

*The ability of  $\beta$  glucuronidase and a small dose of antigen to modify the anaphylactic reaction of previously sensitized mice has been further investigated. Protamine has an important effect on the immunological behavior of the enzyme. A trial on hay fever patients shows that the results in mice are relevant and that the method can produce significant clinical hyposensitization.*

## ENZYME POTENTIATED HYPOSENSITIZATION: IV. EFFECT OF PROTAMINE ON THE IMMUNOLOGICAL BEHAVIOR OF $\beta$ GLUCURONIDASE IN MICE AND PATIENTS WITH HAY FEVER

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### Introduction

THE FIRST THREE papers in this series<sup>1,2,3</sup> have shown that  $\beta$  glucuronidase, administered to sensitized animals or allergic patients with a small dose of specific antigen, is able to alter their responsiveness to subsequent challenge with the antigen.

It has been found that the immunological effect of the enzyme, which can promote hyposensitization or hypersensitization, is controlled by the presence of substances with a 1,3 diol grouping in their chemical structures.

The work reported in this paper shows that protamine can also alter the immunological effect of the  $\beta$  glucuronidase in mice.

Enzyme potentiated treatment of hay fever patients resulted in significant hyposensitization only if a certain minimum dose of protamine was added to the formulation.

### Method

Protamine was obtained from Boots Limited as a 1% aqueous solution without preservative for clinical use. For work with mice, other materials and buffer solutions remain as in the previously published papers.<sup>1,2</sup> The dilutions of 1,3, cyclohexane diol and protamine required for this work are difficult to reproduce because surface adsorption is likely to introduce errors. Glassware treated with silicone "Repelcote" (Hopkins & Williams Limited) is used at all times. This does not reduce adsorption but is likely to make it more constant than if raw glass surfaces are used. The Oxford Laboratories "Ultra Micro Sampler System" (Boehringer Corp.) is used for making dilutions. Protamine is supplied as a 1% solution. Direct transfer of 1  $\mu$ l. to a 10 ml. sample of stock  $\beta$  glucuronidase solution (4000  $\mu$ /ml) achieves a dilution of 1  $\mu$ g/ml. High

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concentrations of protamine in  $\beta$  glucuronidase develop cloudiness and must be avoided. 1,3 cyclohexane diol is weighed as a solid and dissolved in the buffer as a 1% solution ( $10^{-2}$ ). To this is added an equal volume of  $\beta$  glucuronidase stock solution ( $=5 \times 10^{-3}$ ). Now  $1 \mu$ l is transferred to 5 ml of enzyme stock ( $=10^{-6}$ ). This solution of diol, in which enzyme at stock concentration is present to minimize adsorption, is used for making further dilutions.  $1 \mu$ l transferred to 10 ml. of enzyme stock gives  $10^{-10}$  of diol. If it is necessary to make even greater dilutions, the  $10^{-10}$  stock is used for this purpose.

A further precaution is taken against the effects of adsorption when mice are being treated with enzyme and antigen in high dilutions. Dummy samples of antigen and antigen + enzyme are made in parallel with those to be injected and are used in an attempt to saturate the surfaces of syringes and needles before use.

Sterilization of the stock samples of  $\beta$  glucuronidase is now carried out after addition of diol and protamine using "Millex" sterile disposable units, pore size  $0.22 \mu$ m. (Millipore Ltd.). Sample volume is never less than 10 ml. Storage (at  $+4^{\circ}\text{C}$ ) is in sterile, siliconed, 10-ml capacity, screw-topped tubes. Minimum sample volume 2.5 ml. All samples are stored for a minimum of 10 weeks before use. Selection of hay fever patients was carried out using the same criteria as in the preceding work.<sup>3</sup> Treatment was with a formulation similar to the standard hyposensitizing mixture used in the routine clinic:

Buffer 1 ml.

+ Hyaluronidase 1500 u (Fisons Ltd.)

+ 100 Noon units grass pollen extract (as in previous paper)

+ 0.12 mg. chondroitin sulphate (ex

Shark cartilage Type C. Sigma Chemical Co.)

+  $\beta$  glucuronidase 400 u with added protamine and 1,3 cyclohexane diol.

Challenge of hay fever patients was carried out with 0.15 ml. of a solution of cocksfoot pollen extract 700 u/ml instilled into one nostril. (In previous work this solution was 500 u/ml). The subsequent assessment of symptoms was carried out as before.<sup>3</sup>

### Results

Preceding papers<sup>1,2,3</sup> have shown that when mice, sensitized to horse serum four weeks previously, are challenged by pinnal anaphylaxis, their reaction to the challenge can be altered by a treatment dose of antigen and  $\beta$  glucuronidase given one week before the test. In this experiment the presence of 1,3 cyclohexane diol in the sample of  $\beta$  glucuronidase controls the immunological effect of the treatment. Hyposensitization and hypersensitization are produced by different doses of the diol and over a very wide range of doses the dose-response curve is W-shaped.

When protamine 1.1 g/ml is also added to the stock solution of  $\beta$  glucuronidase, the immunological effect of changing the dose of 1,3 cyclohexane diol remains the same but the dose-response curve is displaced; 100-fold less diol is required. In Figure 1 mouse pinnal anaphylaxis has been used to show the effect of different concentrations of 1,3 cyclohexane diol added to a standard treatment dose of  $\beta$  glucuronidase, protamine and horse serum. This dose-response curve has been compared with the corresponding curve (taken from Figure 3 of the preceding paper), which was obtained when protamine had not been used in the formulation.

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Since less 1,3 cyclohexane diol is required for activation in the presence of protamine, it appears that the latter behaves as an activator of the hyposensitizing formulation but it

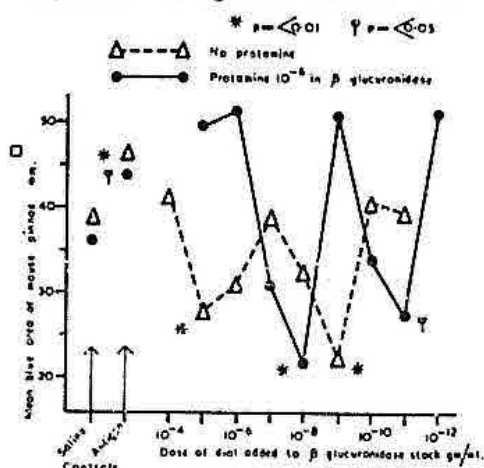


Figure 1. Mouse pinnal anaphylaxis: groups of seven mice sensitized with 250  $\mu$ g horse serum. Treated after three weeks with 1  $\mu$ g horse serum + three 10  $\mu$ g  $\beta$  glucuronidase which had been stored with various doses of 1,3 cyclohexane diol. Mice challenged eight days later. Effect of adding protamine to stored enzyme.

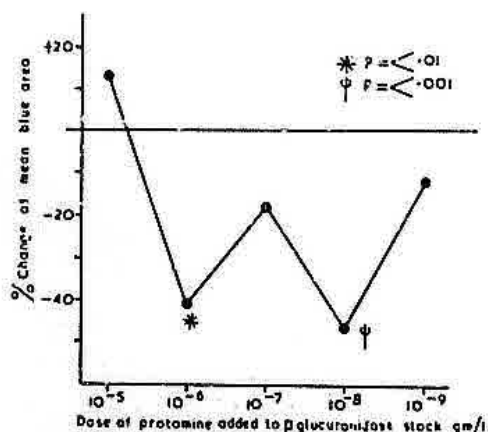


Figure 2. Mouse pinnal anaphylaxis: groups of 10 mice sensitized with 250  $\mu$ g horse serum. Treated after three weeks with 1  $\mu$ g horse serum + 10  $\mu$ g  $\beta$  glucuronidase + 0.1  $\mu$ g 1,3 cyclohexane diol + various doses of protamine. Challenged eight days later. Results expressed as percent of saline treated control.

seems likely that there will also be association between part of the dose of 1,3 cyclohexane diol and the protamine. If this were so it would be expected that in the presence of a standard dose of the diol, changing the dose of protamine would generate a W-shaped dose response curve. Figure 2 shows that this prediction is correct.

Table I shows the effect of intranasal provocation tests with grass pollen extract carried out "blind" on groups of grass pollen sensitive patients who had been treated three weeks earlier with the desensitizing formulation. Different groups were treated with  $\beta$  glucuronidase to which had been added various doses of protamine and 1,3 cyclohexane diol. The percentage of patients who experienced symptoms described as "half" those of real hay fever or less is shown.

Six control patients were treated with saline instead of the enzyme/antigen formulation. The strength of the intranasal challenging dose of grass pollen extract was such that all these reacted with an attack of hay fever nearly equal to the worst they had

TABLE I. EFFECT OF ENZYME-POTENTIATED HYPOSENSITIZATION OF GROUPS OF HAY FEVER PATIENTS USING  $\beta$  GLUCURONIDASE CONTAINING DIFFERENT DOSES OF 1,3, CYCLOHEXANE DIOL AND PROTAMINE. RESULTS AS PERCENTAGE OF GROUPS WITH "HALF" TO NIL SYMPTOMS WHEN CHALLENGED BLIND WITH INTRANASAL GRASS POLLEN EXTRACT 1,000 U.

Protamine 10 <sup>-6</sup>	1,3, Cyclohexane diol 10 <sup>-10</sup>			
	X2	x1	x0.5	x0.1
x2	-	80 <sup>A</sup>	-	-
x1	57 <sup>B</sup>	71 <sup>C</sup>	43	25 <sup>+</sup>
.05	-	14	-	-
x0.1	-	28	-	-

Group of six saline treated control patients 0%

Other groups seven patients, except \*5 and +8

Significance:  $A\chi^2 = 6.9$   $B\chi^2 = 3.8$

$C\chi^2 = 9.4$

ever experienced. Compared with this, the responses to challenge were significantly reduced in three groups of treated patients. It can be seen that certain minimum doses of both diol and protamine are necessary to achieve hyposensitization. Particularly with protamine there was an abrupt loss of effect when the dose was halved.

### Discussion

The ability of a preparation of molluscan  $\beta$  glucuronidase to potentiate the effect of a small dose of antigen, and the control over the immunological outcome which is exercised by substances bearing a 1,3 diol grouping, have been discussed in previous papers.<sup>1,2,3</sup>

During the initial work on sugars reported in papers 2 and 3 successive experiments with mouse pinnal anaphylaxis generated closely similar dose-response curves but there have since been periods when only 50% of mouse experiments have behaved as expected; at other times the dose-response curves would be displaced or distorted in an unpredictable manner. Only part of this variability can be attributed to the influence of adsorption during preparation on the very small doses of the various agents used to treat mice.

Fishman's group has shown<sup>4</sup> that when  $\beta$  glucuronidase is highly purified it loses its enzyme activity. This can be restored by the addition of many substances containing two or more amino groups; 1,10 diamino decane, protamine and gelatin are all effective in very small doses. It was also shown in Paper 2 that gelatin could prevent the hyposensitizing effect in mice of a dose of  $\beta$  glucuronidase and horse serum. It therefore seemed likely that variable protein contamination

of the  $\beta$  glucuronidase and the availability of amino groups in the antigen might seriously affect the performance of the hyposensitizing formulation.

It is obviously impossible to eliminate contaminant protein from the system, so the only way to overcome this difficulty seemed to be to select a substance with a suitable "diamine" effect on the  $\beta$  glucuronidase and, if possible, to find a dose which would be high enough to over-ride the variable effect of contaminants yet would allow the enzyme/diol/antigen formulation to produce hyposensitization. Protamine was chosen for this work because it is possible to obtain preparations which are standardized and suitable for clinical use and also because its structure suggests that it has the best chance of dwarfing the effect of any other amino groups in the formulation. A second possibility is that protamine and diol may associate to some extent, thus providing a "buffer" effect, which might again make the immunological action of a particular formulation more predictable.

The results in this paper show that the premise on which this work started is correct: in order to produce a formulation of  $\beta$  glucuronidase and antigen with a predictable immunological effect it is necessary to standardize the amino groups available in the mixture as well as the dose of diol. Protamine at a concentration of 1  $\mu$ g/ml in the stored  $\beta$  glucuronidase potentiates the immunological effect of the enzyme in the mouse so that lower doses of diol are required for activation.

The W-shaped dose-response curve in Figure 2, produced by changing the dose of protamine in the presence of a standard dose of 1,3 cyclohexane diol, suggests that protamine/diol associa-

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tion is also occurring and the amount of free diol in the formulation is actually less than the dose which was added. It is hoped that this protamine/diol association will, as predicted, produce a "buffer" effect, making it harder for extraneous substances to upset the concentration of free diol in the formulation.

Figure 3 of the preceding paper<sup>3</sup> indicates that mouse pinnal anaphylaxis is a useful experimental model of the changes in clinical hypersensitivity which can be brought about by the enzyme potentiated technique. The figure also shows that unfortunately the mouse model in its present form cannot be used to forecast which dose of diol will be optimal for clinical hyposensitization. It was therefore necessary to conduct a series of clinical experiments on patients who suffered from hay fever, using blind provocation testing by the method also described in the preceding paper.

In this new series of experiments the strength of the challenging intranasal dose of grass pollen extract had been raised. All patients who received treatment with saline instead of the antigen/enzyme formulation reacted to challenge with an attack of hay fever which was nearly as bad, or as bad, as the worst they had ever experienced. For this reason the number of control treated patients was kept to the minimum likely to be necessary to achieve a statistically significant result.

Table I shows the effects of changing the concentration of 1,3 cyclohexane diol and protamine independently in the clinical formulation. Hyposensitization is produced when certain minimum doses of diol and protamine are present. If the concentration of either agent is reduced below these levels hyposensitizing ac-

tivity disappears. The optimal dose-range for 1,3 cyclohexane diol seems to be narrow — a two-fold change in either direction leads to some reduction in effectiveness. Protamine remains fully effective when its concentration is increased two-fold.

The numbers of patients in each of the groups in Table I are not large but in three groups the reactions to intranasal challenge have been reduced to a statistically significant degree by hyposensitizing treatment. The fact that three groups of patients have desensitized, although treated with separately stored samples of  $\beta$  glucuronidase, gives even more weight to the conclusion that these results are not likely to be due to chance. The obvious criticism that the results of this trial can be dismissed because of insufficient numbers is based on clinical tradition but not on logic.

It will be noted that the doses of diol and protamine which must be present in the  $\beta$  glucuronidase if it is to have a hyposensitizing effect are similar, whether the enzyme is to be used in the mouse (Figures 1 and 2) or clinically. In the preceding paper<sup>3</sup> when protamine was not used, the dose of 1,3 cyclohexane diol required for hyposensitization in the mouse model was 10 times higher than the dose required for successful treatment of allergic patients. It seems possible that this divergence was due to the different protein effects of the antigens, which can be rendered unimportant by the addition of protamine.

It is still too early to say whether the mouse model can now be used to forecast the clinical performance of each production batch of  $\beta$ -glucuronidase/protamine/1,3 cyclohexane diol. Work is now directed to this end.

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### 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.

Different doses of protamine altered the immunological effect of the  $\beta$ -glucuronidase.

If protamine was present in the enzyme solution during storage at a concentration of 1  $\mu$ g/ml, the ability of the  $\beta$  glucuronidase to promote hyposensitization was unimpaired and it is suggested that at this dose protamine may be able to minimize the variable effect of contaminant proteins.

In the presence of protamine, 1,3 cyclohexane diol still controls the immunological effect of the enzyme.

Groups of patients sensitive to grass pollen were treated with a mixture of grass pollen extract, hyaluronidase, chondroitin sulphate and aliquots of  $\beta$  glucuronidase which had been stored with different doses of protamine and 1,3 cyclohexane diol.

Subsequently they were challenged with intranasal grass pollen extract.

Three groups of patients reacted to challenge significantly less than saline-treated controls.

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