Enzyme Potentiated Desensitization: Present Views on Mechanisms

Presented by

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ENZYME POTENTIATED DESENSITISATION: PRESENT VIEWS ON MECHANISMS

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OBJECTIVES:

At the end of the presentation the attendee should be able to:

1. Discuss the up-to-date consensus on the mechanisms of immunotherapy.
2. Describe how the mechanisms of immunotherapy apply to EPD.

INTRODUCTION

Enzyme Potentiated Desensitisation (EPD) is a technique of immunotherapy which involves using an ultra low-dose antigen mixed with an enzyme called β-D-glucuronidase (BGLR). This technique was first discovered in the 1960's (McEwen *et al.* 1967) and tested in rats, mice and guinea-pigs. Over the years a number of clinical trials have established its efficacy in treating classical allergic conditions.

The phenomenon was first noticed using mammalian BGLR, but the immunological effect appears to be fairly species non-specific (a molluscan extract is currently used). This is in keeping with the finding that BGLR, along with other hydrolytic enzymes, is highly conserved across many species, and antibodies raised against human BGLR will cross react with molluscan BGLR (Pipe, 1990). It should be noted, however, that only a few different extracts have been tested.

Clinically, the benefit from a dose of EPD arrives about three weeks after the injection. From this it can be surmised that a multiple-stage mechanism is involved: Firstly, the Langerhans' cells of the skin are exposed to the antigen in conjunction with the BGLR. These cells then leave the skin and migrate to the local lymph node, changing into dendritic cells as they go. Finally, the dendritic cells pass a message on to peripheral T-cells in the lymph node, producing some kind of proliferative effect and some phenotypic change which produces either anergy, tolerance or some kind of altered reactivity which reduces the patients' symptoms.

β-GLUCURONIDASE STRUCTURE AND FUNCTION

Although the original desensitising effect was noted in a contaminant of a mammalian extract of hyaluronidase, a molluscan extract was the only one considered "pure" enough for clinical use. In spite of the species difference, the immunological effect appears to be conserved along with the enzymatic activity. This allows for three possibilities:

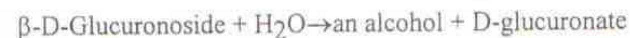
1. The immunological effect is a product of the enzymatic effect.
2. The enzyme is binding to a cellular receptor via the most conserved region of the molecule.
3. The protein structure is highly conserved between species, enough that the immunological effect can remain.

It is already known that BGLR is released by cells which are migrating, especially immunocytes, and it has been used as a marker of immune activation in a variety of studies. In molluscs, where most immunological functions are performed by haemocytes, it has been shown with a range of hydrolytic enzymes, that antibodies raised to human molecules will cross react with the molluscan homologue (Pipe, 1990) including BGLR.

There are two candidate sites that are likely to be most conserved across species, which are the enzymatic cleft and a possible lysosomal targeting motif (Jain *et al.* 1996). Potentially, either one could be involved in cross species reactivity, but in addition, there is high homology between BGLR from all species tested so far including *Escherichia coli*.

Human BGLR is encoded for on chromosome 7 (Speleman *et al.* 1996) and is believed to exist as a homotetramer containing four exposed enzymatic grooves. Each individual subunit of the molecule comprises three separate domains in humans, being a "jelly roll" barrel, a triose phosphate isomerase unit and an immunoglobulin-like domain (Jain *et al.* 1996). It may be speculated that any immunological function of this enzyme may derive from the immunoglobulin domain.

The enzymatic function in acid conditions is to cleave β-glucuronic acid residues from the termini of glycosaminoglycans (GAGs) such as dermatan and keratan sulphates and hyaluronic acid. This action is hydrolytic, the reaction being:



β-D-Glucuronoside + $H_2O \rightarrow$ an alcohol + D-glucuronate

BGLR is normally present in the body, where it is involved in the turnover of the extracellular matrix. In addition to this, a variety of inflammatory cells store the enzyme within lysosomes, from which it is released in large quantities during an inflammatory response. Naive CD4+ T-cells synthesise BGLR *de novo* upon activation, whereas memory T-cells store the enzyme and release it within minutes of antigenic stimulation (Gilat *et al.* 1995).

Although BGLR is an active enzyme at low pH, it has recently been shown that at neutral pH this molecule functions as an adhesion molecule for T-cells, both inducing adhesion to the extracellular matrix (ECM) (Gilat *et al.* 1995) and inducing adhesion to keratinocytes (Hershkoviz *et al.* 1996). It was shown that the presence of the enzyme at a neutral pH induced resting T-cell adhesion, and it was shown that this adhesion was reduced as the pH was lowered. The enzyme has been shown to bind to the ECM and to keratinocyes in a saturable manner.

β-GLUCURONIDASE AND TYPE IV HYPERSENSITIVITY

Although it was first used to treat classical allergies such as hay fever and asthma, anecdotal evidence suggested that a broader spectrum of conditions could be treated in this way. In order to test this hypothesis, a mouse model of chemically-induced (Type 4) contact hypersensitivity was used:

All forms of specific immunity can be divided into three processes which are:-

1. The "cognitive" phase, in which T-cells recognise antigens that have been processed and presented on the surface of antigen presenting cells.
2. The "activation" phase, during which the T-cells proliferate and secrete cytokines.
3. The "effector" phase, where cytotoxic T-cells, antibodies, phagocytic cells, and other effector mechanisms are stimulated by the helper T-cells to remove the pathogen (Abbas, Lichtman and Pober, 1994).

With type I, II or III reactions, various effector functions of antibody molecules cause the pathology either directly (by fixing complement or forming insoluble complexes) or indirectly (by triggering mast cell degranulation). In the case of DTH, the effector phase is largely due to infiltration by inflammatory cells.

It is increasingly apparent that CD4+ T-cell responses, particularly in mice, are polarised into those that produce predominantly interleukin-2 (IL-2) and interferon γ (IFN-γ), called T-helper 1 (T_H1) cells and those that predominantly produce IL-4, IL-5 and IL-10, which are termed T_H2 cells (Mossmann and Sad, 1996). Many different types of immune response have been characterised by the cytokine profiles which can be seen, and it has been confirmed that DTH requires a predominantly T_H1 response (Riemann *et al.* 1996), whilst allergy depends upon a T_H2 response.

There is some evidence that the balance of these cell types is in part controlled by the differential expression of two costimulatory molecules B7.1 (CD80) and B7.2 (CD86) on antigen presenting cells (Kuchroo *et al.* 1995), and in part by cytokines from one cell type inhibiting the other cell type: IL-10 has been shown to inhibit IFN-γ production and IFN-γ has been shown to inhibit IL-4 production (Powrie and Coffman 1993).

An experimental project has used two models of delayed type hypersensitivity, namely the human peripheral blood mononuclear cell (PBMC) response to tuberculin purified protein derivative (PPD), and experimental contact hypersensitivity to 2,4-

dinitrofluorobenzene (DNFB) in mice, to explore some of the processes surrounding EPD.

EXPERIMENTAL MOUSE MODEL

Mice were sensitised to 2,4-dinitrofluorobenzene (DNFB) using the technique described by Phanuphak *et al.* in 1974. The level of sensitivity of these mice was measured by challenging one ear with DNFB and measuring the increase in thickness of that ear compared to the unchallenged control ear over the next 2 days (fig. 1). The mice were then allowed to completely recover from their earlier reaction before being treated using EPD. Three weeks after treatment these mice were challenged again and their responses measured. The response of the treated group vs. untreated controls was abrogated by about 50% ($p=0.01$) (fig. 2).

Figure 1. The effect of antigen challenge on the ear thickness of Balb/c mice. The results are presented as mean increase in ear thickness (mm).

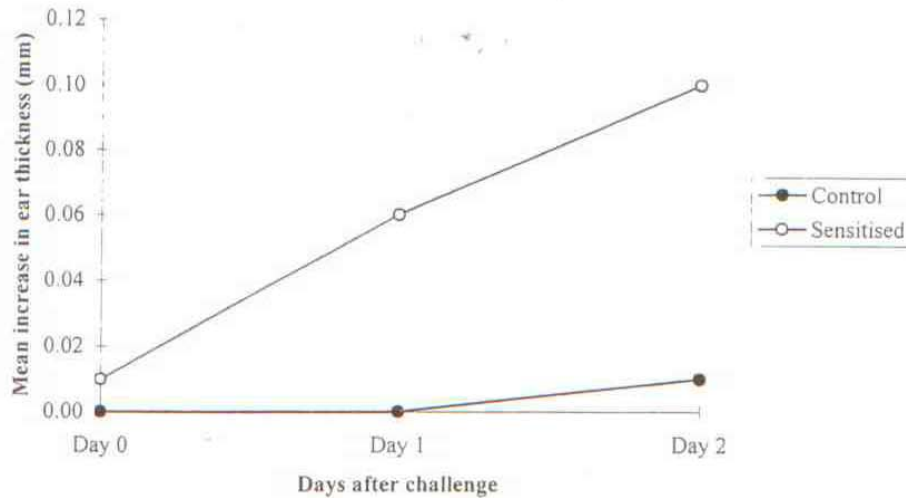
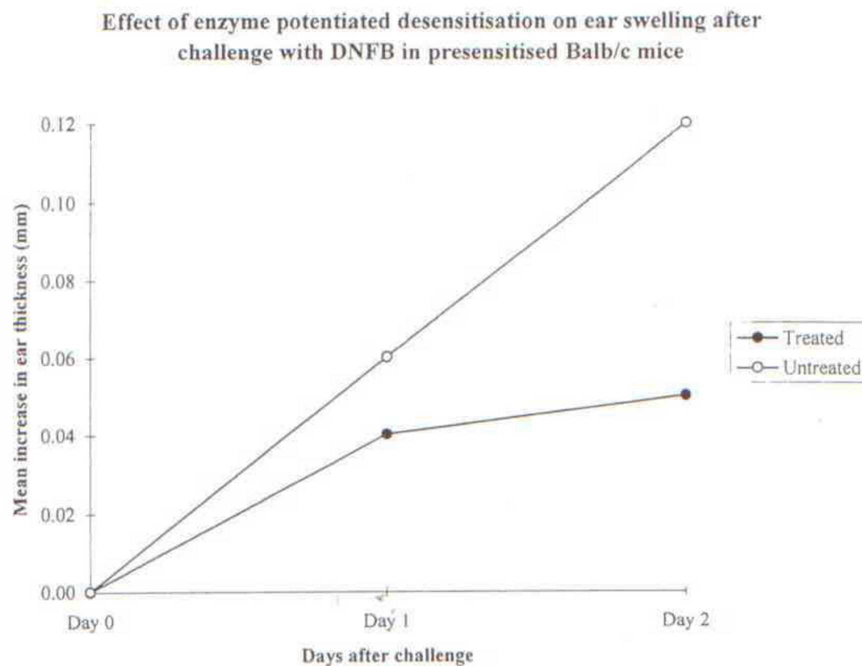


Figure 2. Effect of EPD on subsequent challenge with DNFB of presensitised mice.



HUMAN BLOOD CELL MODEL

In this series of experiments, PPD was chosen as the model antigen because the magnitude of the response is easily measured *in vitro* in terms of proliferation, most of the population responds well due to vaccination and human PBMCs tend to respond within a narrow range of doses. Individuals were bled by venesection and the PBMCs isolated by density gradient centrifugation.

BGLR added to PBMC cultures caused non-specific but weak proliferation after six days in a dose-dependent fashion. The addition of sub-stimulatory amounts of a specific antigen (Tuberculin purified protein derivative (PPD)) had no effect on this response. This indicates that the enzyme acts as a weak mitogen for T-cells (fig. 3.).

When cells were cultured with BGLR for six days and then stimulated for a further six days with stimulatory doses of PPD, proliferation in response to the PPD was inhibited in the same dose-dependent fashion. Again, the addition in the initial incubation period of sub-stimulatory doses of PPD did not significantly alter this effect (fig. 4.).

Figure 3. Responses of PBMCs to incubation with BGLR for six days. At day 5 the cells were pulsed with ^{3}H-thymidine and the cells were harvested onto fibreglass mats on day 6. Incorporation of the ^{3}H-thymidine as measured by radioscintillation is proportional to proliferation. The results are expressed as mean counts per minute (cpm).

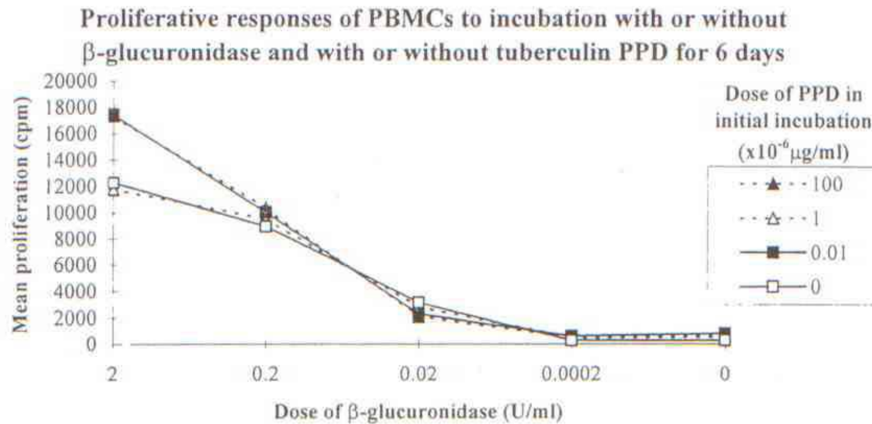
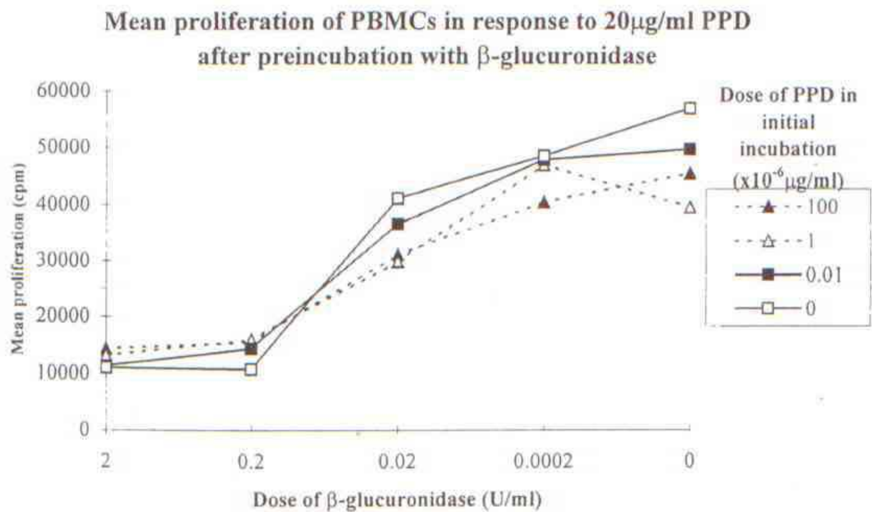


Figure 4. PBMCs were incubated with varying doses of BGLR and PPD. After six days, all of the cells were stimulated with 20μg/ml PPD and incubated for a further 6 days. On day 11 the cells were pulsed with ^{3}H-thymidine and they were harvested on day 12. The results are expressed as mean cpm.



Cultures treated in the same way were analysed by flow cytometry for a variety of markers, but no significant changes attributable to the enzyme treatment were seen with CD3, CD4, CD8 or T-cell receptor γδ. Staining of these samples at day 12 for the interleukin-2 receptor (IL-2R) showed reduced levels for those samples which had been treated with BGLR for the first six days (data not shown). This may or may not be important, because these were the cells that were not proliferating in response to PPD.

and the levels of IL-2R, an activation marker, may simply reflect the low level of activation of these cells rather than being the cause of it. Cells stained for HLA-DR also showed reduced levels in those cells stimulated by BGLR at day twelve (data not shown), but this could be attributable to the same cause.

To further test the reactivity of cells incubated with BGLR, T-cells were incubated with BGLR and then stimulated with freshly harvested antigen presenting cells (APCs) and PHA (a potent T-cell mitogen) for three days. Although this assay was performed only once and cannot therefore be considered statistically significant, the cells incubated with BGLR responded with about twice the proliferative response of untreated control cells (fig. 5).

This result was echoed when cells incubated with BGLR were stimulated with PPD and freshly harvested APCs for six days ($p=0.05$): In this case, cells incubated with BGLR were able to proliferate in response to fresh APCs in the absence of any added antigen (an autologous mixed lymphocyte reaction) (fig. 6). This response indicates that the cells have become hyper-responsive to stimuli.

Figure 5. Responses of purified T-cells to PHA in the presence of freshly purified APCs as measured by ^{3}H-thymidine incorporation and expressed in cpm.

Response of purified T-cells to PHA and fresh antigen presenting cells after preincubation of PBMCs with 2U/ml β-glucuronidase (BGLR) and/or 10^{-8} μg/ml tuberculin PPD for 6 days

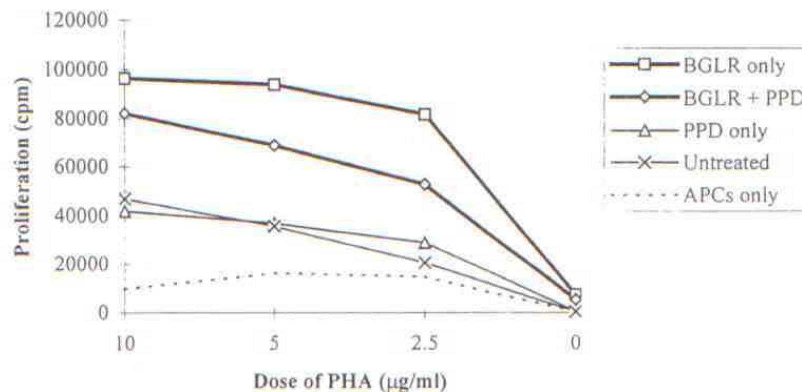
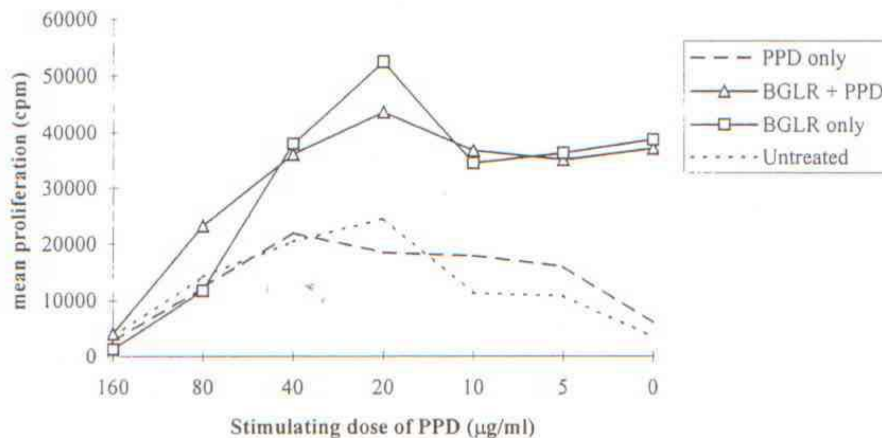


Figure 6. Responses of T-cells which have been incubated with either 10^{-8}µg/ml PPD, 2U/ml BGLR only, 10^{-8}µg/ml PPD and 2U/ml BGLR and PPD or medium alone for six days prior to purification and stimulation with freshly isolated and purified APCs and PPD at a dose of 20µg/ml as measured by ^{3}H-thymidine incorporation. The results are expressed as mean cpm.



DISCUSSION:

Animal models and human studies have shown that EPD can produce clinical hyposensitisation *in vivo* using a type I response (McEwen *et al.* 1973), but no research has been performed on type IV responses *in vivo*, and no *in vitro* studies have been reported with any model system. This series of experiments set out to start the process of dissecting the mechanism of EPD.

The mouse model system shows a clear reduction in the level of sensitivity to DNFB (figure 2.). This is the first time that EPD has been shown to work *in vivo* in an animal model of a type IV response. Although the model worked well, no phenotypic studies were made of the infiltrating cells or their cytokine profile. Because EPD has now been shown to work on both type I and type IV reactions, it seems unlikely that it causes a switch from T_H1 to T_H2 cells or *vice versa*, a mechanism which reduces T-cell responses is more likely. A treatment which induces a change from T_H2-like responses towards T_H1-like responses would not be expected to reduce ear swelling in this model (Kondo *et al.* 1994).

The PBMC model shows that BGLR is a weak mitogen, and the antigen non-specific suppression of responses to a recall antigen could be interpreted as some kind of immune

exhaustion. The data using freshly isolated APCs indicates, however, that the T-cells are far from exhausted, but are hyper-responsive to stimuli (such as autologous cells without extraneous antigen) which would not normally be expected to cause proliferation.

Other mechanisms of regulating immune reactivity have been proposed, and these need to be examined individually to see how they fit into the evidence:

Much recent work has focused on the mechanisms involved in inducing anergy (a profound state of nonresponsiveness) in T-cells. It has been discovered that two molecules in particular called B7.1 and B7.2, which are present on certain APCs, need to bind to a ligand on T-cells called CD28 in order to achieve activation of naive T-cells. Should they bind to another ligand called cytotoxic T-lymphocyte antigen-4 (CTLA-4) anergy is induced. This costimulation can be blocked by antibodies or by a chimeric fusion molecule known as CTLA-4-Ig (which contains CTLA-4 linked to an immunoglobulin Fc portion), and this has been shown to lead to tolerance *in vivo* (Tang *et al.* 1996). This phenomenon has also been seen when the APC lacks expression of costimulatory molecules, for example when using T-cells as APCs (Sidhu *et al.* 1992), or using APC "constructs" (i.e. transfected cell lines) which lack B7 (Gimmi *et al.* 1993). Although B7.1 and B7.2 levels have not been tested on APCs treated with BGLR, there is an indication that this might be the mechanism of action, because the depressed responses seen in figure 4 were reversed when fresh APCs were added.

It may be possible that BGLR is altering the peptides which are presented by APCs. An anergic state can be induced in T-cell clones by so-called "antagonist" peptides: In these cases, a peptide antigen for a specific T-cell clone is mutated in one amino acid. This produces a partial recognition event that fails to induce T-cell activation, even in the presence of costimulation, and instead leads to anergy (Adorini *et al.* 1993, Klenerman *et al.* 1994). However, this does not seem to fit with the clinical evidence and the proliferative responses seen in this study.

Another possible mechanism for EPD is the induction of IL-10 production. This has been identified as a way of reducing T-cell activity through a variety of means. IL-10 is known to reduce the level of expression of HLA-DR on APCs (which might explain the flow cytometry data) but it is also known to increase the level of HLA-DR on other cell types (which seems to contradict the flow cytometry data) (Chadban *et al.* 1998). IL-10 has been shown to act by causing APCs to reduce the level of T-cell responsiveness, but the hyper-responsiveness which was witnessed when the T-cells were stimulated with freshly isolated APCs has not been noted as an effect of IL-10 stimulation. In addition, although IL-10 is known to depress T-cell proliferation, it is also known to upregulate B-cell proliferation (Manickan *et al.* 1998). At least one clinical study has provided evidence that IL-10 levels do increase following EPD treatment (Businco *et al.* 1996).

Further to this, there is some evidence that the expression of IL-10 and the suppression of B7 molecules may be related to one another (Ozawa *et al.* 1996, Dummer *et al.* 1998, Yokote *et al.* 1998). IL-10 has been shown to downregulate B7.1 on Langerhans cells,

and this reduction in costimulatory molecule expression may result in a tolerising signal to T-cells in the periphery.

EPD is an immunologically interesting form of treatment which seems to provide evidence supporting a variety of different theories regarding immunological tolerance. Although it has been used clinically for more than 30 years, its mechanism of action remains largely unexplored and unexplained. The ability to manipulate immune responses using this technique may shed light on many aspects of the immune system in the future.

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