EXHIBIT E

PART 3 of 3

injection. Bovine BI and BI–BSA conjugates are highly immunogenic resulting in antibodies being formed against both molecules. Mice that had received prior injections of BI–MSA did not respond to subsequent injections of BI. This is an important finding in light of our interest in using insulin as a targeting agent (described in the following section). Table 5 lists the number of enzymes which have been rendered nonimmunogenic by the covalent attachment to homologous albumin. We now feel that it is likely that any enzyme can be so rendered providing that we can cross-link it while retaining significant enzyme activity. The exact mechanism whereby the antigenic sites appear to be masked and the question whether the process of tolerance is involved have still to be determined.

5.5. *In Vivo* Targeting

The majority of enzyme deficiency diseases involve substrate accumulation in specific tissues, cell types and intracellular organelles, often lysosomes. In some cases it may simply be possible to lower substrate levels in the plasma producing sufficient concentrations gradients to form and allow substrate to be literally pulled out of accumulating sites within cells not ordinarily accessible to administered enzyme. In many of the lipid and carbohydrate storage diseases which are usually lysosomal in nature, the accumulation of substrate is limited to secondary lyososmes in specific tissue often liver and spleen but frequently muscle and nervous tissue. Table 6 lists a number of the more common or well understood lysosomal storage diseases along with the specific defects and sites of substrate accumulation. The net result of many of these diseases is a gross accumulation of secondary lysosomes packed with undegraded substrate. This can be seen to be choking the cell (sometimes described as Foam Cells in nervous tissue) thereby severely altering cellular and tissue function. Thus consideration of enzyme replacement therapy for the treatment of these lysosomal storage diseases requires specific delivery of the enzyme not only to specific tissues and cells but to the same secondary lysosomes in which substrate is accumulating. One has to imagine both the degradation of accumulated substrate and the prevention of further accumulation in contemplating enzyme therapy.

We have previously discussed a number of clinical trials for the treatment of lysosomal storage diseases where the trials failed because the enzyme administered failed to reach specific organs. In considering targeting enzymes or drugs we have also to consider the importance of steering the carrier–drug/enzyme complex away from the reticuloendothelial tissue of the liver and spleen. In both clinical trials evidence of substrate degradation due to administered enzyme was evident in Kupffer cells of the liver, but not in either respiratory or cardiac muscle or the central nervous system where substrate accumulation is fatal in Pompe's disease and Tay–Sachs disease respectively. We have used two approaches towards the question of targeting enzyme–albumin complexes. We have used antibodies to target enzyme–albumin polymers to specific cells both *in vivo* and *in vitro*. We have also used the insulin molelcule as a targeting agent to direct enzyme–albumin polymers to tissue rich in insulin receptors. One major advantage that enzymes have as therapeutic agents over drugs such as anticancer drugs is the fact that enzymes are generally nontoxic so that even a small degree of targeting to a specific organ may be significant even if the majority of the enzyme is arriving at an ineffective site albeit harmless.

Tables 7 and 8 demonstrate our *in vivo* data for targeting L-asparaginase and α-1,4-glucosidase to tumor cells and hepatocytes respectively. In the case of the anti-tumor agent L-asparaginase we used monoclonal antibodies directed against the H-2^k antigen present on mouse RI tumor cells. The experiments were performed in Balb/Ccr mice which possess the H-2^d antigen so that the enzyme–albumin-anti-H-2^k antibody complex could distinguish between the normal and tumor cells. The *in vivo* data show that the L-asparaginase remains in the mouse for much longer periods of time when it is conjugated with albumin and with the antibody molecule directed against a surface antigen on the tumor cell. Our laboratory (Poznansky *et al.*, 1982) is attempting to direct L-asparaginase to pancreatic tumor cells in an analogous manner using monoclonal antibodies directed against the pancreatic tumor cell line Panc-1. Experiments with α-1,4-glucosidase were

TABLE 6. *Representative List of Inherited Lysosomal Storage Disorders Demonstrable as Enzyme Deficiency Diseases*

Disease	Defective enzyme	Accumulating substrate	Clinical picture
Lipid Storage			
Gaucher (Glucocerebrosidoses)	Glucosylceramidase	Glucosylceramide, lactosylceramide, glucosyl sphingosine	Juvenile or adult onset, hepatosplenomegaly infantile onset, severe retardation, death by 2 years.
Tay–Sachs GM_2 Gangliosidoses	β-*N*-acetylhexosaminidase A	GM_2 Ganglioside	Infantile onset, severe retardation, death by 5 years
Fabry (Ceramidetrihexosidosis)	α-Galactosidase A	Digalactosyl ceramide galactosyl–galactosylglucosyl ceramide	Juvenile onset, renal and cardiovascular insufficiency death by age 30–40 years.
Niemann–Pick Sphingomyelinosis Type A	Sphingomyelin Phosphodiesterase	Sphingomyelin	Infantile onset, severe retardation, hepatosplenomegaly, death by 4 years.
Carbohydrate Storage			
Mannosidosis	Mannosidase A and B	Mannose containing polysaccharides and glycopeptides	Infantile to juvenile onset, severe psychomotor retardation.
Pompe (Type II Glycogenosis	α-1,4-Glucosidase	Glycogen	Infantile onset, cardiomegaly and respiratory distress, muscle weakness, death by 2–3 years.
Hurler (MPS I-H)	DL-Iduronidase	Dermatan sulphate Heparan sulphate	Infantile onset, retardation, hepatosplenomegaly, death by 20 years
Hunter (MPS II Type A)	Iduronate sulfatase	as in Hurler's	Similar to but milder than Hurler's.

TABLE 7. In vivo *Targeting of L-Asparaginase–Albumin Polymers*

Enzyme preparation	% ^{125}I-enzyme remaining 15 hr	24 h	48 hr
Free L-Asparaginase	9	4	3
L-Asparaginase–albumin polymer	41	17	11
L-Asparaginase-anti-H-2^k antibody	60	38	21
L-Asparaginase–albumin-anti-H-2^k antibody	74	511	37

RI tumor cells (which possess the H-2^k antigen) were injected i.p. into Balb/Ccr mice (which possess the H-2^d antigen) at time zero. 24 hr later ^{125}I-enzyme in various preparations was also injected i.p. and the counts remaining determined at 15, 24 and 48 hr. NaI was added to the drinking water of the mice 24 hr prior to enzyme injection. The drop in ^{125}I represents the clearance of the labeled enzyme from the mice.

devised to determine the possibility of targeting the enzyme to the population of liver cells, the hepatocytes, as opposed to the more phagocyctic Kupffer cells, the normal site of clearance of many foreign proteins and particles from the circulation. We had anticipated that albumin itself would act as a targeting agent to direct the α-1,4-glucosidase to hepatocytes which are known to possess specific albumin receptors. Table 8 indicates that the degree of targeting of the conjugate to hepatocytes as a function of the attached albumin is not impressive. We then proceded to produce a heterologous antiserum to rat hepatocytes. This was accomplished by isolating hepatocytes and injecting them into rabbits using a normal immunization protocol. The antiserum was then adsorbed to rat spleen cells and Kupffer cells in an effort to adsorb away the antibodies not directed against hepatocytes (Poznansky and Bhardwaj, 1981). The resultant antiserum was separated into an IgG fraction by ammonium sulphate precipitation (Hudson and Hay, 1976) and the IgG molecules covalently attached to the α-1,4-glucosidase–albumin polymers using glutaraldehydes as the cross-linking agent. Antibodies against human skin fibroblasts were used as the control. Attachment of the anti-hepatocyte antibody to the α-1,4-glucosidase–albumin polymer produced an important shift in the distribution of the enzymes from Kupffer cell uptake to hepatocyte uptake. Thus it is possible in *in vivo* experiments to target these conjugates to hepatocytes while avoiding the more phagocytic Kupffer cells. Figure 4 demonstrated that the targeted enzyme–albumin complex not only bound to the surface of the hepatocytes but also could be found associated with a lysosomal fraction. In the case described in Table 8 and Fig. 4 the polymer was injected intravenously and its distribution studied following clearance of at least 90% of the ^{125}I-labeled enzyme from the circulation.

In the case of Pompe's disease or Type II glycogenosis while glycogen storage occurs in the liver and spleen and the disease is associated with hepatosplenomegaly, the most

TABLE 8. *Targeting Enzyme–Albumin Polymers to Hepatocytes*

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

*Significantly different from all other preparation at $p < 0.001$.

Various enzyme or antibody preparations were injected intravenously. When 80–90% of the injected label had cleared from the circulation, the rats were anaesthetized and the livers were perfused with collagenase to separate hepatocytes from Kupffer cells. The ratio represents the counts per mg cell protein in the hepatocytes over that in the Kupffer cells. The anti-F-IgG represents a control antibody derived from the serum of a rabbits immunized to human skin fibroblasts. The anti-H-IgG preparation was derived from the serum of a rabbit that had received repeated injections of isolated rat hepatocytes. The antiserum was absorbed with rat spleen cells prior to conjugation using glutaraldehyde.

critical site of glycogen storage and the real cause of infant death is cardiac and respiratory dysfunction as a result of glycogen accumulation. It was thus our intention to determine how the enzyme–albumin complex might be targeted to muscle tissue as well as to hepatocytes. Muscle cells have a very high density of insulin receptors. We contemplated the possibility of producing antibodies against the insulin receptor but this has been shown by others to be most onerous task (Kahn *et al.*, 1978). Because it has been shown that anti-insulin receptor antibodies mimic many of the characteristics of the insulin molecule

TABLE 9. *Insulin Mediated Targeting of Enzymes*

Enzyme preparation	% Binding	
	Chick muscle cells	Mouse spleen cells
^{125}I-α-Glucosidase (yeast)	6.7	5.2
^{125}I-α-Glucosidase (human placenta)	8.1	6.8
^{125}I-α-Glucosidase–albumin (yeast)	4.0	3.6
^{125}I-α-Glucosidase–albumin–insulin (yeast)	30.1	28.8
^{125}I-α-Glucosidase–albumin–insulin (human placenta)	37.1	31.4

Binding conditions were as follows: 0.05 μg of ^{125}I-labeled enzyme preparation was incubated with 2×10^6 chick embryonic pectoral muscle cells or 1×10^6 mouse spleen cells for 30 min at 37°C. The cells were then washed and counted. When the cells were also incubated with chloriquine, binding of insulin containing polymers increased while internalization of the polymers was inhibited. Chloriquine had no effect on binding of free enzyme or enzyme–albumin polymer.

itself, we supposed that it might be easier to use insulin as a targeting agent to direct the enzyme–albumin complex to muscle tissue. We were able to conjugate as many as twelve insulin molecule per albumin molecule on an α-1,4-glucosidase–albumin polymer resulting in an enzyme–albumin–insulin molar ratio averaging 1:12:60 with an average molecular weight of 1.2×10^6. Unreacted insulin was separated from the conjugate by gel chromatography using Sephadex G-200 and by extensive dialysis using Spectropore dialysis tubing with a pore exclusion size of 14,000. We have four lines of evidence to indicate that the insulin is conjugated to the enzyme–albumin polymer (Poznansky and Singh, 1982).

1. Anti-insulin antibodies react with enzyme–albumin–insulin polymers but not with enzyme–albumin conjugates.
2. Enzyme–albumin–insulin conjugates are cleared from the circulation with a half-time of 4 hr as compared to 16 hr for the enzyme–albumin complex alone.
3. Enzyme–albumin–insulin polymers retain the hypoglycemic effect of insulin and roughly the same glucose lowering ability that an equivalent amount of free insulin might be expected to produce.
4. Enzyme–albumin–insulin conjugates bind preferentially to mouse spleen cells and to chick embryonic muscle cells both in tissue culture (Table 9). We also have preliminary data to indicate that the insulin conjugate targets preferentially *in vivo* to tissues bearing high densities of insulin receptors. When ^{125}I-labeled α-1.4-glucosidase–albumin polymers are injected intravenously into mice we could not detect *any* label associated with muscle tissue. Conjugating insulin to the polymer prior to injection resulted in a small but significant uptake into muscle tissue including cardiac, respiratory and peripheral skeletal muscle. This difference is seen when careful consideration is made for the amount of polymer remaining in the blood contaminating the various muscle tissue. We also observe a higher uptake of the insulin–conjugated polymer into spleen, another tissue very rich in insulin receptors. Although the extent of uptake of the insulin conjugate into muscle tissue is limited to 2–3% of the total dose of polymer this is a considerable amount of enzyme when compared to the undetectable amount of enzyme delivered to muscle tissue in the absence of insulin as a targeting agent.

6. SUMMARY

It has become apparent in the past few years that a variety of approaches to treating enzyme deficiency diseases and specifically for the delivery of drugs/enzymes to specific sites will be required to overcome the various limitations to simple drug or enzyme administration. It is now evident that no one carrier system will prove a panacea to tackle all of the situations where carrier systems may be required (Gregoriadis, 1976). In one case we may require that an enzyme remain for a prolonged period within the circulation, in another delivery of enzyme to phagocytic tissue may be required whereas in a third case it may be essential that the reticuloendothelial system be avoided in order that delivery to specific tissues be achieved. Under certain circumstances where enzymes may have to move from the plasma past endothelial barriers to tissue such as muscle, it may be important to retain the enzyme within the circulation in order to allow a maximum amount of the enzyme or enzyme–carrier complex to permeate the endothelial barrier. Consideration must also be given to the effects of the enzyme/drug on nontarget tissue. In the case of antitumor agents and perhaps even toxins such as ricin toxin or diphtheria toxin a high efficiency targeting to tumor tissue may be required because of the high toxicity and adverse effects of the product on normal tissue. In other cases the deposition of enzymes or drugs in nontarget tissue will have little adverse effects.

In addition to questions of drug/enzyme delivery, consideration must be given to the rates of biodegradation of enzyme and/or drug following repeated intravenous administrations as well as the immunological complications that may ensue from the administration of foreign proteins or drugs attached to carrier molecules which may now assume the immunological properties of haptens. We now have a sufficient number of different carrier systems with varying properties that we can consider tailoring the carrier system to the specific requirements of the disease condition under consideration be it specific targeting, avoiding biodegradation or immunological attack, alterations in pH characteristics of the carried agent or increasing the circulation half-life of the therapeutic agent. We now have the opportunity to tailor a carriage system to the requirements of a specific disease rather than search for a disease to suit the characteristics of a specific carrier system.

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