Modulation of Antibody Pharmacokinetics by Chemical Polysialylation

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Chemical coupling of a variety of polymers to therapeutic proteins has been studied as a way of improving their pharmacokinetics and pharmacodynamics *in vivo*. Conjugates have been shown to possess greater stability, lower immunogenicity, and a longer blood circulation time due to the chemicophysical properties of these hydrophilic long chain molecules. Naturally occurring colominic acid (polysialic acid, PSA) has been investigated as an alternative to synthetic polymers such as poly(ethylene glycol) (PEG) due to its lower toxicity and natural metabolism. Antibodies and their fragments are a good example of the types of proteins which benefit from pharmacokinetic engineering. Here, we chemically attached differing amounts and differing lengths of short (11 kDa) and longer (22 kDa) chain colominic acid molecules to the antitumor monoclonal antibody H17E2 Fab fragment. Different coupling ratios and lengths were seen to alter the electrophoretic mobility of the Fab fragment but have a minor effect on the antibody immunoreactivity toward the placental alkaline phosphatase (PLAP) antigen. Polysialylation generally increased Fab fragment blood half-life resulting in higher tumor uptake in a KB human tumor xenograft mouse model. One H17E2 Fab-PSA conjugate had over a 5-fold increase in blood exposure and over a 3-fold higher tumor uptake with only a marginal decrease in tumor/blood selectivity ratio compared to the unconjugated Fab. This conjugate also had a blood bioavailability approaching that of a whole immunoglobulin.

INTRODUCTION

The use of polymers to modulate the *in vivo* pharmacokinetics of proteins and drugs for therapeutic applications has been shown for many conjugates to enhance their efficacy (*1, 2*). The primary objective of such conjugation is normally to extend the longevity of therapeutic agents in circulation that would otherwise be cleared too rapidly by renal clearance to give a larger therapeutic window. However, other advantages in polymer conjugation include the masking of antigenic sites that would cause clearance by a variety of immune responses, as well as providing stabilizing features that reduce the risk of degradation under physiological conditions or by protease activity (*3, 4*).

The synthetic polymer poly(ethylene glycol) (PEG) represents the most extensively used and investigated form of polymer conjugation for pharmacokinetic engineering of small proteins or drugs (reviewed in (*1*)). Successful examples in the clinic include Oncaspar (PEG-asparaginase) for the treatment of lymphoblastic leukemia (*5*), PEGasys (PEG-interferon α−2a) for the treatment of chronic Hepatitis C infections (*6*), and Macugen (PEG-aptanib sodium), for the treatment macular degeneration (*7*).

Although considered biologically inert moieties, there have been concerns regarding the long-term safety of PEG *in vivo*, and whether chronic accumulation of PEG over prolonged use could lead to toxicity (*4, 8, 9*). Small length polymers of PEG have demonstrated renal clearance; however, larger molecular weight conjugates cannot be easily broken down through any known natural pathway, and there is evidence to suggest that they can accumulate in tissues once they have been taken up by cells through phagocytic processes. Furthermore, exposure from repeat and/or high doses has been shown to elicit an immune response which may lead to problems where long-term treatment is required (*10, 11*).

Recently, polysialic acid (PSA) has been investigated as an alternative to PEG (reviewed in (*12*)). Naturally occurring PSA polymers are carbohydrate chains that have been identified on the surface of a variety of cells from microbes to vertebrates. A specific form of this polymer, colominic acid, is a homopolymer of α-2,8-linked 5-*N*-acetylneuraminic acid (Neu5Ac) residues normally associated with the bacterial capsule of neuroinvasive bacteria such as *Escherichia coli* K1 (*13*). Gregoriadis et al. was the first to postulate and pioneer the use of PSA (colominic acid) as an alternative to PEGylation for conjugation to improve the stability and *in vivo* pharmacokinetics of small drugs and proteins (*14, 15*). Reductive amination of the nonreducing end of the polymer allowed chemical conjugation via the primary amine groups on proteins. In this manner, the therapeutic benefits of PSA conjugation have been demonstrated with aspariginase (*16*) and insulin (*17*) for the treatment of leukemia and diabetes, respectively, and both have shown an increase in enzyme stability and increased serum half-lives. The prolonged residency of polysialylated conjugates increases the potential of the species to reach its target and provide a therapeutic effect before being cleared from circulation, hence extending the duration of action. It has also been demonstrated that, by varying the length of the PSA chain, the number of PSA chains per conjugate, and the PSA conformation used, the half-life of the of the conjugate can be modulated for

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optimal therapeutic affect (*17*). Furthermore, a study of immunogenicity involving PSA-conjugated and native asparaginase demonstrated that only the native enzyme elicited an immune response in mice, while polysialylated asparaginase. despite having a 3–4-fold longer clearance rate, did not elicit an immune response, suggesting that PSA conjugation can mask antigenic epitopes (*18*). In addition, given that the source of PSA consists of α-2,8-linked 5-*N*-acetylneuraminic acid, which is the natural component of PSA in mammals, it is not thought that the polymer on its own would elicit an immune response in humans.

Although Siglec and Selectin receptors that recognize sialic acid containing glycans are known to exist, ligand recognition appears to be dependent on neighboring sugar residues within the glycan, and as yet, no receptors with PSA specificity have been identified (reviewed in (*19*)). The apparent lack of PSA receptors and low immunogenicity provides molecular stealth and adds to the nontoxic properties of PSA. PSA conjugates must be desialylated by neuraminidase-like enzymes before they can be cleared via the hepatic asialoglycoprotein receptor and metabolized (*20*).

The biodegradable, nontoxic, and highly hydrophilic properties of PSA are therefore being exploited to increase the serum half-life and consequently the action of small peptides and drugs. These observations are of particular pharmaceutical interest, since polysialylation offers a potentially superior method over current conjugation methods using PEGylation. Potentially, the greatest advantage in increasing the longevity of therapeutic agents would be where long-term specific activity was desired, and could be of immediate benefit to already commercially available therapeutic agents such as the neutralizing antibodies Remicade (Infliximab), used to treat rheumatoid arthritis (*21*), and Avastin (*22*), used in many solid cancers. Also, the conjugation technology could be applied to other common therapeutic agents where improved longevity pharmacokinetics would be beneficial such as insulin (*23*) and erythropoietin (*24*) for the treatment of diabetes and anemic conditions, respectively.

In this study, an analysis of the effect of protein polysialylation was investigated using a Fab antibody fragment. This fragment, which is specific for the placental-like alkaline phosphatase (PLAP) antigen associated with germ cell carcinomas (*25, 26*), was modified and immunoconjugates compared *in vitro* and *in vivo*. Our results indicate polysialylation can extend the half-life of antibody fragments leading to improved tumor uptake without compromising on tumor to tissue specificity.

EXPERIMENTAL PROCEDURES

Materials. H17E2 IgG was a gift from Professor Lemoine (CRUK). Polysialic acid (colominic acid) was from Marukin, Japan, supplied by Sigma, UK, and Camida, Ireland. PLAP antigen was supplied by Calzyme, Inc.; PLAP positive KB cell-line supplied by the EACC and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and 50 IU/mL of penicillin and streptomycin (Invitrogen). Unless stated, immunochemicals were from Sigma. Mouse anti-PSA-NCAM monoclonal IgM was from Chemicon. Carrier-free ^{125}I-NaCl was supplied by GE Healthcare, UK.

Production and Purification of H17E2 Fab. H17E2 Fab fragments were derived by papain cleavage using the Immuno Pure Fab preparation kit (Pierce), following the manufacturers instructions. The solublized Fab and Fc fragments were recovered and separated by Protein A. Proteins were further purified using a HiLoad 16/10 Superdex-200 HR 10/30 gel filtration column equilibrated with PBS and compared to previously run protein standards. Samples were further analyzed for the presence of protein by Coomassie staining and Western blotting.

Chemical Polysialylation. The PSA used in this work was colominic acid (linear α-(2,8) linked *N*-acetylneuraminic acid) purified from the capsular coating of *E. coli* K1, supplied in alternative different lengths, 11 and 22 kDa (average molecular weights), which are equivalent to ∼35 and 71 units of sialic acid, respectively. The average molecular weight (nominal mass) of these polymers was determined independently by Viscotek Europe Ltd. (Lipoxen, unpublished; (*17*)).

For the purpose of protein−PSA conjugation by reductive amination, PSA was oxidized with 0.1 M periodate as described (*17*). The oxidized material was dialyzed extensively against a 0.01% ammonium carbonate buffer (pH 7.4) at 4 °C, concentrated by reverse dialysis on a bed of poly(ethylene glycol) (8 kDa), lyophilized, and stored at −80 °C until required. Oxidized PSA was added to 5 mL protein samples to give a 25-fold molar excess in a glass tube. For lower coupling ratios, a 2- to 5-fold molar excess was used. The mixture was mixed gently and supplemented with 20 mg $NaCNBH_3$. The tube was sealed and rotated at 37 °C for 48 h to allow the conjugation reaction to take place whereupon ∼100% conjugation was expected. After incubation, the sample was allowed to cool and the conjugated material was purified from free PSA by addition of $(NH_4)_2SO_4$ to give a 70% saturated solution. The sample was rotated for 5 min at room temperature followed by 30 min on ice. The mixture was then centrifuged at 5000 rpm for 5 min and the resulting pellet resuspended in 1 mL 100% $(NH_4)_2SO_4$. The precipitation procedure was then repeated and the pellet resuspended in minimal PBS. Finally, the conjugated sample was analyzed by PAGE and characterized for polysialylation degree by a resorcinol assay.

Determination of PSA/Fab Ratios. The resorcinol assay, based on the method by Svennerholm (*27*), allowed the amount of colominic acid present in a sample relative to known standards to be measured. The resorcinol agent (0.25 mM cupric sulfate, 0.2% resorcinol, 80% conc HCl made up with dH_2O) was freshly prepared, and at least five colominic acid standards were prepared within the 40–400 μg/mL range. In triplicate, 100 μL of standard and test samples were added to 100 μL of resorcinol agent. The tubes were sealed and the mixture heated for 30 min at 95 °C. Once the tubes had cooled down, 400 μL of ethanol was mixed in. Finally, the absorbance at 560 nm was measured for 250 μL of each sample and standards in a microplate assay. The standard values were used to construct a calibration curve from which the colominic acid content of the test samples could be extrapolated. A molar value was determined according to the PSA length used in test samples, and similarly a protein molar value determined for samples using the Coomassie Plus protein assay kit compared to protein standards. Using these values, a PSA to protein molar ratio was calculated.

Western Blot Analyses. Protein gels for Western blot analysis were transferred to nitrocellulose membrane, blocked in PBS/5% Marvel milk protein made, and then incubated with either anti-mouse Fc-specific or anti-mouse Fab-specific one-step HRP-conjugated antibody. The blot was developed using enhanced chemiluminescence (ECL, GE Healthcre, UK) and exposed to high-performance autoradiography film.

Protein Radio-Iodination. Protein samples were iodinated using the IODO-GEN Iodination Reagent. To precoated tubes, 100 μL of the Tris iodination buffer was added, followed by 10 μL (1.0 mCi) $Na^{125}I$. The activated iodide was then removed and added directly to the protein solution for iodination. The reaction was allowed to take place for 8 min. To end the reaction, 50 μL of scavenging buffer (10 mg/mL tyrosine in PBS) was added and mixed. After 5 min incubation, the iodinated sample was dialyzed against PBS.

Conjugate Stability Assay. Blood was collected from mice, centrifuged at 13 000 rpm for 30 min, and serum was siphoned off for use in protein stability assays. ^{125}Iodine-labeled samples were mixed with an equal volume of serum and incubated at 37 °C over a 24 h period. Aliquots were taken at 0, 0.5, 2, 6, and 24 h points, mixed with SDS-PAGE loading buffer, and frozen until all samples were ready for analysis by SDS-PAGE and autoradiography.

Bioactivity by ELISA Analysis. Flat-bottom 96-well ELISA plates coated with PLAP antigen at 1 μg/mL in PBS were used. After three washes with PBS, one wash with PBS/0.1% Tween, and three more PBS washes, the plate was then blocked with 5% Marvel for 1 h at 37 °C and washed again. Binding activity of the anti-PLAP Fab and conjugates was established by serially diluting samples across test wells in 1% Marvel/PBS for 1 h at 37 °C. For the purpose of testing Fab purity following papain cleavage of whole H17E2 antibody, the plate was then washed as above and incubated with the either antimouse Fc-specific or antimouse Fab-specific one-step HRP-conjugated antibody. For the purpose of PSA detection, the plate was incubated first with primary anti-PSA IgM followed by secondary anti-IgM HRP for 1 h 37 °C. In both cases, incubation with detection antibodies was terminated by washing the plates as described above and binding detected using BM Blue POD substrate (Roche, UK). The reaction was stopped with 10 μL of acid (1 M H_2SO_4) and the absorbance measured at 450 nm.

Sigmoidal curves (four-parameter logistic) were fitted to ELISA data to determine K_d values using *SigmaPlot* using the one-site saturation ligand binding equation.

Pharmacokinetic and Biodistribution Studies. Female nude BALB/C mice, 6–8 weeks old, used for *in vivo* studies were from Harlan UK. All *in vivo* research was carried out under a UK Home Office project license PPL 70/5833. Tumour xenografts were set up by injecting mice subcutaneously into the left flank with 0.1 mL containing up to 10 million KB cells. Tumor growth was monitored and took 3–4 weeks to reach the required 6–8 mm diameter for subsequent work. For tissue analyses, up to 100 μL radiolabeled samples were injected intravenously into the mouse tail vein. At the appropriate time points (typically 0.5, 2, 6, 24, and 48 h), mice were culled by exsanguination under terminal anesthesia. Immediately, blood was collected by cardiac puncture and transferred to a sample tube. The mouse was then dissected, and tissues were collected for biodistribution analysis. Radioactivity was counted using a gamma counter. Values were expressed as a percentage of the initial injected dose per gram of tissue (% id/g). For studying *in vivo* blood clearance pharmacokinetics, data values were fitted using *SigmaPlot* to curves that conform to the two-compartmental intravenous model of clearance, which takes into account the biexponential clearance phases, distribution phase and elimination phase, of single intravenous doses. This is described by the exponential decay, double, four-parameter equation $y = ae^{-bx} + ce^{-dx}$, where the distribution phase clearance rate ($t_{1/2\alpha}$) can be determined by ln 2/b, and the elimination clearance rate ($t_{1/2\beta}$) can be determined by ln 2/d. The area under the curve values were determined using the macros available within *SigmaPlot*.

RESULTS

H17E2 Fab Polysialylation. H17E2 Fab that was free from contaminating Fc portion was used for polysialylation (Figure 1A). Anti-Fab and anti-Fc Western blot confirmed that pure Fab fragment had been obtained (Figure 1B,C). Polysialylations were performed with the 11 and 22 kDa polymers. The PSA/Fab molar ratios for each set of immunoconjugates are shown in Table 1. There are 26 lysine residues available, but not all were expected to be accessible. PAGE analyses of the Fab, Fab-PSA11, and Fab-PSA22H under native conditions showed that both immunoconjugates migrated as a polydispersed smear of higher molecular weight species compared to the unmodified Fab (Figure 1D). This was confirmed by the anti-Fab Western blot which showed a small amount of free noncoupled antibody not visible by Coomassie staining (Figure 1E). A different result was seen when detected with the anti-PSA antibody (Figure 1F): the polydispersed species was again evident, but the larger mass PSA immunoconjugate seemed to migrate more rapidly, possibly due to the higher charge. It is clear from these analyses that polysialylation had altered the size and migration properties of the H17E2 Fab, but their polydispersity and heterogeneity hindered a quantitative analysis of the mass and charge properties.

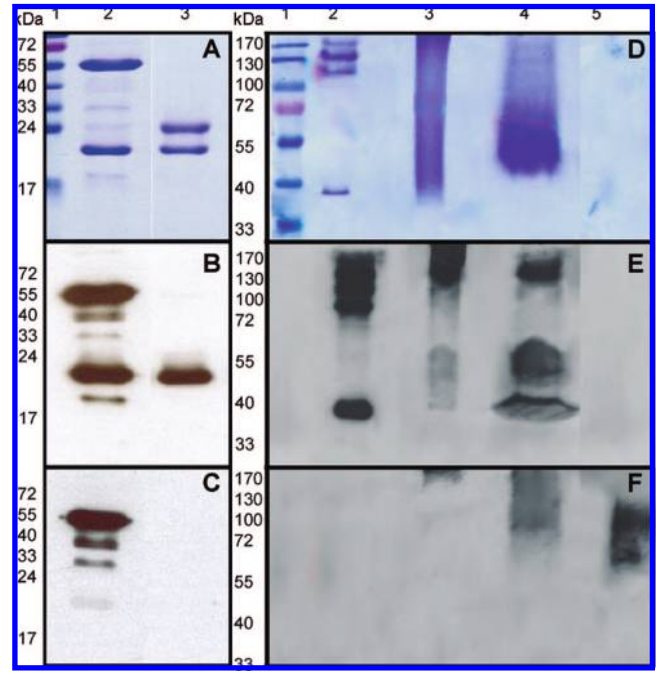

Figure 1. Electrophoretic analyses of H17E2 IgG, Fab, and PSA-Fab conjugates. Left panel: SDS PAGE of markers (lane 1), whole IgG (lane 2), and papain-derived Fab fragments (lane 3) under reducing conditions were analyzed by (A) Coomassie staining, (B) Anti-Fab Western detection, and (C) Anti-Fc Western detection. Right panel: Markers (lane 1), Fab alone (lane 2), and Fab-PSA conjugates (FabPSA11 (lane 3), FabPSA22H (lane 4), and Free PSA22 (lane 5)) were compared by PAGE under native conditions and analyzed by (D) Commassie staining, (E) Anti-Fab Western detection, and (F) Anti-PSA Western detection. Long trail lines observed in conjugates reflect the polydisperse nature of PSA content.

Table 1. Nomenclature and Coupling Ratios of H17E2 Fab-PSA Conjugates[a]

PSA (kDa)	average PSA/Fab molar ratio	abbreviated name
11	9	Fab-PSA11
22	1	Fab-PSA22L (low)
22	5	Fab-PSA22H (high)

[a] The ratios were determined from the resorcinol (PSA) and Coomassie (protein) assays.

Antigen Binding of Immunoconjugates. ELISAs confirmed that the unmodified H17E2 IgG and Fab had binding affinities (K_ds) similar to that already published (*28*). These were compared to the affinities of the PSA immunoconjugates (Figure 2, Table 2). Binding was detected using anti-Fab (Figure 2A) and anti-PSA detection (Figure 2B) antibodies. For the Fab-PSA11 and Fab-PSA22H immunoconjugates detected by anti-Fab antibody, the affinities were slightly reduced by 3.7- and 2.7-fold, respectively.

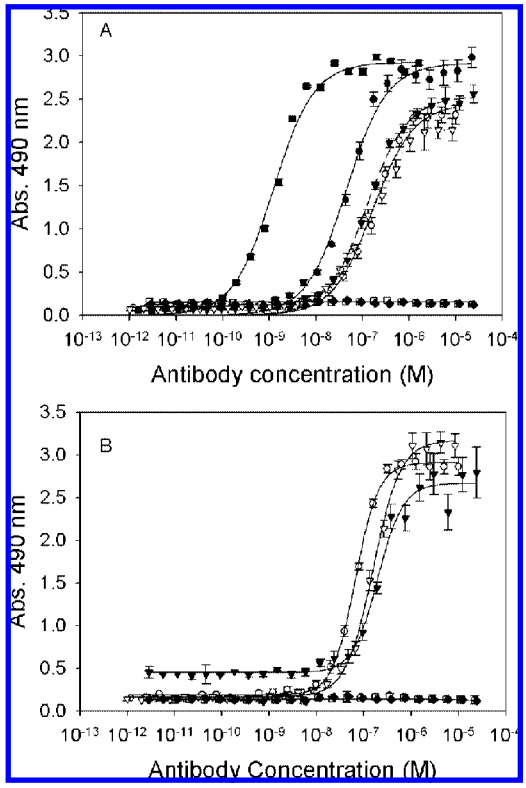

Figure 2. Antigen-binding activity of H17E2 IgG, Fab, and Fab-PSA conjugates. Whole IgG (■), Fab-PSA11 (▽), Fab-PSA22L (○), and Fab-PSA22H (▼) were compared against unconjugated Fab (○) by ELISA. (A) Binding activity was detected by anti-Fab and (B) the presence of PSA on conjugates detected by anti-PSA. Samples were analyzed in triplicate and included control samples MFE-23 (♦), a nonanti-PLAP scFv (detected anti-His), and free PSA22 (□). K_d values were determined by *Sigma Plot*.

Table 2. Immunoreactivity of the H17E2 Fab and PSA Conjugates[a]

antibody	anti-Fab detection K_d (nM)	anti-PSA detection K_d (nM)
unmod. Fab	45	NA
Fab-PSA11	149	61
Fab-PSA22L	145	127
Fab-PSA22H	122	153

[a] The equilibrium dissociation constants for H17E2-PLAP binding were determined by ELISA with both the protein and carbohydrate moieties being detected (Figure 2).

Detection by anti-PSA antibody confirmed that polysialylation had occurred showing a small (1.3–3.4-fold) decrease in affinity. Overall, the addition of long polymers of PSA did not significantly affect the affinity of the Fab antibodies, and the K_ds of the species under study were all very similar among themselves.

Serum Stability. In order to rule out the possibility of protein or carbohydrate degradation occurring, a serum stability assay was carried out *in vitro* using murine serum incubated with a radiolabeled immunoconjugate at 37 °C. Over a 24 h period, no change in the SDS-PAGE banding profile was observed for either the Fab or Fab-PSA22H immunoconjugate, indicating that they were stable in serum (Figure 3).

***In Vivo* Pharmacokinetics.** The effect of polysialylation on the pharmacokinetic behavior of the H17E2 Fab was studied by analyzing blood samples at various time points after IV injection of Fab-PSA conjugates. Data from nude BALB/C mice and tumor-bearing nude BALB/C mice were obtained and pooled. Due to the nature of the coupling chemistry, it was not possible to produce exactly matched immunoconjugates of

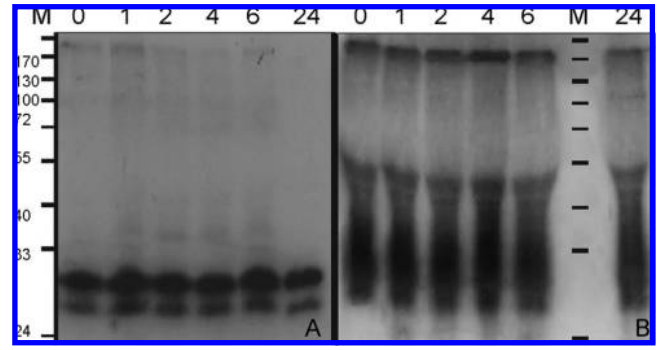

Figure 3. Stability assay of ^{125}I- radiolabeled (A) Fab and (B) Fab-PSA22H samples were incubated with mouse serum at 37 °C. Samples were taken at 0, 1, 2, 4, 6, and 24 h time points and analyzed by SDS-PAGE under reducing conditions. Protein was visualized by exposure of the gel to autoradiographic film.

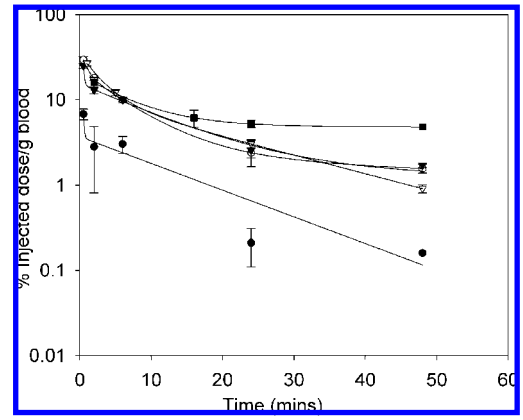

Figure 4. Blood clearance analyses of H17E2 IgG, Fab, and Fab-PSA conjugates. Radioactive cpm values for each sample were measured and calculated as a percentage of the injected dose per gram of blood. Each time point is represented by the mean ± SD of between 3 and 6 mice. The blood clearance of H17E2 (■) whole IgG and (•) Fab is compared graphically with (○) Fab-PSA11, (▽) Fab-PSA22L, and (▼) Fab-PSA22H.

predefined coupling ratios (Table 1). The immunoconjugate present in the blood calculated as the percentage of remaining radioactivity per gram of blood was plotted against time (Figure 4). Pharmacokinetic parameters such as beta-phase clearance rates and total blood exposure (as measured by the area under the curve) were determined and are summarized in Table 3. The stability of the conjugate in serum indicated that the radioactivity represented the intact Fab-PSA conjugate. The results show that, in all cases, polysialylation generally leads to longer blood clearance times compared to the unmodified Fab, resulting in higher blood exposure and hence greater bioavailability. This modulatory effect was generally related to increasing degree of polysialylation, with the Fab-PSA11 having the greater $t_{1/2b}$ (3.2-fold increase compared to Fab) and a high tumor exposure (2.8-fold increase compared to Fab). Similar mass immunoconjugates (Fab-PSA11–150 kDa and Fab-PSA22H-160 kDa) had broadly similar pharmacokinetic properties. Increasing the degree of polysialylation 5-fold using the same length polymer chain (22 kDa) led to an almost 2-fold increase in beta-phase blood clearance half-life with little change in antibody affinity (Figure 2, Table 2). Compared to whole immunoglobulin, which has a very slow blood clearance profile, the immunoconjugates compared favorably, with blood bioavailability (exposure) almost as high as the whole antibody but with better tissue specificity ratios (see below) and potentially lower cross-reactivity. The alpha (distribution) blood

Table 3. Summary of the Pharmacokinetic and Biodistribution Data[a]

antibody	$t_{½(\alpha)}$ (h)	rel. to Fab	$t_{½(\beta)}$ (h)	rel. to Fab	blood AUC (% h/g)	rel. to Fab	tumor AUC (%h/g)	rel. to Fab	tumor/blood ratio @ 48 h	rel. to Fab
IgG	4.6	46	>72 h	>7.5	324	6.8	343	3.5	1.5	0.14
Fab	0.1	1	9.6	1.0	48	1.0	98	1.0	10.8	1.00
Fab-PSA11	1.7	17	30.5	3.2	249	5.2	273	2.8	5.3	0.49
Fab-PSA22L	2.0	20	17.1	1.8	223	4.7	178	1.8	2.6	0.24
Fab-PSA22H	0.7	7	29.1	3	201	4.2	319	3.3	3.5	0.32

[a] Pharmacokinetic beta-elimination constant, total blood exposure (blood area under the curve), total tumor exposure (tumor area under the curve), and tumor/blood selectivity (ratio) were determined from plots of the biodistribution data in Figures 4 and 5.

clearance rates are presented for guidance and are approximate, as only a few early time points were recorded.

Tumour and Tissue Biodistribution. The uptake or retention of the different immunoconjugates in tumors and tissues, measured as the percentage of injected radioactive material per gram, was determined and presented as an absolute value and a tissue/blood ratio (Figure 5). The key parameters are summarized in Table 3 with total tumor exposure as determined from the area under the curve. These were compared to whole IgG and unmodified Fab. As seen for other whole immunoglobulins, the H17E2 IgG exhibited at least a 7.5-fold increase in blood retention resulting in a 3.5-fold increase in tumor uptake compared to the H17E2 Fab. The tumor/blood ratios, typical for IgGs, were poor with a value greater than 1 not being reached until after 16 h. The Fab demonstrated rapid clearance leading to lower absolute uptake with high tumor/blood ratios (greater than 10) by 24 h.

The Fab-PSA22H immunoconjugate, which had one of the slowest blood clearances, yielded the highest absolute uptake, 3.3-fold higher than the unmodified Fab and approaching that of the whole IgG. The tumor/blood ratio was superior to that of whole IgG and only one-third that of Fab (3.5:1), demonstrating improved selectivity (compared to whole immunoglobulin) toward the tumor with almost as good tumor exposure as whole immunoglobulin. The Fab-PSA11 conjugate, although not as effective as the Fab-PSA22H immunoconjugate, still had higher tumor uptake and better specificity ratios compared to the Fab, resulting from the altered blood pharmacokinetics, which was slightly slower than that of the Fab-PSA22H immunoconjugate. Generally, increased blood exposure led to increased tumor uptake. Increasing the PSA content 5-fold (Fab-PSA22L and Fab-PSA22H) led to an almost 2-fold increase in beta-phase blood clearance rate and a 1.8-fold increase in tumor exposure.

There was no significant uptake by any of the normal tissues, showing that polysialylation had not resulted in undesirable and nonspecific cross-reaction.

DISCUSSION

This research showed that antibody fragments such as the anti-PLAP H17E2 Fab species can be polysialylated to different degrees with little significant change to the binding affinities. The polysialylated immunoconjugates migrated as highly charged heterogeneous species by gel electrophoresis. This is due to the highly heterogenic nature of the naturally isolated colominic acid. Further refinement of the colominic acid purification process to generate species with a smaller range of molecular weights should lead to the generation of more defined immunoconjugates. Polysialylation led to a modulation in the *in vivo* pharmacokinetics demonstrated by decreased blood clearance rates and increased blood bioavailability, tumor uptake, and tumor exposure values. This outcome is similar in principle to the effects of conjugation to other polymers (*29–31*).

The H17E2 anti-PLAP antibody/KB tumor xenograft model was chosen because of our past experience and the reliability of the antigen expression. The affinity of the monovalent Fab was approximately 40 nM, which is deemed moderate for typical recombinant antibodies. This affinity value, not being excessively high, permitted a study of the pharmacokinetic influence of polysialylation. It has been shown that very high affinity antibodies reach a ceiling in terms of tumor uptake at around 1 nM (*32*). The degree of polysialylation was determined using the resorcinol assay after repeated ammonium sulfate precipitation to remove the free, unconjugated PSA. Conjugations were performed at higher molar ratios giving higher subsitituted immunoconjugates, but this also resulted in a high degree of noncovalently associated PSA, which was difficult to remove by repeated ammonium sulfate precipitation. Polysialylation did have a small affect on the affinity of the Fab, but the affinities of the immunoconjugates being compared were all similar in value, allowing a valid comparison between the polysialylated species. Site-specific conjugation is an obvious extension of this approach, especially in cases where there are lysine residues in key positions (such as CDRs) where their modification could lead to loss of function (*33*). This was not a problem for the H17E2 antibody, but we have employed our approach with other antibody fragments and found a loss of binding (Constantinou et al., manuscript in preparation). Recombinant antibodies can easily be engineered to replace lysines which are detrimental to coupling (*33*) or engineered to possess one or more residues for site-specific coupling. One example is the use of cysteine residues for thiol coupling onto therapeutic Fab antibodies (*34*).

Gel electrophoresis suggested that the immunoconjugates' mobility was more influenced by the PSA charge properties than the Fab size and charge. The PSA polymers were supplied as polydispersed mixtures of an average molecular weight, which is reflected in the range of different mobilities when observed by gel electrophoresis. Anti-PSA Western blot again confirmed that the Fab had been covalently modified with the PSA polymer.

Prior to any *in vivo* analyses, it was important to show that the polysialylated immunoconjugates were stable in serum at 37 °C. This was indeed the case, as there was no change in the banding pattern across the times analyzed. During the time of the incubation, serum glycosidases did not remove the PSA chains from the conjugate, which would have been seen as a reduction in the banding pattern back to that of the unmodified Fab. The linkage of the PSA chain to the lysine residue of the protein was expected to be robust, as it consists of the stable secondary amine bond and is attached in the reverse orientation to that of naturally occurring PSA.

The pharmacokinetic values obtained for the control proteins such as the unmodified Fab and whole IgG were comparable to what had been published already (*28*). The immunoconjugates showed an up to 3.2-fold decrease in the blood clearance beta-rate compared to the Fab. This slower clearance leads to greater bioavailability (as measured by the area under the curve, AUC), key to prolonging the action of some drugs. However, the greatest blood bioavailability was seen for the Fab-PSA22L, 5.2-fold higher than for unmodified Fab and 80% that of the whole antibody. This was due to the much slower alpha clearance phase.

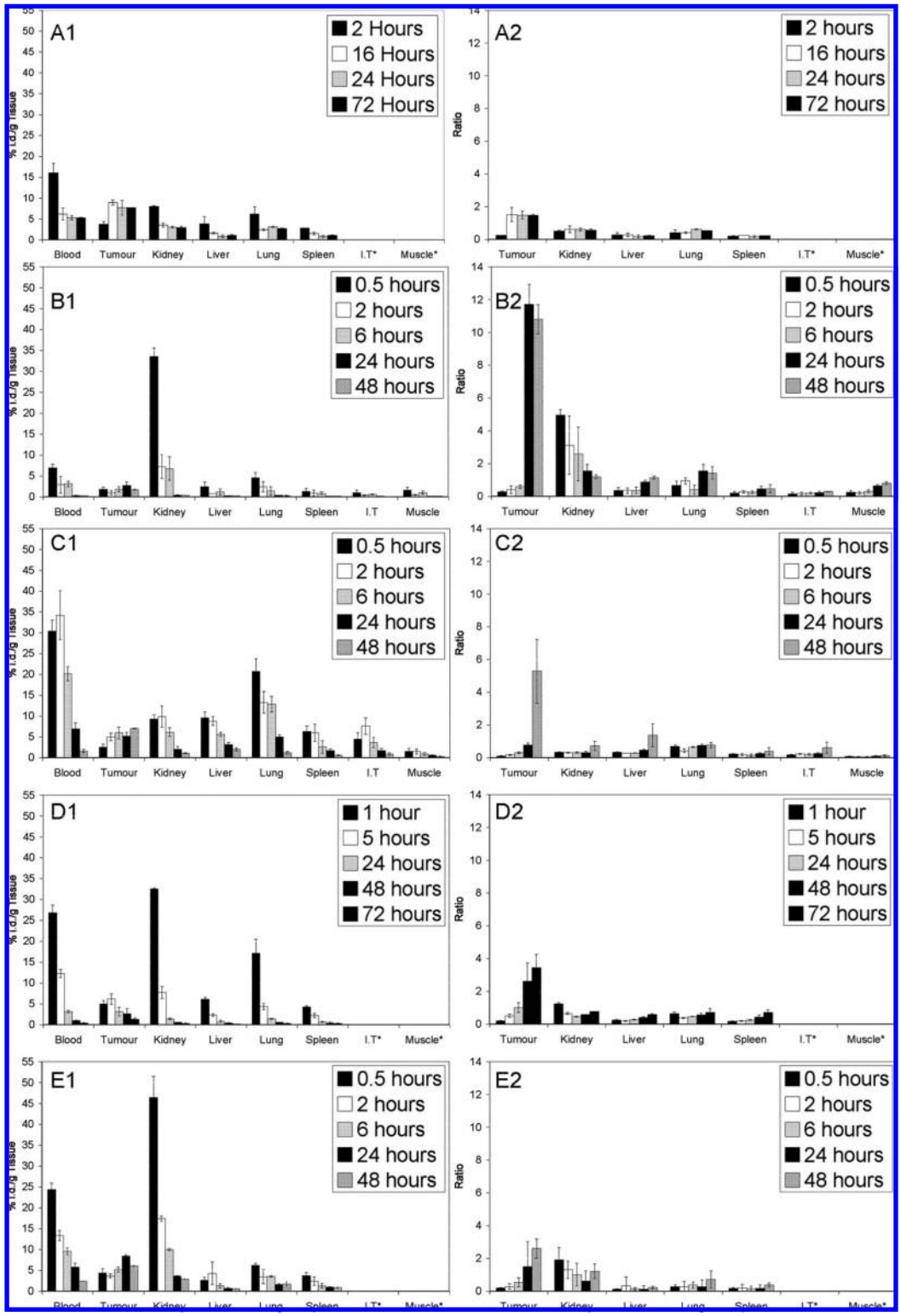

Figure 5. Biodistribution analyses of H17E2 IgG, Fab, and Fab-PSA conjugates. Radioactive cpm values for each sample were measured and calculated as a percentage of the injected dose per gram of tissue. Each bar represents the mean ± SD for three or four mice. Bar charts on the left show the absolute uptake values by each tissue over time, while bar charts on the right show the uptake by each tissue as a ratio to the amount of sample remaining in the blood over time. The bar charts show the biodistributions for (A) whole IgG, (B) unconjugated Fab, (C) Fab-PSA11, (D) Fab-PSA22L, and (E) Fab-PSA22H. IT* = intestinal tract.

In general, increased polysialylation led to increased bioavailability, which led to increased tumor exposure approaching that of whole immunoglobulin. The heterogenic nature of these conjugates makes it difficult to identify clear trends; therefore, this work points the way toward site-specific conjugation with polymers of a more defined nature. Polymer conjugation of antibody fragments is seen as a way to increase protein stability and bioavailability (*5–7, 29–31*) without the limitations of Fc-mediated cross reaction. In addition, less expensive protein production methods available for recombinant fragments such as *E. coli*- and *Pichia pastoris*-expressed single-chain Fvs and Fabs makes polymer conjugation more attractive practically and commercially. However, polysialylation may supersede PEGylation due to its more favorable properties.

Like PEGylation, polysialylation may achieve the observed effects by allowing the protein to possess large hydrodynamic radii by formation of a hydrophilic "watery cloud" (*35, 36*). This property slows down the blood clearance and prevents excretion through the kidneys. The *in vivo* data did show some high kidney radioactivity at the early time points, which could be attributed to free radioactive iodine which may have been complexed with the PSA and not removed during dialysis. This level quickly reduced. Polysialylation did not lead to aggregate formation, which would have been seen by high liver and spleen uptake caused by metabolism through the reticulo-endothelial system (Figure 5).

The increased blood residency time seen with all immunoconjugates all lead to a corresponding increase in tumor uptake without significant cross-reaction with normal tissues (Figure 5). This supports the premise that PSA is an inert polymer with no natural receptor. The pharmacokinetic distribution (alpha)-phase was not investigated, and it is likely that polysialylation has an effect on how the antibody species distributes within the blood compartment. This needs further investigation.

In conclusion, this study has shown that Fab antibody fragments can be successfully polysialylated with little detriment to its *in vitro* binding properties. We have shown that polysialylation leads to increased blood bioavailability by modulating the clearance rates resulting in increased tumor uptake. Although polysialylation has been used successfully to manipulate drug and protein behavior, this study marks the start of a program of work leading to the better design of PSA antibody conjugates.

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