme preparation used by Drs Gaciong and Heidland also selectively cleaves CD44 [7], possibly potentiating trypsin's effect.

Strikingly, the three molecules that trypsin cleaves from living cells (CD44, CD4, and B7-1) are among the 'hottest' targets of modern immunotherapy. All three are central for regulating the threshold for T-cell activation [8]. T cells do not recognize soluble antigen molecules (as antibodies do) but encounter the antigen on antigen-presenting cells, usually macrophages, dendritic cells, or B cells. In this recognition process, the T cell's antigen receptor (TCR) specifically binds to the complex of a major histocompatibility molecule (MHC/HLA) and a processed peptide fragment excised from the native antigen (MHC molecules are 'silver trays' on which antigen fragments are displayed to T cells for recognition). The TCR–MHC/peptide–antigen interaction determines the specificity of the T-cell recognition event. In order to become activated, a single T cell has to recognize more than 100–300 MHC/peptide–antigen complexes, i.e. there is a threshold for T-cell activation [9]. This threshold is not fixed, however, but is modulated by a number of accessory molecules, including the trypsin sensitive CD4, CD44, and B7-1 molecules. High expression of accessory molecules on T cells or APCs lowers the activation threshold (requiring fewer MHC/peptide–antigen complexes for activation) while decreased expression of accessory molecules increases the threshold (requiring more MHC/peptide-antigen complexes for activation). Thus enzymatic cleavage of the trypsin sensitive accessory molecules should raise the T-cell activation threshold. Indeed, when T cells are isolated from enzyme-treated mice and tested freshly *ex vivo* for their antigen-specific recall response, their dose response curve is significantly right-shifted, i.e. their activation threshold is increased [6]

Upregulation of accessory molecules regularly occurs at sites of T-cell-driven inflammation, promoted primarily by the local release of interferon gamma (IFNγ) by T cells. This facilitates T-cell responses. Our work has shown that this upregulation results in a critical amplificatory cascade of T-cell-mediated inflammation, called determinant spreading [10]. Without this amplificatory event, full-blown T-cell-mediated pathology may not develop [11]. Therefore cleavage of accessory molecules at sites of inflammation by trypsin could locally reset the T-cell activation threshold, acting as a physiological regulator of the inflammatory response. Analogously, trypsin's therapeutic effect on T-cell-mediated disease may be achieved by raising the T-cell-activation threshold at the sites of immune injury. Thus, low-affinity T cells, which constitute the majority of the diversified repertoire, will be locally silenced, but high-affinity T cells will continue to respond at a reduced level. Importantly, the result would not be generalized immunosuppression, but local immune modulation. While the regulation of T cell activation thresholds is apparently a key mechanism for the enzyme's immunomodulatory action, additional mechanisms may also play a role.

The news on the enzymes' immunomodulatory effects comes at a time when the demand for immune modulators is higher than ever. Although in the last two decades there has been an explosion in our knowledge of how T cells function, down to molecular detail, little of this knowledge has been translated into clinical practice. The molecules to be targeted are well known, but the issue has been how to target them. Present technology does not yet permit the design of low-molecular-weight chemicals (classical, orally applicable drugs) that would specifically target key molecules or functions of the immune system. There are few such drugs on the market and the discovery of new ones largely relies on random screening. Even the most potent of immunosuppressive drugs in this category, cyclosporin, antimetabolites, and corticosteroids, ultimately fail to halt autoimmune diseases or graft rejection. The alternative approach is the use of monoclonal antibodies as immunotherapeutic agents. Monoclonal antibodies can be readily generated against virtually any molecule of the immune system. Most probably the effect seen after enzyme therapy in Drs Gaciong and Heidland's studies might also have been achieved by injecting anti-CD4 antibodies alone, or in conjunction with anti-CD44 and anti-B7-1 antibodies (it has

been seen in most rodent models of T-cell-mediated disease). Can the enzyme therapy compete with this?

Treatments with monoclonal antibodies frequently fail in the long run because the immune system produces neutralizing antibodies against the injected antibody. Even human or humanized monoclonal antibodies injected into humans are likely to induce the production of neutralizing antibodies, jeopardizing the therapeutic effect and occasionally causing severe side-effects. Perhaps the most exciting perspective of enzyme therapy is that trypsin, because of its active resorption and enteropancreatic recirculation, can be administered orally; monoclonal antibodies and most other therapeutic proteins given this way would not be resorbed in significant quantities. Most importantly, antigens that the immune system encounters after oral exposure induce a specific state of immune tolerance, called oral tolerance [12]. In contrast, systemic exposure to the same antigen, after injection, usually induces an immune response. Thus, when injected, both monoclonal antibodies and trypsin should be immunogenic in the long run. However, oral administration of trypsin entails induction of specific immune tolerance, preventing the generation of neutralizing antibodies. With the increasing need for tolerogenic delivery of therapeutic proteins like monoclonal antibodies, efforts should be made to engineer those molecules to mimic the trick that trypsin employs.

In conclusion, studies such as Dr Gaciong's and Dr Heidland's suggest that enzyme therapy is a promising approach to the treatment of T-cell-dependent diseases in humans. The evaluation of its efficacy awaits randomized, controlled therapeutic trials, which should be facilitated by the fact that the enzymes are already on the market, are relatively inexpensive, and have proven to be virtually free of side-effects.

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The management of the failed renal allograft

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Introduction

Patients returning to dialysis after a failed renal transplantation represent about 10% of the population starting dialysis each year [1]. Although a significantly higher mortality has been described for these patients compared to age- and disease-matched stable dialysis patients [2], the optimal management of the failed allograft has been poorly studied.

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Mortality and morbidity of the allograft nephrectomy

In the pre-cyclosporin era, graft nephrectomy was considered a risky intervention. Mortality was reported to range between 7.3% [3] and 16.3% [4]. A paper from India by Sharma and co-workers even reported a mortality rate as high as 38.7% [5]. At that time only the Maastricht group reported on a mortality of no more than 0.9% [6]. Death was mainly due to septic complications. Severe wound infection was also a frequently reported complication [7].

Since the more widespread use of cyclosporin and the lower doses of steroids, graft nephrectomy has