Immunological Evaluation of Repeated Administration of Erythrocyte-Entrapped Protein to C3H/HeJ Mice*

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The immunogenicity of heterologous protein entrapped in autologous erythrocytes was evaluated in a mouse system. Multiple intravenous administrations of an erythrocyte-entrapped bovine β -glucuronidase preparation resulted in little, if any, immunological response to the entrapped protein or erythrocyte carrier. Erythrocyte entrapment also protected protein from circulating antibody elicited by prior subcutaneous sensitization with unentrapped enzyme in adjuvant. Thus erythrocyte entrapment may protect potentially immunogenic agents from immunological surveillance.

One of the requisites for enzyme therapy is the need to protect the administered enzyme from various mechanisms of bioinactivation, particularly by the immunological system (Boyer *et al.*, 1973; Desnick *et al.*, 1976). The initiation of an immune response after repeated administrations of an enzyme may further endanger the health of the recipient as well as deter the catabolism of pathogenic substrates by the administered enzyme. Although the use of erythrocytes as enzyme delivery vehicles has been encouraging (Thorpe *et al.*, 1975; Ihler *et al.*, 1975), the possible expression of new antigen determinants induced by the lysis and resealing procedure for enzyme entrapment (Cabantchik *et al.*, 1975), as well as the possible leakage of enzyme from the resealed 'ghosts' (Bodemann & Passow, 1972), and/or the adsorption of the molecule being entrapped on the erythrocyte surface, make an immunological assessment of these vesicles imperative despite their autologous origin.

We report here the immunological evaluation of repeated administrations of an erythrocyte-entrapped protein preparation, containing primarily the bovine glycoprotein β -glucuronidase, to C3H/HeJ mice.

Materials and Methods

Purification of bovine liver β -glucuronidase (final specific activity 187 μ mol/h per mg of protein) was carried out by the method previously described (Fiddler & Desnick, 1977) with the addition of Sepharose–concanavalin A (Pharmacia, Piscataway,

NJ, U.S.A.) chromatography immediately before the final anion-exchange step. This preparation was assayed fluorimetrically for other lysosomal glycosidase activities, including α -mannosidase, α -galactosidase, β -glucosidase, β -galactosidase and β -N-acetylhexosaminidase; only the last activity was detected at more than 5% of the β -glucuronidase activity. In addition, two protein-staining bands other than β -glucuronidase were observed by polyacrylamide-gel electrophoresis (Swank & Bailey, 1973); therefore the enzyme preparation is referred to as the 'protein preparation' in the present paper so as not to imply homogeneity of the antigen.

C3H/HeJ *gus*^h (low β -glucuronidase) male mice (6–10 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.). Entrapment of the protein preparation in mouse erythrocytes as well as determination of the tissue uptake and distribution of β -glucuronidase activity after intravenous administration to mice have been detailed elsewhere (Thorpe *et al.*, 1974, 1975). One unit of enzymic activity is 1 nmol of substrate cleaved/h. All protein preparations were filtered through Nuclepore membrane filters (Nuclepore Corp., Pleasanton, CA, U.S.A.) and stored at 4°C under sterile conditions in phosphate-buffered saline (0.02 M-sodium phosphate/0.15 M-NaCl), pH 7.2.

Sensitization of mice to erythrocyte 'ghost' preparations was carried out by intravenous or subcutaneous administration of the protein preparation entrapped in erythrocytes (1000–1500 units in 0.15 ml of erythrocytes) or 'empty' (buffer-loaded) erythrocyte 'ghosts'; sensitizations were carried out six times over 14 days, followed by another injection at 24 days, 24 h before the initiation of a time-course experiment. A low dose of entrapped activity (250 units) was injected on day 24 to contribute negligible

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activity to the time-course experiment. Sensitizations with untrapped protein were carried out with 100 μ g of protein administered subcutaneously in 0.1 ml as a 1:1 (v/v) mixture with incomplete Freund's adjuvant by following the same sensitization schedule.

The time-course experiment was designed as follows: sensitized mice (two to four) were intravenously injected with erythrocyte-entrapped protein preparation (1000–1500 units), killed at three time points (1, 4 and 24 h) and assayed individually for β -glucuronidase activity. The fate of administered enzyme was compared with previous time-course data in unsensitized mice (Thorpe *et al.*, 1975). Statistical analysis utilized the two-sample *t* test and significance levels were accepted at a power function ≥ 0.75 .

Tests for antibody to the protein preparation, protein-loaded mouse erythrocytes and 'empty' mouse erythrocytes were carried out by using pooled serum from the sensitized mice of this inbred strain killed during the time-course experiments. Complement lysis was carried out as follows: the protein preparation and [14 C]inulin (100 mCi/g; New England Nuclear, Boston, MA, U.S.A.) were entrapped in erythrocytes; 200 μ l of the loaded erythrocytes (3900 c.p.m.), 620 μ l of human serum as complement source, 180 μ l of serially diluted de-complemented (56°C, 30 min) mouse serum being tested and 3 ml of 0.15 M-NaCl were incubated at room temperature (24°C) for 30 min. The suspension was centrifuged at 4000g for 20 min at 4°C. The supernatants were counted for radioactivity in a Beckman LS-100 scintillation counter with Aquasol (New England Nuclear) as the scintillation 'cocktail'. Substitution of the appropriate incubation components by de-complemented human serum (620 μ l), normal mouse serum (180 μ l) or 0.9% NaCl (180 μ l) respectively provided control values.

Haemagglutination was done as follows: 25 μ l of a 2% suspension of mouse erythrocytes (intact erythrocytes, protein-loaded 'ghosts' or 'empty' 'ghosts') was incubated with 25 μ l of serially diluted test antisera at room temperature and examined for agglutination after 2.5 h. Serum from unsensitized mice was incubated with the various protein-entrapped erythrocyte preparations, 'empty' erythrocyte 'ghosts' or intact erythrocytes as controls.

For passive haemagglutination, equal volumes of washed sheep erythrocytes, protein and 0.05% CrCl₃ were incubated at room temperature for 5 min and then washed four times with 8–10 vol. of phosphate-buffered saline, pH 7.2. Binding of protein to the sheep erythrocytes, as monitored by β -glucuronidase activity, was maximized by prior manipulations of volume ratios and incubation times. A portion (20 μ l) of a 5% (w/v) suspension of protein-coated sheep erythrocytes in 0.9% NaCl and 20 μ l of serially diluted sera from the sensitized mice or commercial

goat anti- β -glucuronidase (RPI, Elk Grove Village, IL, U.S.A.) were incubated at 25°C for 1 h and scored for agglutination. The commercial anti-enzyme was prepared against rat preputial-gland β -glucuronidase, but was demonstrated to be cross-reactive to the bovine enzyme by Ouchterlony double diffusion in our laboratory.

Ouchterlony double-diffusion plates were utilized to test the various antisera against the protein preparation (Campbell, 1970).

Results and Discussion

An immune response was assessed by the fate *in vivo* of entrapped enzymic activity (time-course experiment) and antibody determinations. To test the capacity of erythrocyte entrapment to prevent an immunological reaction, mice were first intravenously sensitized to protein-loaded and 'empty' autologous erythrocytes, then challenged with erythrocyte-entrapped protein. As a control, the immune competency of the C3H/HeJ mice was assessed by their ability to respond to subcutaneous injections of 'empty' or protein-loaded erythrocytes.

Table 1 shows the results of these time-course experiments. Compared with the unsensitized control, the fate of erythrocyte-entrapped protein as monitored by β -glucuronidase activity after intravenous sensitization by erythrocyte-entrapped protein was essentially unaltered at the three time points indicated. There was a slightly lower activity recovered in hepatic and renal tissues and blood; however, these were within the range observed in previous control time-course experiments (Thorpe *et al.*, 1975) and not significant. The ranges of recovered activity in the sensitized mice varied within $\pm 10\%$ of the mean value. In contrast, the time-course observed after subcutaneous sensitization with erythrocyte-entrapped enzyme was markedly altered, particularly for hepatic and blood recoveries, which were lower at all time points ($P < 0.05$). Similar results were observed when mice were sensitized with 'empty' erythrocyte 'ghosts'. Thus subcutaneous administrations demonstrated that the mice could raise an immune response to the erythrocyte preparations used.

The identical results observed in time-course experiments for intravenously administered protein-loaded erythrocytes and 'empty' erythrocytes suggest that little, if any, enzyme adhered to the surface of the erythrocyte 'ghosts' after entrapment and washing; however, it is possible that extremely low amounts of the protein preparation at the surface could escape detection by the immune system.

Finally, the fate of erythrocyte-entrapped enzyme in mice subcutaneously sensitized with untrapped enzyme is shown in the last portion of Table 1. Before erythrocyte administration, these mice ex-

Table 1. *Time-course study: recovery of intravenously administered erythrocyte-entrapped bovine β -glucuronidase activity in previously sensitized C3H/HeJ mice*

Sensitization of mice was carried out as described in the Materials and Methods section. Recovery values are the means of two to four mice for sensitized mice and four to six mice for the unsensitized controls.

Sensitization	Route for sensitization	Tissue	Mean recovery (% of dose)		
			1 h	4 h	24 h
Unsensitized control	—	Liver	38	72	28
		Kidney	15	0	0
		Spleen	10	7	0
		Lungs	0	0	0
		Blood	28	0	0
Erythrocyte-entrapped enzyme	Intravenous	Liver	34	64	20
		Kidney	7	0	0
		Spleen	9	6	0
		Lungs	0	0	0
		Blood	22	0	0
Erythrocyte-entrapped enzyme	Subcutaneous	Liver	26	42	6
		Kidney	9	0	0
		Spleen	6	6	0
		Lungs	0	0	0
		Blood	8	0	0
'Empty' erythrocyte 'ghosts'	Intravenous	Liver	40	66	20
		Kidney	10	0	0
		Spleen	9	9	0
		Lungs	0	0	0
		Blood	25	0	0
'Empty' erythrocyte 'ghosts'	Subcutaneous	Liver	19	35	9
		Kidney	8	4	0
		Spleen	5	4	0
		Lungs	0	0	0
		Blood	8	0	0
Unentrapped enzyme	Subcutaneous (in Freund's incomplete adjuvant)	Liver	38	64	29
		Kidney	6	2	0
		Spleen	12	11	0
		Lungs	0	0	0
		Blood	20	0	0

Table 2. *Complement lysis tests of sera from C3H/HeJ mice sensitized with autologous erythrocytes with and without entrapped heterologous proteins*

Experimental details are given in the text.

Route of erythrocyte administration	Enzyme entrapped	Serum dilution	[¹⁴ C]Inulin released (%)
Intravenous	+	1:1	34
Control		1:1	33
Subcutaneous		1:1	53
		1:2	52
		1:8	30
		1:64	34
		Control	1:1
Intravenous	—	1:1	32
Control		1:1	33
Subcutaneous	—	1:1	49
		1:8	40
		1:64	34
Control		1:1	33

hibited a demonstrable antibody to the protein preparation by Ouchterlony double diffusion. Compared with unsensitized mice, no significant differences in tissue distribution or retention were observed. This demonstrated the capacity of erythrocyte entrapment for protection of protein from an antibody already present in the circulation. These data also suggest (a) that there is minimal adsorption of enzyme on the erythrocyte surface which could have initiated antibody binding and subsequent disposal through the immunological system, and (b) that there is undetectable leakage of enzyme from the erythrocytes into the circulation. Had there been leakage, a significantly lower recovery of activity should have been observed, particularly at later time points, as the fate of free β -glucuronidase in the circulation would follow the pattern of liver uptake and clearance described previously for unentrapped enzyme (Thorpe *et al.*, 1974).

Table 2 summarizes the results of the complement lysis test. Values are expressed as the means of duplicate determinations on the pooled sera from the sensitized mice, and the ranges were within 2 percentage points of that value. Intravenous administration of either protein-loaded or 'empty' erythrocytes resulted in no detectable complement-dependent antibody. The control values were mean values for the de complemented serum incubation; identical values were observed for the other control incubations described in the Materials and Methods section. However, serum obtained from mice sensitized subcutaneously by loaded or 'empty' erythrocytes induced complement lysis significantly above the control values, again providing a positive control for evaluating the protection offered by erythrocyte entrapment.

When mice were sensitized intravenously with 'empty' or protein-loaded erythrocytes, no antibody was detected by haemagglutination against either enzyme-loaded or 'empty' erythrocyte 'ghosts'. A low titre (1:1), however, was detected against intact mouse erythrocytes when incubated with serum from mice sensitized intravenously with either protein-loaded or 'empty' erythrocytes; this suggests that low titres of antibody may be produced. It is not clear why haemagglutination was observed with intact erythrocytes and not with the erythrocyte 'ghosts' used to sensitize the mice, although it is possible that intact erythrocytes provide more antigenic sites for binding of bivalent antibody(ies) than the 'ghosts' and suggests that the loading procedure does alter the erythrocyte membrane. This result also suggests that clinical trials of enzyme therapy utilizing erythrocyte entrapment should be carefully monitored for antibody production to the patients' erythrocytes as well as to the entrapped preparation. Haemagglutination of 'empty', loaded and intact erythrocytes was detected with sera obtained after subcutaneous sensitization; the low titres observed, 1:1-1:2, were not unexpected, owing to the autologous origin of the erythrocytes and the lack of an adjuvant in the administration protocol. Normal mouse serum resulted in no haemagglutination against 'empty' or loaded erythrocyte 'ghosts' or intact cells.

Repeated intravenous administration of erythrocyte-entrapped protein elicited no detectable anti-protein response by double diffusion or passive haemagglutination. This further indicated that there was undetectable leakage of enzyme from the erythrocyte 'ghosts' after intravenous administration within the sensitivity limits of these tests.

Finally, antibody production to the protein preparation, as determined by double diffusion, was demonstrated only when it was administered subcutaneously in Freund's adjuvant (1:4 titre) or in erythrocytes

(1:1 titre); only one line of precipitation was observed.

The results of these studies suggest that the repeated intravenous administration of erythrocyte-entrapped protein fails to stimulate a detectable immune response to either the entrapped protein or the erythrocyte carrier. Moreover, the capacity of erythrocyte entrapment to protect protein from antibodies already present in the circulation was also demonstrated. It should be noted that these experiments were limited to testing in general the response *in vivo* to repeated erythrocyte administration and that individual variation in immune response as detected by antibody tests was not assessed. However, the results of the antibody determinations on pooled sera were consistent with the data of the time-course experiment, monitored in individual animals.

The procedure of hypo-osmotic exchange used for protein entrapment appears to be satisfactory for both preventing leakage of entrapped molecules and minimizing surface adsorption detectable within the limits of this test system. It is expected, however, that the nature of the enzyme or protein preparation to be entrapped (water-soluble or insoluble), as well as the entrapment method (Updike *et al.*, 1976; Dale & Beutler, 1976; Deloach & Ihler, 1977), may be important factors in the potential immunogenicity of this carrier system.

These studies in a mouse system provide support for the immunological safety of erythrocyte entrapment as a means of administering enzyme or other therapeutic agents for the treatment of storage diseases, clearance of circulating metabolites or delivery of drugs. It is possible that antibody(ies) may be elicited after longer periods of sensitization, owing to alterations of the erythrocyte membrane induced by loading and small amounts of entrapped protein on the surface. Thus caution should be used in extrapolating these results in mice to potential human recipients without further long-term investigations. Studies on the clearance of erythrocyte-entrapped bacterial asparaginase in monkeys (Updike *et al.*, 1976) suggest that further evaluation of erythrocyte-entrapped enzyme in primates may be an appropriate prelude to human trials of enzyme therapy.

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