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Enzyme Potentiated Hyposensitization III

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Control by sugars and diols of the immunological effect of Beta glucuronidase in mice and patients with hay fever

Abstract: The ability of Beta glucuronidase and a small dose of antigen to modify the anaphylactic reaction of previously sensitized mice has been further investigated. A 1, 3 diol structure appears to be optimal to control the effect of the enzyme. The dose response curve for the diol is W-shaped. A clinical trial on hay fever patients confirms that the results for the diol in mice are clinically relevant.

Introduction

The first report that hyposensitization of allergic patients could be potentiated by Beta glucuronidase was published in 1967.

The first paper in the present series showed that Beta glucuronidase administered with a small dose of antigen to sensitized animals was able to reduce their anaphylactic responsiveness to a subsequent challenge. The second publication reported that hyposensitizing of the enzyme in mice was influenced by glucose and could be blocked by gelatin.

This paper gives an account of experiments in mice aimed at identifying the chemical structure which controls the immunological behaviour of the Beta glucuronidase. A double blind trial in patients with hay fever suggests that the work in mice is clinically relevant.

A summary of part of this work has already been published.

Method

The methods used for sensitizing, treating and challenging the mice have been described in the two preceding papers.

As in the work described in the last paper, the Beta glucuronidase was passed through a Bio Gel P6 column, sterilized by millipore filtration and stored in buffer at a concentration of 5,000 Fishman Units/ml in siliconed tubes at +4°C.

For the second part of this work, in which 1,3 cyclohexane diol at various dilutions was stored for 10 weeks with aliquots of Beta glucuronidase, it was feared that the greater dilutions of diol might be adsorbed onto a sterilizing membrane in significant quantities. Accordingly, a solution of the diol, 1 mg/ml in distilled water, was sterilized by millipore filtration. Buffer for dilution and the sample of enzyme were similarly treated. Thereafter the dilutions were made up and the aliquots dispensed under sterile conditions.

The clinical trial was conducted between January and the end of March. One hundred and seventy-three subjects with hay fever were selected by their histories and confirmatory skin tests. Those who gave histories suggesting that their responses to grass pollen were delayed were excluded.

Patients were treated with 1 ml of buffer

containing 100 Noon units of grass pollen extract, 500 I.U. of hyaluronidase ("Hyalsae" Fisons LTD) and 500 U. of the Beta glucuronidase containing 1,3 cyclohexane diol. The grass pollen extract and hyaluronidase had each been passed through a Bio Gel P6 column to remove contaminants of low molecular weight and were stored separately in sterile buffer at +4°C. The buffer was that used for the work with mice.

A small area of skin on each patient's arm was lightly scarified to remove the cornified layer but not sufficiently deeply to cause capillary bleeding. A plastic "cup" of 1 ml capacity was then strapped over the raw area and filled with the treatment mixture. The "cup" was worn for 24 h and then removed by the patient, who was instructed to wipe the scarified area with clean tissue and then to expose it until it became dry. This technique for the administration of enzyme/antigen mixtures has now been used more than 10,000 times. The inherent safety of the method makes it preferable to the injection of antigens which might provoke anaphylaxis. If the "cups" are reused, as they were in this work, rigorous precautions must be taken to prevent transmission of serum hepatitis virus.

Because 10 different doses of 1,3 cyclohexane diol were to be tested, it was impractical to randomize the treatment completely. Accordingly, at each session three or four different doses were randomized among the subjects, depending on the numbers available. So far as was possible treatments which were expected to give contrasting effects were used at each sessions.

Saline control treatments were included at four separate treatment session.

Three weeks after treatment the patients were challenged by instillation into one nostril of 0.15 ml containing 750 Noon units of grass pollen extract. Thirty minutes later each patient was asked to compare his reaction with the nasal symptoms he had experienced during the summer. At this time neither the patients nor the observer knew which treatment each subject had received. The patients' subjective assessment was scored as follows:

1 - "Very slight"
2 - "Moderate"
3 - "Equal to severe hay fever"
4 - "Worse"

At a subsequent visit each patient was given a further treatment with a formulation which was known to hyposensitize to grass pollen in order to give protection during the summer.

Results

The preceding paper showed a graph of the changes in the anaphylactic responsiveness of groups of mice caused by adding different doses of glucose to the Beta glucuronidase/antigen mixture with which the previously sensitized animals were treated one week before challenge. Very similar dose-response curves were obtained using other substances with chemical structures based on the pyranose ring.

This work has now been extended to cover a wider range of concentrations of the activating substances and it has become apparent that the dose-response curves are usually W-shaped, not V-shaped. Figure 1 shows these results for glucosamine, N-acetyl-glucosamine and galactosamine. Figure 2 shows that ascorbic acid and a polysaccharide, heparin, may have similar effects and comparable results have been obtained with a wide variety of substances.

Figure 3 shows that diols can control the immunological effect of the Beta glucuronidase in the same way of the more complicated substances above. Propylene glycol is effective but propane and butane 1,3 diols are affective at lower concentrations. When this series is extended it is found that pentane 1,5 diol has no effect. Cyclohexane 1,2 diol, 1,3 diol and 1,4 diol were also investigated and again the greatest activity was shown by the 1,3 diol.

When the work with diols was repeated on further groups of mice it was found that the extent of the changes produced

by 1,3 propane and butane diols varied from experiment to experiment. Cyclohexane 1,3 diol appeared to be more reliable and was chosen for the next stage of the work.

Figure 4 shows the effect in mice of samples of Beta glucuronidase which had been stored for 10 weeks at +4°C in the presence of different doses of 1,3 cyclohexane diol. The scale indicates the concentrations of diol present in the stored Beta glucuronidase and each mouse will have received 1/500th of this dose. It is evident that the diol/enzyme mixture retains its immunological effect over a long period of time. Further, there is no progressive change in the critical doses of diol during prolonged storage.

Also in figure 4 in a graph showing the percentage of groups of hay fever patients who suffered moderate or worse symptoms when they were challenged with an intranasal dose of 750 Noon units of grass pollen extract. Three weeks prior to challenge each group of patients had been treated with grass pollen extract, hyaluronidase and further aliquots of the same 1,3 cyclohexane diol/Beta glucuronidase mixtures. The dose of the enzyme/diol mixture used clinically is 50 times greater than the appropriate dose for a mouse.

It can be seen that the dose-response curve for the diol in the treatment of allergic patients appears to be W-shaped also. Since the method of scoring is arbitrary, a quantal assessment is better for statistical purposes: six out of seven patients in the group treated with enzyme which had been stored with diol 10-9 gm/ml suffered a reaction to challenge which was as bad as a severe attack of hay fever. Only two in 18 saline-treated patients reacted similarly. This result is higly significant ($p=<.001$). The groups of patients represented by the two "throughs" of the "W" had mean symptom scores and quantal responses which were lower than the control values and appear to represent trends towards hyposensitization, but neither result reached statistical significance.

Discussion

These results confirm and extend the work reported in the first two papers in this series. The last publication showed that if mice, previously sensitized to horse serum, were treated with a standard mixture of antigen and Beta glucuronidase, and one week later were challenged by pinnal anaphylaxis, then the response to the challenge would be reduced if glucose 10-7 gm/ml had been added to the treatment mixture.

A higher or lower dose of glucose was ineffective. Further, it was shown that glucosamine, N acetyl glucosamine, N acetyl-galactosamine and N-acetyl neuraminic acid all behaved in a similar way.

It has now been shown that if a wider range of doses of these substances is investigated, not one but two critical concentrations will be found which will cause the enzyme/antigen mixture to hyposensitize the mice. An intervening dose is ineffective or may induce hypersensitization, so the full dose-response curve is W-shaped. This unusual curve might be explained in terms of progressive activation of the enzyme/antigen stimulus to some antigen-sensitive target cell by the changing dose of "sugar". In the well-studied "In vitro" system of antigen stimulated histamine release from sensitized human leukocytes it has been shown that with increasing doses of antigen a critical level is reached at which maximum histamine release occurs. As the dose of antigen is increased further histamine release is reduced and a point of "high dose paralysis" is reached at which antigen releases very little histamine. By analogy it is possible to postulate that with high doses of "sugar" in the enzyme/antigen mixture a potent stimulus to the target cell results in "high dose paralysis" of the immune response to the antigen. As the dose of "sugar" is reduced the enzyme/stimulus to the target cell produces more response until at the optimum dose the cells may be disorganized as a result of their excessive response thus accounting for a

second phase of hyposensitization. Experiments to test this hypothesis will be undertaken when the necessary work with direct clinical application has benn completed.

The investigation designed to reveal the chemical structure critical for the control of the immunological effect of the enzyme/antigen mixture led from glucose to a simple 1,3 diol. In this paper some significant intermediate results are also reported.

The molecule to which the hydroxyls are attached seems to be remarkably unimportant, since propane and heparin are comparable in the role of carrier for the critical grouping. 1,2 diols also have some effect and the clue to the possibility that a pair of hydroxyls was all that was required stemmed from the observation that the substituted pyranoses behaved similarly to glucose. In the series tested only the hydroxyls on carbons 3 and 4 were left unchanged. As pointed out in the last paper, cis-trans-isomerism on carbons 3 and 4 makes no difference in the present system.

This surprising degree of non-specificity for the molecule of the potentiating agent, provided it carries the relevant grouping, is parallelled by the wide range of alcohols and diamines which Fishman and his collaborators have found to act as potentiating agents for the enzyme's action on phenolphtalin glucuronide: Molecules as dissimilar as gelatin and propylene glycol are both effective in the latter role.

Many alcohols can also act as glucuronide acceptor, and propylene glycol is active in both these respects, but propane and butane 1,3 diols are almost inactive. On the other hand the 1,3 diols are more potent than propylene glycol in the control of immunological effect of the enzyme. The present evidence suggests that the immunological role of the enzyme does not involve its ability to split glucuronides.

Until the discovery of the way in which the immunological activity of the enzyme could be controlled the use of highly purified enzyme in this research was bound to yield meaningless negative results. At the present time a reinvestigation of this subject is called for.

The second part of the work reported here is important in several ways. First, it has been shown that enzyme and diol can be dispensed and stored for 10 weeks without change of their immunological effect. This means that it will be possible to make and distribute suitably treated enzyme for widespread clinical use.

Secondly, the dose-response curve obtained with the hay fever patients suggests that the animal work reported in this series of papers has direct clinical relevance. The striking feature of the result of the clinical trial is the hypersensitization which was produced in the middle of the dose-range of the 1,3 cyclohexane diol. This is in marked contrast to the effects of doses of diol on either side, which appear to have induced trends towards hyposensitization.

Six out of seven patients who had been given enzyme stored with 1,3 cyclohexane diol 10^{-9} gm/ml reacted violently to the challenging dose of intranasal grass pollen extract. Four patients in the "10^{-7}" group reacted with equal strength. The groups treated with enzyme plus diol 10^{-6}, 10^{-7}, 10^{-9} and 10^{-11} gm/ml were treated alternately at the same session, doses being made from the same stocks of buffer, hyaluronidase and grass pollen extract. They were subsequently challenged as a single group. At the time of challenge neither the patients nor the observer knew the precise treatment which each had received.

In view of the statistical significance of the result achieved at this session it was not considered justifiable to increase the number of patients subjected to hypersensitization followed by challenge and the enzyme stored with diol 10^{-8} gm/ml was never tested. In a future trial it is intended to confirm these results using a smaller challenging dose of polen extract.

The groups of patients treated with Beta glucuronidase which had been stored with 1,3 cyclohexane diol 10^{-5} and 10^{-10}

gm/ml had less severe symptoms than the control group when they were challenged with intranasal grass pollen extract but the differences were not statistically significant in spite of the trends which are apparent in Figure 4. This experiment was the first clinical trial of a new activating system and it is to be expected that the doses of enzyme

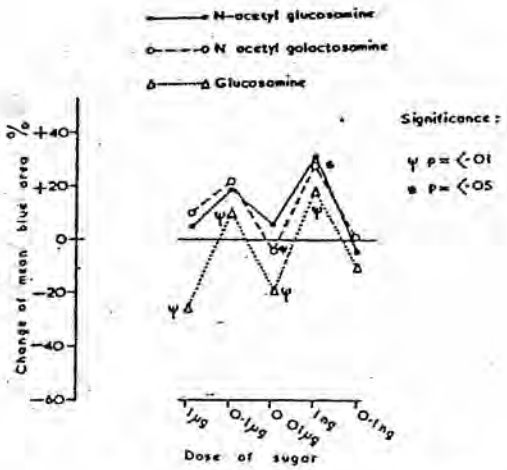

Fig. 1. Mouse pinnal anaphylaxis: groups of seven mice sensitized with 250 µg horse serum. Treated after three wocks with 1 µg horse serum + 10 U β-glucuronidase + various doses of amino-sugars and challenged eight days later. Results expressed as percentage of result after treatment with antigen + enzymes.

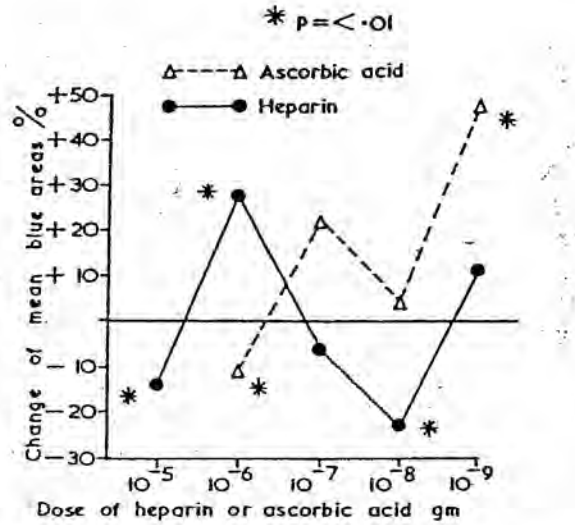

Fig. 2. Mouse pinnal anaphylaxis: groups of seven mice sensitized with 250 µg horse serum. Treated after three wocks with 1 µg horse serum + 10 U β-glucuronidase + various doses of eparin or ascorbic acid and challenged eight days later. Results expressed as percentage of result after treatment with antigen + enzymes.

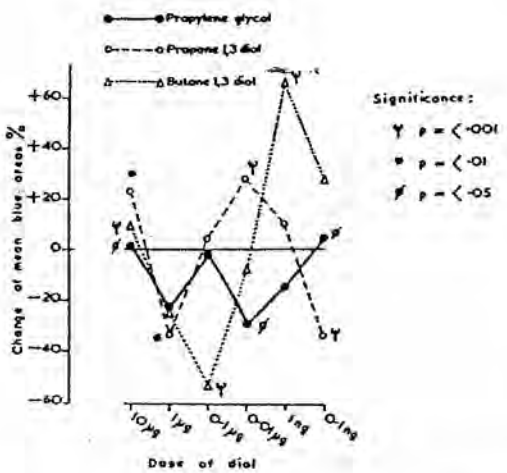

Fig. 3. Mouse pinnal anaphylaxis: Groups of seven mice sensitized with 250 µg horse serum. Treated after three weeks with 1 µg horse serum + 1 OU β-glucuronidase + various doses of diols and challenged eight days later. Results expressed as percentage of result after treatment with antigen + enzyme.

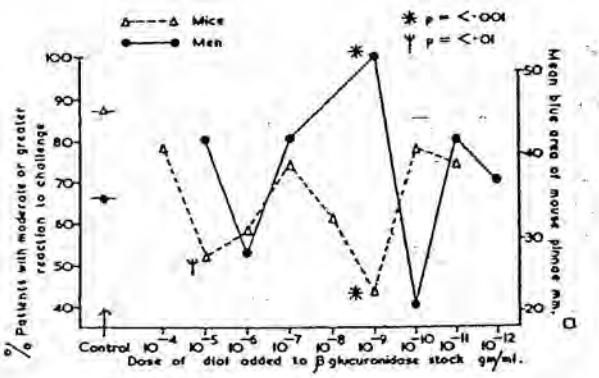

Fig. 4. Effect of different concentrations of 1.3 cycloherans diol on the ability of β glucuronidase + antigen to after the response of hay fever patients to intranasal challenge with grass polten extract three wiiks later and to alter the response of mice previously sensitized with horse serum to challenge by pinnal anaphylaxis one week later.

and antigen will now have to be altered to arrive at the best hyposensitizing formulation for clinical use.

A previous blind clinical trial of this treatment, using an early formulation and the same method of challenge, was carried out in 1967. Patients who received active treatment reacted to challenge less than those who received placebo and the result was statistically significant ($p = <0.02$). Since at that time it was impossible to account for the variable effect of different samples of Beta glucuronidase, the result was not published.

Although several patients were hypersensitized to grass pollen, we have no qualms about the morality of the present trial: a similar trial with a different formulation of Beta glucuronidase was carried out on 120 patients in 1971. Many patients were hypersensitized. A single treatment with a hyposensitizing formulation (at an earlier stage of development than at present) was given a few weeks after challenge and this proved sufficient to reverse the effects of the trial. When the summer came patients who had been hypersensitized to grass pollen during the trial fared no worse than those who had only been members of control groups.

At the time of writing these patients have been followed through a second summer, again preceded by a single hyposensitizing treatment. At follow up, more than 75% of the patients said their hay fever was more than 50% improved and 50% said it was more than 75% improved. This compares most favorably with the results of alternative methods of treatment.

It should be noted that the hypersensitizing dose of pollen extract and enzyme, which the patients in the 10^{-9} group received, produced no side-effects. It was not until the subjects were challenged that their increased sensitivity to antigen was revealed.

There is, therefore, reason to suppose that the hypersensitizing effect of the enzyme may also be exploited clinically. The possibility of using this technique as part of cancer immunotherapy is being considered.

Summary

Mice previously sensitized to horse serum were treated with a mixture of Beta glucuronidase and a small dose of horse serum. One week later the animals were challenged by pinnal anaphylaxis.

The changes in anaphylactic responsiveness produced by the treatment with antigen and enzyme could be controlled by the addition to the mixture of small doses of various pyranose derivatives, ascorbic acid, heparin or a number of diols. Among the compounds tested a 1,3 diol grouping appeared to confer the greatest activity.

The dose-response curve for these substances was W-shaped; two doses induced hyposensitization while an intervening dose caused hypersensitization. Using aliquots of Beta glucuronidase stored with 1,3 cyclohexane diol, it was shown that the diol's effect was not lost after storage for 10 weeks at +4°C.

Patients sensitive to grass pollen were treated with a mixture of grass pollen extract, hyaluronidase and further aliquots of Beta glucuronidase stored with differing doses of 1,3 cyclohexane diol.

Subsequently they were challenged with intranasal grass pollen extract. The responses to challenge of groups of subjects treated with different doses of diol parallelled the W-shaped dose-response curve from the mouse experiment; patients treated with Beta glucoronidase stored with 10^{-9} gm/ml of the diol were significantly hypersensitized.

Patients treated with Beta glucuronidase which had been stored with 10^{-6} and 10^{-10} gm/ml of the diol reacted to challenge less than the saline-treated controls but the results did not reach statistical significance.

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