technical note

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Streptavidin-biotinylated IgG Conjugates: a Simple Procedure for Reducing Polymer Formation

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Disulfide links of the IgG2ak anti-ovarian carcinoma antibody, 5G6.4, were site-specifically biotinylated [≈2 biotins/IgG2a] using a novel crosslinking procedure using the biotin derivatized ETAC (equilibrium transfer alkylation crosslink reagent) **1a**. Complexation of ETAC **1a** biotinylated 5G6.4 on a column of immobilized protein A at high dilution, followed by passage of [^{125}I]streptavidin, washing and pH change leads to elution of a streptavidin-free product with a molecular mass in the 200–300 kDa range. By contrast, direct mixing with [^{125}I]streptavidin rapidly gave larger oligomers of ⩾669 and ≈440–669 kDa molecular mass, respectively. The biodistribution of the 200–300 kDa complex showed significantly diminished liver, kidney and spleen uptake as well as higher blood activity than the 440–669 kDa complex. The methodology represent the first application of ETAC chemistry to disulfide-bond directed biotinylation of antibodies and the synthesis of streptavidin antibody conjugates which minimizes their polymerization.

Developments in the use of biotinylated antibodies and pre-formed streptavidin biotinylated antibody complexes may have applicability to using such approaches in radioimmunoconjugate delivery (Hnatowich *et al.*, 1987; Paganelli *et al.*, 1990d; Pimm *et al.*, 1988; Sinitsyn *et al.*, 1989). Successful clinical studies have been reported by at least two groups (Rowlinson *et al.*, 1988; Paganelli *et al.*, 1990a,b,c). Recently, we reported our findings concerning the potential applications of sulfhydryl site-directed biotinylation as a means of better preserving the immunoreactivity of biotinylated antibodies and their corresponding radiolabeled conjugates (del Rosario and Wahl, 1989, 1990). These earlier data grew from our initial observation that reduced interchain disulfide links of antibodies were readily mono-alkylated with an iodoacetamide derivative of biotin (del Rosario and Wahl, 1989). Following complexation with radio-streptavidin, the oligomeric conjugates retained *in vivo* immunological activity. However, we and others have recognized that proper control of molecular size and purification remains a key problem in the synthesis of homogeneous streptavidin-biotinylated–monoclonal IgG complexes for use in radioimmunoimaging (Hnatowich *et al.*, 1987). Here we describe a simple method for minimizing the molecular size of the conjugate formed upon reaction with streptavidin which allows excess streptavidin to be easily separated from product without HPLC purification.

Biotinylation was accomplished using a novel procedure using the "ETAC" (equilibrium transfer alkylation) crosslink reagent **1a**, prepared in our laboratories (del Rosario *et al.*, 1989, 1990) (Fig. 1). In previous work (del Rosario *et al.*, 1989, 1990; Liberatore *et al.*, 1990) we have shown that ETAC reagents provide a unique site-specific pathway for attaching a wide range of bioprobes to antibodies, i.e. through insertion and formation of a stable three carbon crosslinked disulfide bridge (del Rosario *et al.*, 1990). In contrast to our earlier papers, ETAC biotinylation proceeds through a bis-alkylation as opposed to a mono-alkylation (as in the reaction of haloacetyl derivatives) of disulfide bonds (del Rosario and Wahl, 1989, 1990).

We have recently reported the synthesis of the parent compound carboxy ETAC **1** and a biotin derivative, **1a** (del Rosario *et al.*, 1989). Biotinylated ETAC **1a** (Fig. 1) was prepared by carbodiimide-mediated derivatization of carboxy-ETAC **1** with commercially available 5-(((*N*-biotinoyl)aminohexanoyl)amino)pentylamine (Molecular Probes) (del Rosario *et al.*, 1989). The anti-ovarian carcinoma IgG2ak antibody, 5G6.4 (Wahl *et al.*, 1986) was chosen for the present study. 5G6.4 (produced from mouse ascites and purified by protein A chromatography) in 0.1 M sodium phosphate (pH ≈ 7) solution was reduced (37°C, 5 h) with a ≈150 molar excess of DTT (Aldrich Chemical Co.). The mixture was treated with a DMSO solution of ETAC **1a** (1:1 molar ratio with respect to DTT), vortexed and the emulsion allowed to react for 15–24 h at 37°C. The reaction mixture was vortexed (15 min) and the clear supernatant desalted over Sephadex G-25 using 0.1 M sodium phosphate buffer (pH ≈ 7).

Site-specific crosslinking and biotinylation of interchain disulfide links of 5G6.4 was confirmed by reducing SDS–PAGE analysis (Pharmacia Phast System) of the crosslinked products. Since the new —S—{biotinoyl-ETAC}—S— chemical bonds were stable even at 100°C and not cleaved by reducing thiols, the crosslinked species appeared as new bands of ≈H_2L_2, H_2L, H_2 and HL mass units in addition to uncrosslinked heavy (H) and light (L) chains on electrophoresis. These observations were identical to our previous findings (del Rosario *et al.*, 1989, 1990; Laemmli, 1970; Liberatore *et al.*, 1990). Laser densitometric measurement (Pharmacia Ultrascanner) of the Coomassie blue content on each band allowed calculation of an average of ≈2 interchain H–H and H–L disulfide attached biotins per biotinylated 5G6.4 molecule (del Rosario *et al.*, 1989, 1990). As expected the extended "spacer arm" of the new biotin derivative **1a** allowed facile complexation with streptavidin as has been observed with derivatives of *N*-hydroxysuccinimide esters of biotin (Hnatowich *et al.*, 1987). Reaction of biotinylated 5G6.4 (MW ≈ 150,000) with [^{125}I]streptavidin (MW ≈ 60,000) in approximately equimolar ratios led to formation of complex oligomeric mixtures covering the ≈400,000 to ⩾669,000 molecular mass region. The molecular weights of these conjugates were estimated from calibration plots of known standards using Pharmacia Superose 6 and 12 size-exclusion radio-FPLC as previously described (del Rosario and Wahl, 1990). Figure 2(a) shows a representative product profile. With excess biotinylated 5G6.4, the predominant species was mass ⩾669 kDa (species A), whereas ≈2 or more molar equivalents of streptavidin per mole of biotinylated 5G6.4 favored species in the lower 400–669 kDa mass range (Species B).

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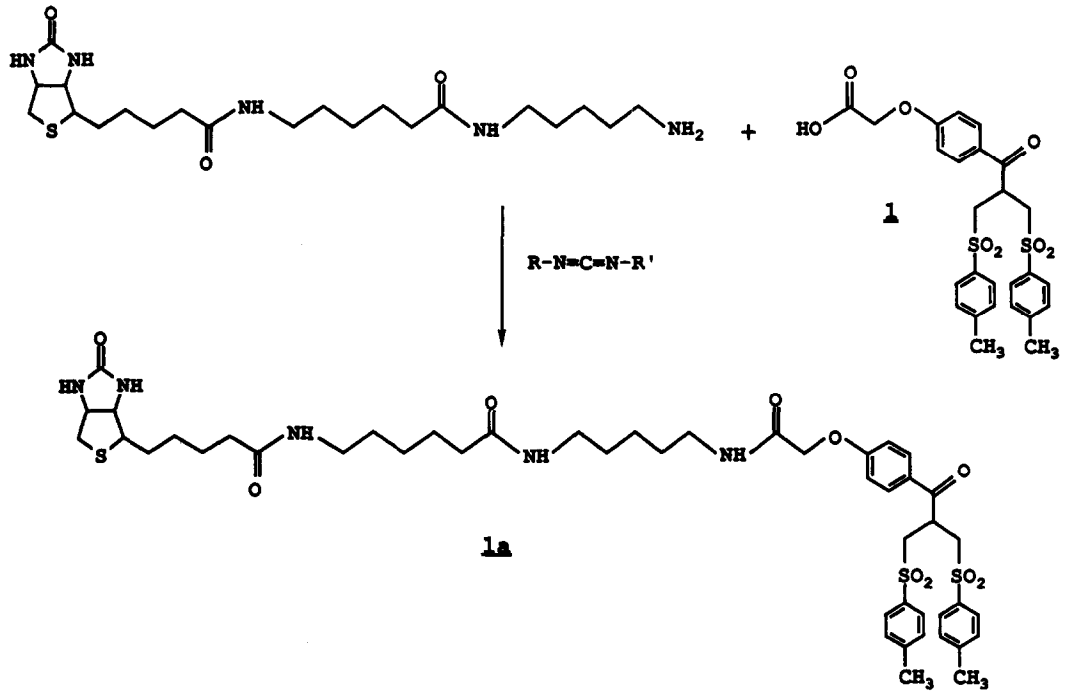

Fig. 1. Chemical structures of ETAC **1** and biotin derivative **1a**.

Retention of immunoreactivity of the biotinylated and streptavidin-biotinylated 5G6.4 pre-formed complexes was verified by direct cell binding assays of the radiolabeled complex using human 77 IP3 target cells (del Rosario and Wahl, 1990). Direct binding of biotinylated 5G6.4 was indicated by specific binding (60%) of [^{125}I]streptavidin on target cells pre-incubated with the biotinylated 5G6.4 conjugate. Preformed oligomeric species A ($\geqslant$669 kDa) or B ($\approx$400–> 669 kDa) [Fig. 2(a)] both exhibited comparable or better (60–80%) specific binding than [^{125}I]5G6.4 (50–65%) labeled via the iodogen method (at substantial antigen excess). Flow cytometry results using fluorescent streptavidin were identical with those previously reported for the iodoacetyl–biotin conjugate of 5G6.4 (del Rosario and Wahl, 1989, 1990). Species A [Fig. 2(b)] showed excellent stability in serum (5 days) as assessed by radio-FPLC. Similarly, complex formation was observed when ETAC **1a** biotinylated 5G6.4 was allowed to mix with radiolabeled streptavidin in serum ($\approx$1 μg biotinylated 5G6.4/mL serum) instead of buffer. The preservation of immunoreactivity and stability data are consistent with the hypothesis that ETAC conjugation occur at sites distant from the antigen binding sites and leads to relatively stable biotin ETAC **1a** crosslinks in 5G6.4. However, a preliminary biodistribution study (mice, $n = 3$/group) of product complex enriched in species A ($\geqslant$669 kDa) showed large liver (0.263* $\pm$ 0.016) and spleen (0.539 $\pm$ 0.134) uptake with very little blood (0.006 $\pm$ 0.001) pool activity by 48 h in comparison to a 150 kDa radiolabeled non-specific IgG2a antibody (UPC-10) [liver: 0.055 $\pm$ 0.006; spleen: 0.167 $\pm$ 0.026; blood: 0.111 $\pm$ 0.066, $P \leqslant 0.0005$, $P \leqslant 0.025$, $P \leqslant 0.10$, respectively.*] The above results closely parallel our previous studies concerning the effect of added anti-mouse antibody on the *in vivo* behavior of circulating intact radiolabeled UPC-10 and 5G6.4 (Wahl and Fisher, 1987) and implied that large complex size would present a substantial problem in targeting these antibody conjugates to tumors.

*All values are in % kg ID/g ($\pm$SEM).

To minimize formation of very large inter-antibody streptavidin oligomers (and their undesired non-target uptake in the liver and spleen), an alternative approach was devised using a protein affinity matrix. The strategy begins by first "anchoring" the same lot of biotinylated 5G6.4 on protein A (via its Fc portion) at pH 8–9 (Ey *et al.*, 1978). [^{125}I]Radio-streptavidin was passed through the column briefly at high dilution to allow contact with the protein A-bound IgG. Unbound [^{125}I]streptavidin was then washed away and the 5G6.4-biotinylated streptavidin conjugate was saturated by passing a saturated buffer solution of biotin through the column to block available biotin binding sites on the streptavidin while still being bound to protein A. The product complex was conveniently eluted from off the column by washing at pH 5.

A representative procedure is as follows. The above ETAC **1a**-biotinylated 5G6.4 was thoroughly mixed with immobilized Protein A (Repligen®, $\approx$200–300 μg IgG2a/5 mL gel) suspended in 0.1 M sodium phosphate buffer (pH $\approx$ 8–9). The slurry was poured into a column. [^{125}I]Streptavidin (30–40 μCi/μg, 100–300 μCi) was allowed to run through the column once. The column was washed several times with a saturated solution of biotin in 0.1 M (pH $\approx$ 8–9) sodium phosphate buffer until the eluent showed no change in background activity. Product complex was then eluted with 0.1 M citrate buffer at pH 5 ($\approx$80 μCi) and examined for free streptavidin by size-exclusion radio-FPLC [Pharmacia Superose 6, Fig. 2(c)] analysis.

Products of significantly lower molecular weight ($\approx$200,000–300,000) with radio-FPLC profiles such as C [Fig. 2(c)] were eluted at pH 5. The shoulder of peak C (higher molecular mass) in Fig. 2(c) was evident even when the radio-streptavidin used was partially blocked with <4 equivalents of biotin prior to contact with the immobilized biotinylated IgG. The biodistribution of species C was then compared with species B in rats ($n = 3$) and is summarized in Fig. 3. The biodistribution (rats, $n = 3$/group, $t = 48$ h) of species C showed significantly diminished liver, kidney and spleen uptake as well as higher blood activity than the 440–669 kDa complex {liver $\approx$2.4 [ratio of 440–669 kDa/200–300 kDa activity], $P \leqslant 0.025$; kidney $\approx$2.7, $P \leqslant 0.005$;

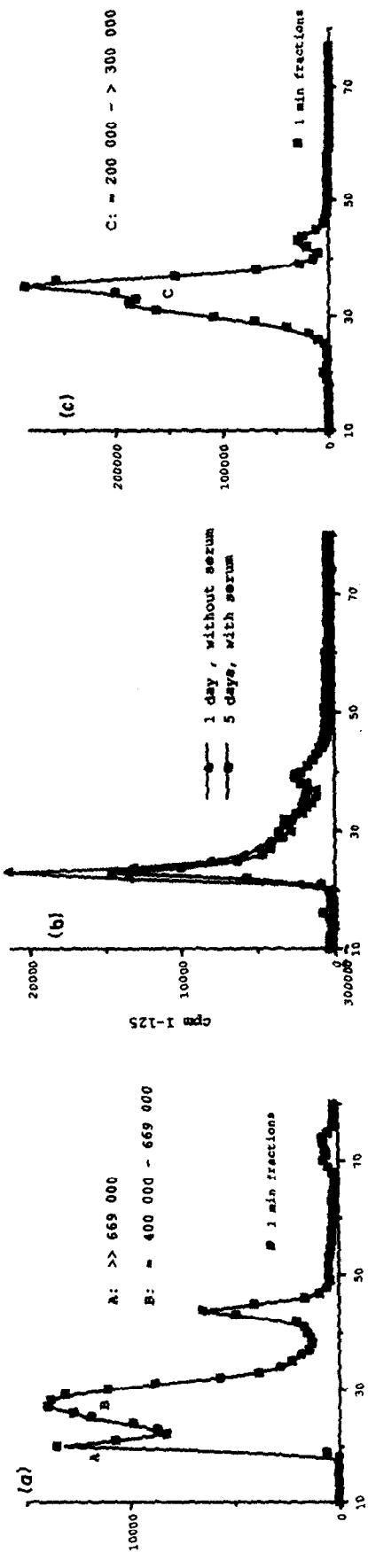

Fig. 2. Size exclusion radio-FPLC analysis of biotinoyl-ETAC-5G6.4 + streptavidin reaction products. (a) Reaction product mixture of equimolar mixture of [^{125}I]streptavidin + biotinylated 5G6.4, (b) serum stability of [^{125}I]streptavidin *biotinylated* 5G6.4 species "A" and (c) reaction product complex using a protein A affinity *support* column.

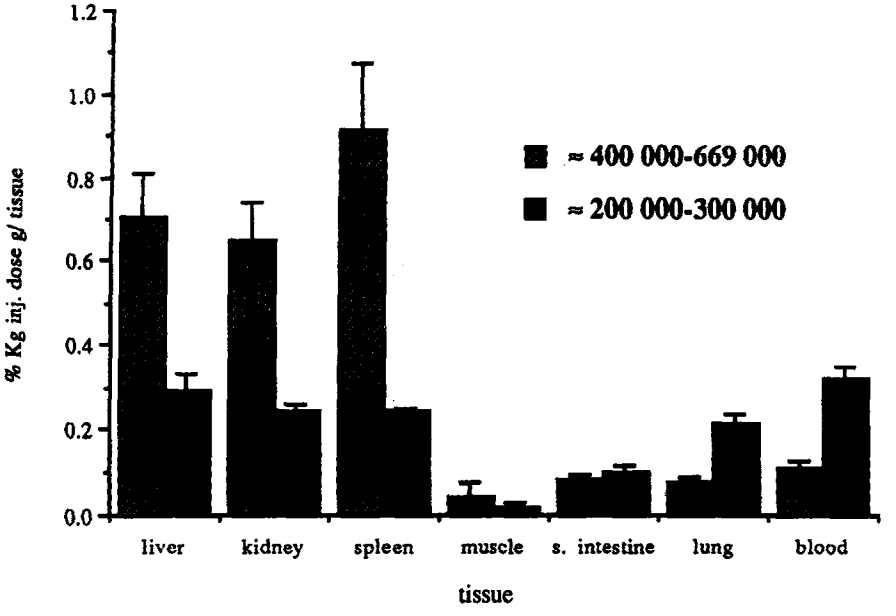

Fig. 3. Biodistribution of biotinylated 5G6.4 streptavidin complexes in rats ($n = 3$) as a function of size (species B: hatched; species C: solid).

spleen ≈ 3.8, $P \leqslant 0.01$; blood [440–669 kDa 200–300 kDa] ≈ 0.35, $P \leqslant 0.005$}. Liver and spleen to blood ratios were decreased by $\approx 8 \times (P \leqslant 0.05)$ and $\approx 13 \times (P \leqslant 0.05)$ for the 200 kDa complex relative to the 440–669 kDa product. The improved retention of the lower molecular weight complex in the blood appears due to avoiding high level delivery to the liver and spleen. Furthermore, this should result in better contact between the antibody and antigens present on tumor targets thus improving immunoconjugate targeting (Hnatowich *et al.*, 1987).

In conclusion the above results provide (a) the first example of site-specific biotinylation via a unique mechanism of concomitant crosslinking of reduced disulfide links, (b) further illustrates the relevance of immunoconjugate size in tissue localization, (c) demonstrates that improvements in biodistribution can clearly be achieved with a smaller complex and (d) illustrates a practical approach for avoiding high molecular weight oligomers. A limitation of the technique is that it may not be directly applicable to pre-targeting strategies if all streptavidin sites are pre-blocked by biotin. This can be addressed by using lesser quantities of biotin to allow only partial blocking (Green, 1975). The chemistry of the new biotin derivative, ETAC **1a** further widens the scope and utility of immunologic applications of the biotin–streptavidin couple. Furthermore, the immobilization strategy described herein may find similar applicability in the synthesis of other antibody conjugates where polymer formation is undesirable (Alam *et al.*, 1989).

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