Antibody-mediated targeting of α -1,4-glucosidase–albumin polymers to rat hepatocytes

A model for enzyme therapy

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Chemically cross-linking α -1,4-glucosidase, homologous albumin and antibody (immunoglobulin G, IgG) molecules raised against isolated rat hepatocytes yields an active and stable soluble enzyme–polymer complex of mol.wt. approx. 10^6 . After intravenous injection, the ^{125}I -labelled complex is seen to be preferentially associated with hepatocytes when compared with labelled free α -1,4-glucosidase, enzyme–albumin polymers without IgG or polymer linked to a non-specific IgG molecule, all of which are associated to a much larger extent with the Kupffer cells. The procedure offers several advantages for targeting of enzymes to specific tissues and cells and for the possible lowering of hepatocyte glycogen content in Type II glycogenesis (Pompe's disease).

The success of enzyme therapy as a means of chemotherapy, or enzyme replacement in certain inborn errors of metabolism, may be limited primarily by: (a) rapid bioinactivation of injected enzyme, (b) immunological complications after repeated injection of a foreign protein, and (c) difficulties in delivering enzyme to specific tissue or cell sites for therapy or replacement (Desnick *et al.*, 1976). We have utilized a technique (Pailot *et al.*, 1974; Poznansky, 1979) of chemically cross-linking an enzyme to homologous albumin to produce a water-soluble polymer that is more stable than the native enzyme to both heat and proteolytic degradation. The use of homologous albumin was shown to render pig liver uricase both non-immunogenic and non-antigenic, thus avoiding immunological complications (Remy & Poznansky, 1978). In the present paper, we report data to show that it is possible to target an enzyme–albumin polymer to specific cell types by the use of antibodies against cell-surface antigens (Poznansky & Cleland, 1980).

Pompe's disease, or Type II glycogen-storage disease, represents a deficiency in α -1,4-glucosidase (acid maltase) causing death, usually by cardio-respiratory failure, before the age of 1 year (Howell, 1978). Although patients with this disease have a variable clinical phenotype, the biochemical defect appears similar in all forms of the disease, with intravacuolar accumulation of glycogen in what are

believed to be lysosomes in both liver and cardiac muscle. This type of accumulation has been observed most often in hepatocytes in infants with α -1,4-glucosidase deficiency (Baudhuin *et al.*, 1964). Attempts at enzyme therapy in Pompe's disease (Hug, 1978) and other lysosomal storage diseases (Brady, 1976) have met with only limited success, demonstrating the difficulties associated with enzyme replacement as an effective therapy. The present paper represents an attempt to direct or target α -1,4-glucosidase, cross-linked with homologous albumin forming a stable polymer, to rat hepatocytes. The reticuloendothelial cells of the liver (Kupffer cells) and spleen are the usual sites of uptake of foreign proteins and a number of different enzyme-carrier systems (Gregoriadis, 1974). We alter the site of uptake of the complex by covalently attaching antibody molecules directed against isolated hepatocytes.

Materials and methods

Synthesis of enzyme polymer

Albumin– α -1,4-glucosidase polymers were made by using an albumin/enzyme molar ratio of 20:1 with glutaraldehyde as the cross-linking agent. The polymer was prepared by co-incubating 50 mg of rat albumin, 2.5 mg of α -1,4-glucosidase (350 units from yeast; Sigma), 6 mg of *p*-nitrophenyl α -D-glucopyranoside, 3 ml of phosphate-buffered saline (0.067 M-potassium phosphate, 0.15 M-NaCl, pH 7.4)

Abbreviation used: IgG, immunoglobulin G.

and 50 μ l of 25% (v/v) glutaraldehyde (Sigma) at 4°C for 3½–4 h. The reaction was stopped by the addition of 30 mg of glycine, followed by a 24 h dialysis against phosphate-buffered saline containing 0.05% glycine. The polymer was separated from albumin that had not reacted, enzyme or small polymers by ultrafiltration with an Amicon XM300 filter to exclude polymer or molecules with mol.wts. below 300 000. Substrate (*p*-nitrophenyl α -D-glucopyranoside) was included in the reaction mixture to protect the active site of the enzyme during the cross-linking step.

Cross-linking the polymer to IgG was accomplished by incubating 3 ml of α -1,4-glucosidase–albumin polymer (30 mg) with 3 mg of IgG (from rabbit antisera to either human skin fibroblasts or rat liver hepatocytes) in the presence of 6 mg of substrate and 50 μ l of 25% glutaraldehyde and incubated as above for 2 h. After dialysis against phosphate-buffered saline containing glycine, the IgG–polymer complex was separated from unchanged IgG by ultrafiltration through the Amicon XM300 filter. The anti-hepatocyte IgG preparation was partially purified by adsorption against isolated rat spleen cells.

Gel chromatography of polymer complexes

α -1,4-Glucosidase–albumin polymers and α -1,4-glucosidase–albumin–IgG polymers were applied to a Bio-Gel A-50m column and the elution profiles determined. Pharmacia standards (catalase, ferritin, thyroglobulin and Blue Dextran 2000) were used to construct the plot of molecular weight versus fraction number of the elution peak shown in Fig. 1.

Preparation of rat hepatocytes and Kupffer cells

Rat livers (from 250 g Sprague–Dawley males) were exposed and perfused with collagenase (Sigma, C5138) via the portal vein at a flow rate of 40 ml/min and recirculated from the inferior vena cava as described (Seglen, 1973). After 30 min the liver was easily teased apart and gently filtered through cheesecloth. Individual hepatocyte and Kupffer-cell populations are then separated by differential centrifugation (Seglen, 1973), the hepatocytes appearing in the pellet produced by centrifugation for 2 min at 800 *g*. Washed hepatocytes were free of Kupffer cells as determined by light microscopy. The contamination of the Kupffer-cell population was not determined, although it contained no intact hepatocytes.

IgG preparations

Antisera were produced in rabbits to either isolated rat liver hepatocytes or human skin fibroblasts grown in tissue culture. Rabbits were given subcutaneous injections of either cell preparation (20 mg of cell protein/injection) on days 1, 8 and 16,

and antisera were collected on day 23. IgG was prepared by (NH₄)₂SO₄ precipitation and fractionation as described (Hudson & Hay, 1976). ¹²⁵I labelling of enzyme, polymer or antibody was performed by using chloramine-T by established techniques (Hudson & Hay, 1976). The IgG preparations were partially purified by incubating 10 mg of IgG with 5 × 10⁶ isolated rat spleen cells for 15 min at 37°C. This resulted in an adsorption of approx. 5 mg (50%) of the IgG molecules.

Results

Fig. 1 represents a molecular-weight determination of α -1,4-glucosidase–albumin polymer and α -1,4-glucosidase–albumin–IgG polymer complex by gel chromatography on Bio-Gel A-50m. The glucosidase–albumin polymer has an average mol.wt. of 8 × 10⁵, representing on an average 1 enzyme molecule to 11 molecules of albumin, considering that the glucosidase molecule has a mol.wt. of 120 000. The elution profile indicates that 80% of the polymer protein is eluted with mol.wts. between 6 × 10⁵ and 10 × 10⁵. Subsequent cross-linking of IgG to the polymer increases the average molecular weight to 10 × 10⁵ (range 8.5 × 10⁵–12 ×

Fig. 1. Molecular-weight determinations of α -1,4-glucosidase–albumin polymers and of α -1,4-glucosidase–albumin–anti-hepatocyte-IgG polymers

Gel chromatography was performed with a 1.6 cm × 40 cm column of Bio-Gel A-50m (Bio-Rad) at a flow rate of 0.4 ml/min. Pharmacia standards (catalase, ferritin, thyroglobulin and Blue Dextran 2000) were used to construct the semilogarithmic plot of molecular weight versus fraction number (0.4 ml) of the elution peak.

10⁵). After the two cross-linking procedures, the enzyme retains approx. 50% of its native enzyme activity. With exposure to serum, however, this activity loss is quickly regained, considering the greatly increased heat-stability and increased resistance to proteolytic inactivation of the polymer-enzyme complex compared with free enzyme (Paillot *et al.*, 1974; Poznansky, 1979; Poznansky & Bhardwaj, 1980).

The plasma clearance and tissue distribution of enzyme, albumin, polymer and polymer-IgG complex were then determined. IgG molecules prepared from rabbit antisera against human skin fibroblasts were used as the control for the anti-hepatocyte IgG preparations. Plasma clearance half-time values varied from over 4 h for homologous albumin to 45 min for the albumin-enzyme polymer complex and 15 min for the native enzyme (Fig. 2). Cross-linking anti-(human fibroblast) IgG to the polymer had little effect on its clearance rate, whereas the attachment of anti-(rat hepatocyte) IgG decreased the clearance half-time to less than 5 min. After clearance of 90% of the ¹²⁵I-labelled preparations, between 45% and 65% of the radioactivity could be located in the liver and spleen. This value held for all of the preparations.

Tables 1 and 2 describe experiments which attempt to ascertain whether the anti-hepatocyte-antibody-polymer complex was in fact being targeted to liver parenchymal cells (hepatocytes as opposed to the more phagocytic Kupffer cells). α -1,4-Glucosidase, albumin and glucosidase-albumin polymer were all taken up preferentially by Kupffer cells rather than hepatocytes at a ratio (per mg of protein) ranging from 10:1 to 5:1. Similarly,

anti-fibroblast IgG, both free and conjugated to polymer, was preferentially taken up by the Kupffer cells. The attachment of anti-hepatocyte IgG to the glucosidase-albumin polymer produces a dramatic alteration in the site of polymer uptake with an approximately equal distribution of labelled polymer between hepatocytes and Kupffer cells. The distribution of anti-hepatocyte-antibody-polymer appears

Fig. 2. Plasma clearance of ¹²⁵I-labelled enzyme, albumin and polymer preparations after intravenous injection in rats

▲, ¹²⁵I-labelled albumin; △, ¹²⁵I-labelled α -1,4-glucosidase; ○, ¹²⁵I-labelled α -1,4-glucosidase-albumin polymer; □, ¹²⁵I-labelled anti-fibroblast-IgG-enzyme-albumin polymer; and ●, ¹²⁵I-labelled anti-hepatocyte-IgG-enzyme-albumin polymer. IgG preparations to human skin fibroblasts and to rat hepatocytes were prepared as described in Table 1.

Table 1. Distribution of ¹²⁵I-labelled preparations between hepatocytes and Kupffer cells

IgG was prepared by (NH₄)₂SO₄ precipitation of rabbit antisera against skin fibroblasts (F) or isolated rat hepatocytes (H) by standard immunization procedures (Hudson & Hay, 1976). Hepatocytes and Kupffer cells were separated by liver perfusion with collagenase (Seglen, 1973). Protein was determined in the separated and washed cell preparations by the technique of Lowry *et al.* (1951). ¹²⁵I labelling of enzyme, albumin and IgG was done with chloramine-T (Hudson & Hay, 1976). The 'ratio' is of ¹²⁵I in hepatocytes to that in Kupffer cells in each experiment. All values represent means $\pm$ S.D. for at least three cell separations, except for the anti-F IgG preparations, where only two were done.

¹²⁵ I-labelled protein preparation	¹²⁵ I radioactivity (c.p.m./mg of cell protein)		Ratio
	Hepatocytes	Kupffer cells	
α -1,4-Glucosidase	108 $\pm$ 22	1030 $\pm$ 131	0.10 $\pm$ 0.01
Albumin	42 $\pm$ 20	177 $\pm$ 49	0.25 $\pm$ 0.05
Enzyme-albumin polymer	95 $\pm$ 31	508 $\pm$ 133	0.20 $\pm$ 0.03
Anti-F IgG	109 $\pm$ 15	629 $\pm$ 120	0.16 $\pm$ 0.03
Anti-H IgG	233 $\pm$ 32	280 $\pm$ 40	0.85 $\pm$ 0.09*
Anti-F-IgG-polymer	182 $\pm$ 39	1067 $\pm$ 189	0.17 $\pm$ 0.02
Anti-H-IgG-polymer	317 $\pm$ 80	259 $\pm$ 11	1.23 $\pm$ 0.15*

* Significantly different from all other preparations at *P* < 0.01.

Table 2. *Relative distribution of ^{125}I -labelled preparations between liver and spleen*

Preparations and abbreviations are described in Table 1. Values given are means $\pm$ s.d. for the numbers of experiments indicated in parentheses. As indicated from this Table and Table 1, the liver/spleen distribution ratio appears to be an accurate indicator of parenchymal (hepatocyte) cell to reticuloendothelial (Kupffer) cell uptake of proteins.

^{125}I -labelled protein preparation	Total ^{125}I c.p.m. ratio liver/spleen
α -1,4-Glucosidase	11.7 $\pm$ 3.0 (6)
Albumin	19.8 $\pm$ 3.3 (6)
Glucosidase-albumin polymer	18.7 $\pm$ 3.8 (6)
Anti-F IgG	10.0 $\pm$ 4.0 (3)
Anti-H IgG	59.3 $\pm$ 7.6 (6)*
Anti-F-IgG-polymer	19.2 $\pm$ 1.2 (3)
Anti-H-IgG-polymer	60.9 $\pm$ 15.1 (10)*

* Significantly different from other preparations at $P < 0.01$.

similar to the distribution of the anti-hepatocyte antibody itself after intravenous administration and plasma clearance. The data in Table 2 suggest that measuring the liver/spleen ratio of the ^{125}I -labelled preparation may give a good approximation of the relative distribution of the various macromolecular preparations between hepatocytes and Kupffer cells. By using the same ^{125}I -labelled preparations as in Table 1, it appears that preferential targeting to hepatocytes of the anti-hepatocyte IgG alone or the α -1,4-glucosidase-albumin polymer conjugated with the anti-hepatocyte IgG greatly increases the ratio of total radioactivity (c.p.m.) in the liver to that in the spleen. This might be expected, since the Kupffer cells of the liver and many of the spleen cells are phagocytic reticuloendothelial cells and would be the likely site of uptake of foreign macromolecules, unless they can be specifically targeted and bound to hepatocytes. The liver/spleen ratio is a convenient index, since a greater number of rats may easily be assayed for preferential uptake without the necessity of separating hepatocyte and Kupffer-cell populations. It is interesting that albumin alone is taken up to a greater extent by hepatocytes than is α -1,4-glucosidase, as seen in both Tables 1 and 2. This preferential shift in site of uptake may be at least partially imparted to the enzyme-albumin polymer.

By assuming that all ^{125}I -labelled polymer in the liver is associated with either hepatocytes or Kupffer cells, an estimate of the efficiency of the targeting to the different liver cells may be made. In the absence of anti-hepatocyte antibodies, the hepatocyte population received 8.1% of the total injected α -1,4-glucosidase-albumin polymer, whereas the Kupffer-cell population received 38.2% of the

injected polymer. Cross-linking anti-hepatocyte IgG to the polymer complex alters these values, to 27.8% of the polymer being delivered to the hepatocytes, and 24.2% of the polymer appearing in the Kupffer-cell population. By subtracting the amount of polymer bound to hepatocytes in the absence of anti-hepatocyte IgG from that bound in the presence of that IgG, it is possible to estimate that 19–20% of the anti-hepatocyte-IgG-enzyme-albumin polymer possesses some specificity for binding to the hepatocyte population. These calculations assume that the total percentage of labelled polymer appearing in the liver in experiments described in Table 1 (where total liver radioactivity could not be measured) was the same as the percentage tissue distribution seen in Table 2 when total tissue radioactivity was determined without the separation of hepatocyte and Kupffer-cell populations.

Discussion

Although phagocytic uptake by cells of the reticuloendothelial system is the most common fate of many foreign macromolecules after intravenous injection, the ability to target enzyme-albumin polymers specifically to less phagocytic hepatocytes has definite advantages. In a number of defects of carbohydrate, lipid and amino acid metabolism, the defect has either been shown or is assumed to be associated with an enzyme deficiency (often lysosomal) within the metabolic fraction of the liver, namely the hepatocytes. In any attempt at enzyme replacement for such diseases, where substrate accumulation is intracellular, it may be critical to be able to bypass the phagocytic Kupffer cells and effect uptake directly into hepatocytes (Baudhuin *et al.*, 1964; Howell, 1978). However, it should be noted that in these experiments, although we have shown that cell-specific antibodies may be used to target the enzyme-albumin polymer effectively, we have not shown whether the polymer-antibody complex is in fact internalized by the hepatocytes. The rates of uptake and intracellular localization of enzyme-polymer complex have been investigated (M. J. Poznansky & R. Singh, unpublished work) and preliminary experiments have localized ^{125}I -labelled polymer to a population of lysosomes after binding to the plasma membrane. The efficiency of the targeting may also be greatly enhanced by the use of monospecific monoclonal antibodies directed against cell-surface-specific antigens (Kohler & Milstein, 1975; Yeh *et al.*, 1979), which might in effect increase the efficiency of targeting of the enzyme-albumin polymer above the 20% suggested in the present paper.

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