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The effect of polysialylation on the immunogenicity and antigenicity of asparaginase: implication in its pharmacokinetics

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Abstract

Erwinia carotovora L-asparaginase was conjugated via the ∈-amino groups of its lysine residues with colominic acid (CA) (polysialic acid) of average molecular mass of 10 kDa by reductive amination in the presence of $NaCNBH_3$. Polysialylation using 50-, 100- and 250-fold molar excess CA relative to the enzyme led to an increasing proportion of the enzyme's ∈-amino groups (5.8, 7.6 and 11.3%, respectively) being conjugated to CA. Polysialylated and native (intact) asparaginase were used to immunize mice intravenously. Results (total IgG immune responses) indicate that all preparations elicited antibody production against the enzyme moiety but not against the CA of the conjugates. Moreover, antibody titres appeared highest for the native enzyme and were generally reduced as the degree of polysialylation increased. In other experiments mice pre-immunized with native or polysialylated asparaginase, with anti-asparaginase antibodies in their blood, were injected intravenously with the corresponding enzyme preparations. Results revealed that polysialylation reduces the antigenicity of asparaginase thus leading to circulatory half-lives ($t_{\frac{1}{2}}\beta$) that were 3–4-fold greater than that of the native enzyme, and similar to those observed in naive, non-immunized mice. Our data suggest that polysialylation of therapeutic enzymes and other proteins may be useful in maintaining their pharmacokinetics in individuals with antibodies to the therapeutic proteins as a result of chronic treatment.

Keywords: Asparaginase; Polysialic acids; Protein delivery; Antibody response

Abbreviations: ANOVA, one way analysis of variance; BSA, bovine serum albumin; CA, colominic acid; FCS, foetal calf serum; mPEG, monomethoxypoly(ethyleneglycol); MPS, mononuclear phagocyte system; OPD, *o*-phenylenediamine; PBS, 0.15 M sodium phosphate buffered saline, pH 7.4; dpm, disintegrations per minute; $t_{\frac{1}{2}}\beta$, terminal circulatory half-life; PBS-T, PBS containing 0.05% v/v Tween 20.

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1. Introduction

Asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) is an anti-neoplastic agent, currently used in the treatment of acute lymphoblastic leukaemia. The enzyme exploits a biochemical difference between malignant and normal cells, namely the inability of the former to produce asparagine owing to a deficiency in L-asparagine synthetase (Keating et al., 1993). However, because of the high molecular weight of the asparaginase and its bacterial origin (Ruyssen and Lawers, 1978), prolonged use leads to hypersensitivity, ranging from mild allergic reactions to life-threatening anaphylaxis (Reynolds, 1993). Moreover, antibodies against asparaginase greatly accelerate its clearance from the circulation and thus reduce its therapeutic effectiveness (Peterson et al., 1971; Wahn et al., 1983).

Previously proposed strategies to reduce immunological clearance of therapeutic proteins include entrapment into liposomes which prevents interaction of preformed antibodies with the proteins (Gregoriadis and Allison 1974; Neerunjun and Gregoriadis, 1976). Drawbacks of this approach are the relatively rapid uptake of the carrier system by the mononuclear phagocyte system (MPS) and the immunoadjuvant action of liposomes (Gregoriadis, 1990; Gupta et al., 1993) which can further promote immune responses to the proteins. Grafting of hydrophilic macromolecules such as monomethoxypoly(ethyleneglycol) (mPEG) (Nucci et al., 1991) onto the surface of asparaginase and other enzymes has proved successful not only in prolonging enzyme presence in the blood circulation (Park et al., 1981) but also in reducing or abrogating immunogenicity (Wada et al., 1990) and antigenicity (Kodera et al., 1992), with consequent increase of therapeutic efficacy (Fuertges and Abuchowski, 1990). However, conjugation of mPEG to enzymes often leads to substantial reduction of their activity (Park et al., 1981). Moreover, the non-biodegradable mPEG is expected to accumulate in the lysosomes following endocytosis of the conjugates, possibly leading to toxicity on chronic use.

We have recently proposed the use of the highly hydrophilic and biodegradable polymers of *N*-acetylneuraminic acid (polysialic acids) as an alternative to mPEG in prolonging the circulatory half-lives of proteins (Gregoriadis et al., 1993; Fernandes and Gregoriadis, 1996, 1997). The rationale of this approach is that polysialic acids may not only render the proteins more stable and highly hydrophilic and thus augment their circulatory half-lives, but also mask immunogenic determinants that would otherwise elicit antibody production. Polysialic acids may also sterically hinder the approach of pre-formed antibodies to the antigenic sites. However, covalent coupling of polysialic acids to a carrier protein may also enhance their immunogenicity by converting them to thymus-dependent antigens (Devi et al., 1991). Although low molecular weight polysialic acids as such (Wyle et al., 1972) or as protein conjugates (Jennings and Lugowski, 1981) are poor immunogens, their conjugation to proteins is expected to alter the latter's net surface charge and, possibly, enhance their immunogenicity by altering their three-dimensional structure. Here we have evaluated the immunological properties of polysialylated asparaginase as compared to the native enzyme. To that end, a low molecular weight polysialic acid (namely colominic acid; CA) was covalently linked to asparaginase and the pharmacokinetics of the native and polysialylated enzyme injected intravenously in pre-immunized mice was monitored. Results suggest that polysialylation reduces the antigenicity of asparaginase and as a result prolongs its circulation in the blood even in the presence of anti-asparaginase antibodies.

2. Materials and methods

2.1. Materials

Erwinia carotovora L-asparaginase was kindly provided by Dr C.N. Wiblin (Microbiological Research Establishment, Porton Down, UK). CA (sodium salt) from *Escherichia coli* K1 (average molecular mass 10 kDa), bovine serum albumin (BSA), Tween 20, sodium cyanoborohydride ($NaCNBH_3$) and *o*-phenylenediamine (OPD) were purchased from Sigma (Poole, Dorset, UK).

Horseradish peroxidase-labelled goat antibodies against mouse IgG and IgM, and foetal calf serum (FCS) were purchased from Sera-lab (Crawley Down, Sussex, UK). Sodium cyanoboro [^{3}H]hydride (NaCNB[^{3}H3]) (2.2 Ci/mg) was obtained from Amersham International (Amersham, Buckinghamshire, UK). All other reagents were of analytical grade.

2.2. Preparation of polysialylated asparaginase

Asparaginase was polysialylated with oxidized CA by reductive amination in the presence of $NaCNBH_3$ and isolated by ammonium sulphate precipitation (Fernandes and Gregoriadis, 1997). Use of CA in molar amounts of 50-, 100- and 250-fold greater than those of the enzyme resulted in preparations (designated here as 50:1, 100:1 and 250:1) with increasing number of substituted amino groups (5.8, 7.6 and 11.3%, respectively). After extensive dialysis against 0.15 M sodium phosphate buffer (supplemented with 0.9% NaCl, pH 7.4) (PBS) at 4°C, samples of polysialylated asparaginase were filtered through a low protein binding 0.45 μm filter (Whatman Scientific, Maidstone, Kent, UK) to remove insoluble material. Asparaginase concentration (Bradford, 1976) and enzyme activity (Ruyssen and Lawers, 1978) in the filtrates were determined in a Wallac CompuSpec UV–visible spectrophotometer (Wallac UK, Crownhill, Milton Keynes, UK). The half-life of native and polysialylated asparaginase in pre-immunized mice, was determined by monitoring its enzyme activity (Ruyssen and Lawers, 1978) and also its radioactivity following tritiation with sodium cyanoboro^3[H]hydride as already reported (Fernandes and Gregoriadis, 1997).

2.3. Immunization protocol

Aliquots containing 200 μg of native or polysialylated asparaginase were freeze-dried (Edwards Modulyo, Sussex, UK) and stored at 4°C. Immediately before use, 1 ml of filter (0.2 μm diameter)-sterilized PBS was added to the freeze-dried material to obtain a 200 μg/ml asparaginase solution. Male Balb/c mice (28–30 g body weight; purchased from Bantin & Kingman Universal, North Humberside, Hull, UK) in groups of five were injected intravenously (tail vein) on days 0, 8 and 15 with 0.1 ml (20 μg of protein) of the solution and bled from the tail vein on days 7, 14 and 21. Blood samples (50 μl) were then placed into tubes containing 450 μl PBS, spun at 5000 × *g* for 10 min (Biofuge 13, Heraus Equipment, Brentwood, Essex, UK) and the diluted plasma (corresponding to about 25 μl plasma assuming a 50% haematocrit) in the supernatants kept frozen at −40°C.

2.4. Determination of asparaginase clearance from the blood of pre-immunized mice

Freeze-dried samples of tritiated native and polysialylated asparaginase were dissolved in filter-sterilized PBS immediately before use. Male outbred T/O mice (25–30 g body weight; obtained from Harlan-OLAC UK, Bicester, Oxon, UK) in groups of four were each injected intramuscularly (hind leg) with the equivalent of 20 μg of protein on days 0, 7 and 14. Serum IgG titers were measured in plasma samples obtained on days 7 and 14 (prior to boosting) and on day 28 when animals were subsequently injected intravenously (tail vein) with 1 mg of radiolabelled native or polysialylated enzyme in 0.1 ml PBS. Samples of blood (50 μl) taken at time intervals were immediately diluted in 450 μl PBS, centrifuged as above and the diluted plasma was assayed for residual enzymatic activity (Ruyssen and Lawers, 1978) and ^{3}H radioactivity (Fernandes and Gregoriadis, 1997). The terminal half-lives ($t_{\frac{1}{2}}\beta$) of asparaginase given as a single intravenous dose in the pre-immunized mice, were estimated as already described (Fernandes and Gregoriadis, 1997) for naive animals. Statistical analysis of the results was performed with GraphPad InStat version 1.15 (GraphPad, Software, 1990) and ANOVA tables were omitted from the text (only the Bonferroni *P*-values are reported). Owing to the non-homogeneity of the variances, circulatory half-lives obtained in pre-immunized mice were compared by a Kruskal–Wallis test (non-parametric ANOVA) with Minitab Release 7.2 (Minitab, 1989).

2.5. Determination of antibody titers

Antibody titers in immunized Balb/c and T/O mice were measured by an indirect enzyme-linked immunosorbent assay (ELISA) (Catty and Raykundalia, 1989). Polystyrene microtiter plates (Immulon 1 and 4; Dynatech Laboratories, Billingshurst, W. Sussex, UK) were coated with 60 μl of the native or polysialylated asparaginase solution (2 μg/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.6) and kept at 20°C for 1 h or overnight at 4°C. After washing three times with PBS containing 0.05% v/v Tween 20 (PBS-T), 60 μl of 1% BSA solution in PBS-T was added to each well to prevent non-specific antibody binding. Appropriately diluted serum (60 μl) was added into the top well and doubly diluted down the plate. After incubation at 20°C for 2 h, plates were again washed three times with PBS-T and tap dried. Then 50 μl of diluted (1/10 000 in PBS-T supplemented with 5% FCS and 1% BSA) anti-mouse IgG and IgM goat antibodies conjugated to horseradish peroxidase were added to each well and the plates incubated for 2–3 h at 20°C. After washing three times with PBS-T, the plates were again tap dried. Citrate-phosphate buffer (200 μl), pH 5.0, containing OPD and 30% H_2O_2 as the substrates of the enzymatic reaction, were added to each well and incubated again at 20°C for 30 min. The reaction was stopped by the addition of 25 μl of a 1.5 M H_2SO_4 solution and the absorbance read at 492 nm in a microplate reader (Titertek Multiskan® MCC/340). Antibody titers were estimated from the serum dilution required to obtain absorbance readings around 0.20 and expressed as log 10 of that dilution. In every run, control wells containing only coating buffer, blocking protein solution, secondary antibody solution or diluted plasma from naive mice were included.

3. Results and discussion

3.1. Immunogenicity of polysialylated asparaginase

In initial experiments the immunogenicity (IgG and IgM) of native and polysialylated asparaginase was studied in Balb/c mice injected intravenously with the enzymes three times, at weekly intervals. Results (Fig. 1), especially important in the present work because of their relevance to

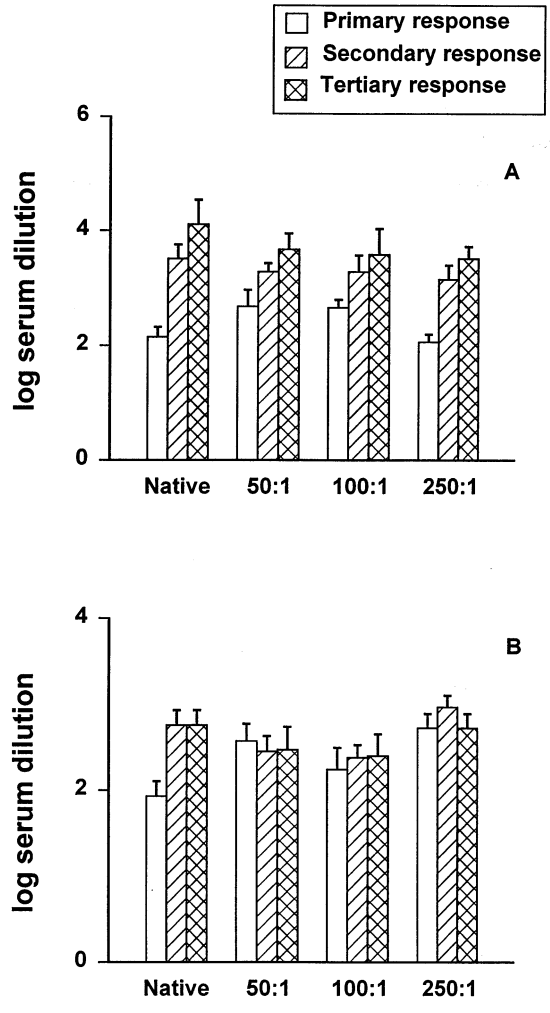

Fig. 1. Immunogenicity of native and polysialylated asparaginase (50:1, 100:1 and 250:1 polysialic acid to enzyme molar ratio in the coupling reaction). Balb/c mice were immunized intravenously with 20 μg of native or polysialylated enzyme on days 0, 8 and 15 and bled on days 7, 14 and 21 (primary, secondary and tertiary responses respectively). Serum IgG (A) and IgM (B) titers were estimated by ELISA in plates coated with the corresponding antigen. Results are mean ± S.D. of four or five animals.

chronic intravenous therapy with the enzyme, show that after the third injection sera IgG and IgM titers for the native and polysialylated asparaginase are, respectively, similar indicating that, at least at this level of polysialylation, the immunogenicity of the conjugates on chronic usage is not reduced. Since the secondary amine bond formed between CA and asparaginase is chemically stable (Gray et al., 1978) and not susceptible to enzymatic hydrolysis (Francis et al., 1996), premature cleavage of the conjugate in vivo is unlikely to account for the failure of polysialylation to reduce the enzyme's immunogenicity. A more plausible explanation is the low degree of polysialylation achieved (a maximum modification of 11% of amino groups for the 250:1 preparation). For instance, it has been reported that the ability of mPEG to reduce the immunogenicity of proteins (Abuchowski et al., 1977; Sasaki et al., 1993) is dependent on the degree of pegylation. In the case of asparaginase, its immunogenicity was reduced when either 56% (Kamisaki et al., 1981) or 70% (Park et al., 1981) of the enzyme's free amino groups had been pegylated. It is thus conceivable that more extensive polysialylation of asparaginase would reduce its immunogenicity.

3.2. Effect of the coating antigen on antibody titers

In order to establish the antigen specificity of the antibodies formed in the immunization experiment (Fig. 1), antisera were tested by ELISA against CA, native enzyme and polysialylated asparaginase (50:1, 100:1 and 250:1). This would also allow us to evaluate antibody cross-reactivity and its relation, if any, to the degree of polysialylation and also to predict as to whether polysialylated asparaginase can be an alternative to native asparaginase in the therapy of patients already immune to the latter enzyme and thus resistant to treatment.

Initially, antisera to polysialylated asparaginase were tested for anti-CA antibodies. As effective adsorption of substrate (antigen) on the microtiter plate is essential to the success of ELISA, Immulon 4 plates known to adsorb hydrophilic macromolecules optimally, were also used in addition to Immulon 1. Absorbance readings with both types of CA-coated plates were low and similar to those obtained with negative controls (results not shown). Although covalent coupling of poor immunogens to carrier proteins can increase the former's immunogenicity, this did not appear to occur with CA, in agreement with work by others (Jennings and Lugowski, 1981) on polysialylated tetanus toxoid. On the other hand, the linkage area of the polysialylated construct (also prepared by reductive amination) was found (Jennings and Lugowski, 1981) to be a strong determinant for the production of antibodies in mice. However, our results do not support the presence of antibodies specific for the linkage area in the polysialylated asparaginase: antisera against the three constructs did not crossreact with polysialylated catalase prepared (Fernandes and Gregoriadis, 1996) by the same method (results not shown).

Subsequently, the antibody titers of antisera raised against each of the asparaginase preparations (native, 50:1, 100:1 and 250:1) were determined by ELISA on plates coated with each of the antigen preparations. Results (total IgG) from plates coated with native asparaginase suggest that all preparations elicited antibody production against the enzyme moiety of the constructs (Fig. 2A–D). Moreover, judging from the IgG titers from plates coated with each of the enzyme preparations, there was cross-reactivity with antisera obtained with all preparations. However, titers appeared consistently highest when plates were coated with the native enzyme and were, generally, reduced as the degree of polysialylation of the coating antigen increased (Fig. 2A–D and legend). A similar reduction in the antigenicity of the enzyme with increasing degree of polysialylation was also apparent for IgM (data not shown).

A possible reason for the lower anti-asparaginase antibody titers seen in ELISA plates coated with the polysialylated preparations is that the presence of polysialic acid interferes with the ability of these preparations to adsorb onto the plates. This, however, was deemed unlikely since polysialic acids per se do adhere onto polystyrene microtiter plates (Jennings and Lugowski, 1981). Results thus suggest that polysialic acid chains on asparaginase sterically hinder the binding of IgG

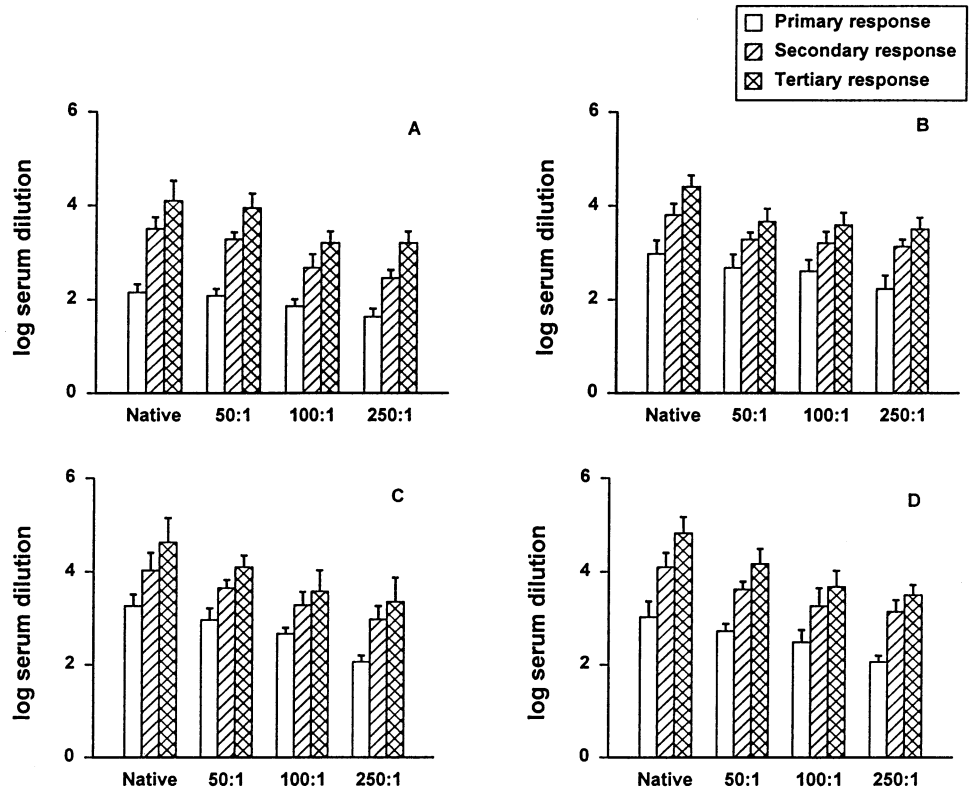

Fig. 2. The effect of coating antigen on IgG titers. Antisera raised in experiment of Fig. 1 against native (A) and polysialylated asparaginase prepared with 50:1 (B), 100:1 (C) and 250:1 (D) CA:asparaginase molar ratios in the coupling reaction were sequentially tested by ELISA in plates coated with each of the antigenic asparaginase preparations (shown in the abscissa). Results are mean ± S.D. of four or five animals. Statistics: antibody titers for each preparation in A–D were compared by ANOVA and P-values were corrected by the Bonferroni method. For clarity, only the significant differences (tertiary response) between each of the coatings with polysialylated asparaginase to the coating with native enzyme, are presented. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

molecules to the relevant antigenic sites, leading in turn to reduced enzyme antigenicity. This is consistent with the finding (Roitt, 1994) that immune complexes are formed by spatial complementarity and that the forces that bind antigen and antibody together are weakened by an increased distance between the two entities. Another factor that may have contributed to the reduction of antigen–antibody binding could be the generation of repulsive forces due to the negative charge of the enzyme-bound polysialic acids. In agreement with our results, Marshall (1978) and Miyata et al., (1988) were able to lower the antigenicity of dextran-conjugated α-amylase and superoxide dismutase, respectively, although immunogenicity was not suppressed.

3.3. Pharmacokinetics of asparaginase in immune mice

Antibodies raised against protein drugs can be neutralizing or non-neutralizing (Working, 1992). Neutralizing antibodies lead to the loss of biological properties of the protein, including its therapeutic action. This is effected either by the binding of antibodies to the protein's active site or to a different site but altering the protein's tertiary structure. With non-neutralizing antibodies the

therapeutic efficacy of the protein may also be impaired as a result of rapid clearance of the immune complexes formed (Working, 1992). In view of such findings, it was of interest to study the pharmacokinetics of native and polysialylated asparaginase in mice, previously immunized with the corresponding enzyme preparations. At first, T/O (outbred) mice were immunized by the intravenous route (tail vein) on days 0, 7 and 14 with native or polysialylated asparaginase. However, granulomas appeared in the tails of the animals, regardless of the treatment. After the course of immunization, the tail veins appeared collapsed, thus preventing the withdrawal of blood at time intervals and the study the enzyme's blood clearance (interestingly, no adverse effects were observed on intravenous immunization of Balb/c mice with the same preparations). T/O mice were, therefore, immunized intramuscularly, according to the schedule described in Section 2.4. Although the development of immune responses is known to depend not only on the animal species (Van Regenmortel, 1992) but also on the strain (Working, 1992) as well as the route of administration, (e.g. asparaginase is more immunogenic in humans by the intravenous than the intramuscular route (Keating et al., 1993)), antibody levels (Table 1) for T/O outbred mice immunized intramuscularly were not different than those (Fig. 1) seen in intravenously immunized Balb/c mice. As observed with animals immunized intravenously, after the third injection, differences between IgG titers obtained with the unmodified and polysialylated asparaginase (Table 1) were not significant.

Animals sensitized with a given (native or polysialylated) asparaginase preparation (all groups of animals had similar antibody titers, regardless of the preparation used; Table 1) were then injected intravenously with 1 mg of the corresponding tritiated preparation. Similarly to what was observed previously in naive animals injected with identical preparations (Fernandes and Gregoriadis, 1997), both native and polysialylated asparaginase exhibited a biphasic clearance of tritium (Fig. 3A) and asparaginase activity (Fig. 3B), consistent with a two-compartment distribution model. Immune clearance probably contributed to the initially faster removal of the enzyme from the circulation (α phase) of pre-immunized mice, since all animals had significant antibody titers against the homologous antigen. For example, 30 min post-injection only 28% (Fig. 3B) of native asparaginase was present in the circulation of immune mice compared to a value of 37% (Fernandes and Gregoriadis, 1997) in naive animals. Immune clearance was less marked for the polysialylated enzyme and decreased with increasing polysialylation. Thus, 30 min after injection, values of enzyme presence in the blood of immune animals (Fig. 3) injected with preparations 50:1 and 100:1 were 31% and 42% respectively, as compared with corresponding values of 46 and 51% in naive animals (Fernandes and Gregoriadis, 1997). Preparation 250:1 however did not appear to suffer immune clearance, as ascertained by the same percentage value (48% at 30 min) in naive (Fernandes and Gregoriadis, 1997) and immune (Fig. 3) animals. The pharmacokinetics of enzyme clearance is summarized in Table 1: mice immunized with native asparaginase, cleared the same enzyme from the plasma much more rapidly ($t_{\frac{1}{2}}\beta = 7.04$ h) than did naive animals ($t_{\frac{1}{2}}\beta = 15.27$ h). Moreover, blood enzyme

Table 1
Terminal half-lives of asparaginase injected intravenously into pre-immunized mice[a]

Preparation	Log IgG titers	$t_{\frac{1}{2}}\beta$ (h)	
		Immune mice	Naive mice[d]
Native	3.98 0.38	7.04 0.41	15.27
50:1	3.88 0.29	22.28 0.60	23.46 1.32
100:1	3.88 0.15	24.91 1.97	27.19 3.05
250:1	3.51 0.25	27.50 3.04	37.76 1.39
Significance	n.s.[b]	$P = 0.013$[c]	$P < 0.01$
			−0.001

[a] T/O mice in groups of four immunized intramuscularly with native or polysialylated (50:1, 100:1, 250:1) asparaginase (3×20 μg) were injected intravenously with 1 mg of the corresponding enzyme preparations. Results of IgG titers (after the third injection) and terminal half-lives denote mean ± S.D.

[b] ANOVA (native vs. other groups).

[c] Kruskal–Wallis (native vs. other groups).

[d] Data from Fernandes and Gregoriadis (1997).

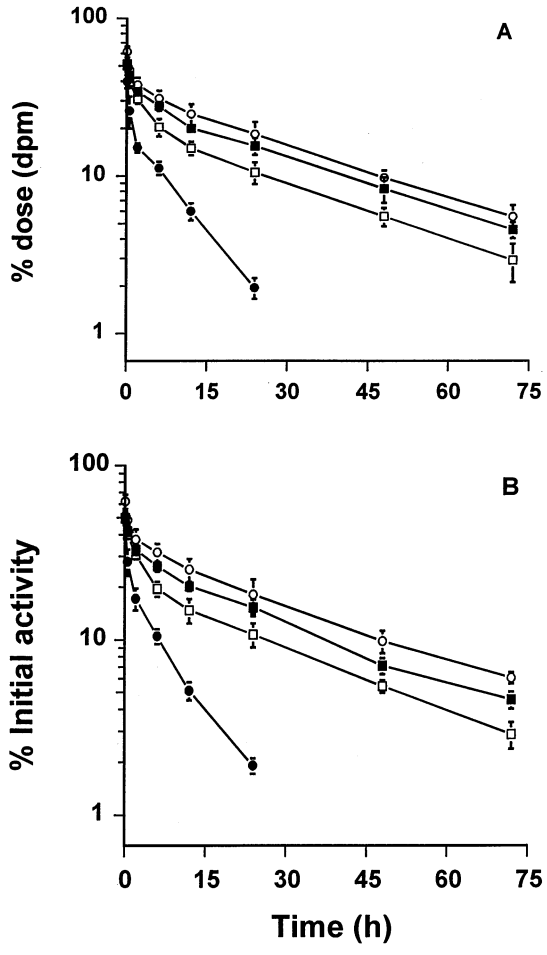

Fig. 3. Clearance of asparaginase from the blood circulation of pre-immunized mice. Animals were intravenously injected with radiolabelled native (●) and polysialylated asparaginase prepared with 50:1 (□), 100:1 (■) and 250:1 (○) CA:asparaginase molar ratios in the coupling reaction. Plasma was assayed for residual ^{3}H label (A) and asparaginase enzyme activity (B). Results are mean ± S.D.; $n = 4$ animals. Native asparaginase (^{3}H and enzyme activity) was not detectable after 24 h.

levels were undetectable beyond 24 h post-injection in immune mice (Fig. 3) whereas in naive animals, 3% of the injected asparaginase activity was still present after 48 h (Fernandes and Gregoriadis, 1997). Polysialylated preparations, on the other hand, circulated in the blood of immune animals for longer periods of time (3–4-fold greater half-lives ($t_{\frac{1}{2}}\beta$)) in comparison with the native enzyme ($P = 0.013$, Table 1), with half-lives of two of the preparations being almost identical to those in naive animals (Table 1).

The presence of anti-asparaginase antibodies in the blood limits the chronic use of asparaginase in antitumour therapy (Goldberg et al., 1973) either as a result of allergic reactions or because of reduced (in direct relation to the antibody titers) residence of the injected enzyme in the blood circulation (Wahn et al., 1983). For instance, asparaginase coupled to poly-(DL-alanine) was cleared rapidly (as immune complexes) from the blood of highly immune animals whereas in mice with low antibody titers, the modified enzyme circulated for extended periods albeit not as extended as in the naive animals (Uren and Ragin, 1979). Native asparaginase in immune mice was short-lived, independently of the level of antibody titers (Uren and Ragin, 1979). As already discussed, immune clearance can account for the initial faster removal of the polysialylated enzyme from the blood of pre-immune mice. However, it is of interest that, in the presence of similar sera IgG titers (Table 1), native asparaginase is removed much faster than any of the polysialylated preparations (Fig. 3). This could be explained by the reduced antigen-antibody affinity anticipated from the ELISA assays for the latter preparations. It is conceivable that the chains of CA grafted to the protein, prevent anti-asparaginase antibodies from interacting with the antigen effectively. In vivo, this would lead to blood clearance levels that are more similar to those observed in naive mice. Moreover, once formed, circulating immune complexes of polysialylated asparaginase may escape interception by the MPS due to a shielding effect of the CA chains, similarly to what is believed to occur with the polysialylated asparaginase in naive mice (Fernandes and Gregoriadis, 1997). Since asparaginase activity (at least 50% of the activity of the intact enzyme) appears to be maintained in the immune complexes (Peterson et al., 1969, 1971), these could by themselves be therapeutically effective.

Although immunological response to both native and polysialylated asparaginase was developed, allergic reactions upon repeated challenge with the antigens were not observed. The lack of

success in abrogating the immunogenicity of polysialylated asparaginase is probably due to the relatively low degree of polysialylation. The present results are, however, encouraging in that they suggest a decrease in antigenicity on polysialylation in vitro (ELISA) as well as in vivo. Since both half-life in the circulation and antigenicity appear to be related to the degree of polysialylation, it is legitimate to expect that a greater degree of enzyme polysialylation will further increase the former and reduce the latter. The enhanced biological properties shown by the polysialylated asparaginase, particularly improved resistance to proteolysis (Fernandes and Gregoriadis, 1997), extended half-lives in naive (Fernandes and Gregoriadis, 1997) and immune animals (Fig. 3) and, as Table 1 and Fig. 3 suggest, reduced antigenicity in vivo, could contribute to improve therapeutic efficacy. Investigation of the anti-tumour efficacy of the polysialylated constructs is therefore warranted.

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