Fig. 1. *N*-iodoacetyl-*N*′-biotinylhexylenediamine (1).

Site-specific Radiolabeling of Monoclonal Antibodies With Biotin/Streptavidin

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Sulfhydryl groups of a murine IgG2a monoclonal antibody, 5G6.4, were site-specifically biotinylated with *N*-iodoacetyl-*N*′-biotinylhexylenediamine (compound 1) following partial reduction of disulfide bonds. Antibody labeling using fluoresceinated or [^{125}I]streptavidin were consistent with sulfhydryl group-specific incorporation of label with good retention of immunoreactivity, mainly in the form of high molecular weight oligomers (molecular weight $\approx > 400,000$). *In vivo* localization of the radioantibody complex to xenografts of ovarian carcinoma was achieved. This approach appears to be a promising method of labeling monoclonal antibodies.

Attachment of radiolabels to monoclonal antibodies at some distance from the antigen binding sites appears to be a rational method to minimize radiolabeling related decrements in immunoreactivity arising from random labeling methods (Rodwell *et al.*, 1986). Recently we began a study of the site-specific biotinylation reagent 1 (Fig. 1) which targets biotin to reduced sulfhydryl residues of antibodies located at some distance from the antigen combining sites (Wakubayashi *et al.*, 1984). The use of non site-specifically biotinylated antibodies as biochemical probes in conjunction with labeled avidin (or streptavidin) has found wide application in immunoassays (Bayer and Wilchek, 1980), immunohistochemical staining (Guesdon *et al.*, 1979) and protein blotting (Bayer *et al.*, 1987) techniques. Of interest was the recent report by Hnatowich and co-workers who showed that monomeric streptavidin–biotin–polyclonal IgG complexes localized to staphyloccal protein A through binding of the antibody Fc portion (Hnatowich *et al.*, 1987). Their study illustrated the potential of using biotinylated monoclonal antibodies in radioimmunoimaging.

In order to fully exploit the potential of the site-specific biotinylation technique it is first necessary to determine how biotinylation and subsequent complexation with streptavidin would affect the antigen binding ability of the antibody *in vitro* and *in vivo*. The size and molecular mass of streptavidin suggests that steric factors will be important particularly if complexation to biotin occurs near the antigen binding sites. If site-specific biotinylation can be directed so as to minimize steric interference in antibody–antigen binding, biotinylation provides an effective bridge for attaching a radiolabel away from the hapten binding sites. The tetravalency of streptavidin also raises the possibility of forming multi-valent dimeric or oligomeric antibody species which could exhibit higher immunoreactivity than divalent antibodies. If higher immunoreactivity results from regiospecific biotinylation and formation of oligomers with increased immunoreactivity, enhance-
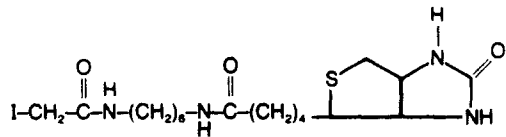
ment of tumor uptake and target to background ratios might occur. To evaluate new methods of site-specific labeling of monoclonal antibodies via alkylation of thiols (Bayer *et al.*, 1987; Packard *et al.*, 1986), we investigated the dithiothreitol (DTT) reduction of the murine monoclonal antibodies 225.28S (Wilson *et al.*, 1981) and 5G6.4 (Wahl *et al.*, 1986) and their reactions with iodoacetamide and *N*-iodoacetyl-*N*′-biotinylhexylenediamine (compound 1). Here, we present direct evidence that biotinylation not only led to retention of immunoreactivity *in vitro* but that these biotinylated antibodies formed oligomeric species with streptavidin capable of tumor localization to human ovarian carcinoma xenografts *in vivo*.

We found that alkylation was best performed by quenching the reduction mixture with a stoichiometric quantity of iodoacetamide or 1 with respect to DTT. Typically, a solution of IgG2a [$\approx 2 \times 10^{-3} \mu$mol (Braford, 1976)] in 0.1 M
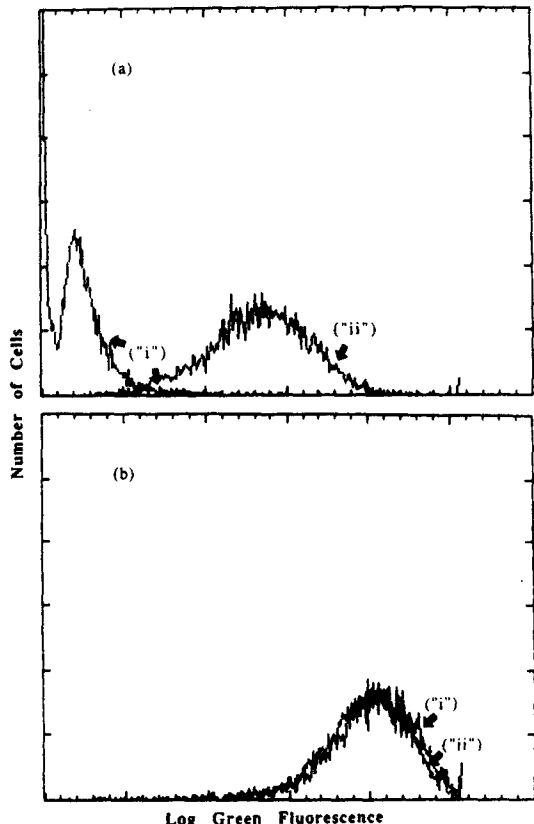

Fig. 2. Flow cytometry analysis of 1-alkylated 5G6.4: (a) plots of observed fluorescence from IP3 cells incubated with unreduced 5G6.4 + 1 ("i") and reduced 5G6.4 + 1 product ("ii") after treatment with fluorescein–streptavidin; (b) fluorescence data from reduced 5G6.4 + 1 product ("i") superimposed with data from unreduced and unalkylated 5G6.4 ("ii") following treatment with fluorescein–IgG2a specific antimouse.
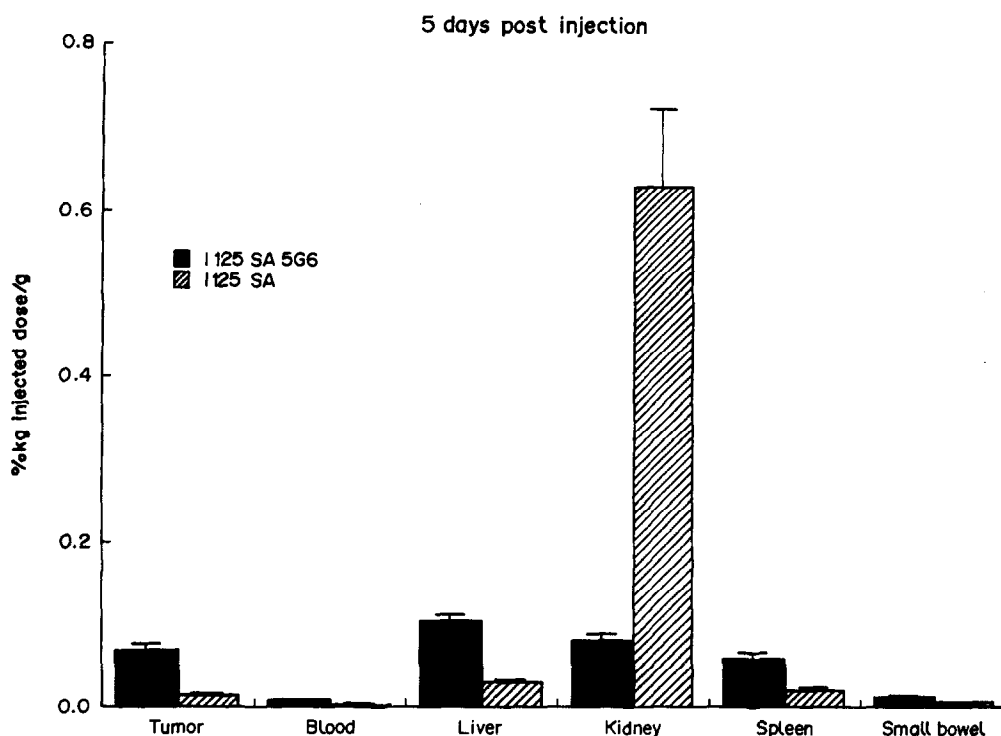
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Fig. 4. Comparative biodistribution data for [^{125}I]streptavidin + 1-alkylated 5G6.4 and [^{125}I]streptavidin.

sodium phosphate (pH ≈ 7-7.4) was incubated with DTT (≈ 5 × 10^{-2} μmol, 5 h, 37°C), treated with 1 (0.01 μmol, 4–5 days, 4–8°C) and purified by gel filtration chromatography (Sephadex G-25). A [DTT]/[IgG2a] molar ratio of ≈ 25 was eventually adapted in view of the limited solubility of 1 in aqueous media. In view of our great interest in the radiotherapeutic potential of 5G6.4 in ongoing ovarian carcinoma studies and its availability, all subsequent experiments were focused on this antibody.

Fluorescence microscopy and flow cytometry analysis of 77 IP3 human ovarian carcinoma target cells (Wahl et al., 1987) incubated with 1-alkylated 5G6.4 (≈ 15 μg/mL) followed with fluorescein conjugated streptavidin confirmed positive binding using an identical unreduced 5G6.4 + 1 preparation as a negative control [Figure 2(a)]. Similarly, flow cytometry analysis of target cells incubated with unreduced and 1-alkylated 5G6.4 probed with fluorescein conjugated IgG2a specific antimouse showed essentially no difference in the observed mean fluorescence [Fig. 2(b)]. SDS gel electrophoresis in the absence of reducing reagents of 1-alkylated 5G6.4 gave a product mixture profile very similar to those

with the reaction with iodoacetamide, consisting mainly of fragments of L, H, HL, H$_2$ mass (Sutton et al., 1984; Nisonoff, 1984). The above observations are consistent with site-specific biotinylation of 5G6.4 via in-situ generated sulfhydryls with retention of immunoreactivity.

The immunoreactivity of 1-alkylated 5G6.4 was also retained by preformed streptavidin-1-alkylated-5G6.4 complexes in vitro and in vivo. In views of the relatively slow clearance rate of streptavidin (Bayer, 1987) and to minimize the unwanted formation of free radiolabeled streptavidin we decided to examine the reactions of [^{125}I]streptavidin in the presence of excess 1-alkylated-5G6.4. Treatment of 1-alkylated 5G6.4 with [^{125}I]streptavidin (5:1 molar ratio with respect to 5G6.4) gave oligomeric mixtures. The major products were a species consistent with a dimeric antibody–streptavidin complex (molecular mass of ≈ 440,000) and polymers (mass > 669,000) by comparison with molecular weight standards as evidenced by native gel electrophoresis and autoradiography (Fig. 3). Despite the heterogeneously larger size of these products over monomeric species, a cell binding assay of these preparations gave ≈ 20% specific and ≈ 1–7%

Table 1. Biodistribution activity data for [^{125}I]streptavidin and 1-alkylated 5G6.4 + [^{125}I]streptavidin

Tissue	[^{125}I]Streptavidin (%kg inj. dose/g)	Tissue/blood	1 + 5G6.4 + [^{125}I]streptavidin (%kg inj. dose/g)	Tissue/blood
Tumor	0.012 ± 0.002[a]	4.4	0.068 ± 0.009[b]	7.8
Blood	0.0027 ± 0.001	1.0	0.0087 ± 0.001	1.0
Liver	0.029 ± 0.002	10.7	0.10 ± 0.007	11.5
Kidney	0.63 ± 0.097	233	0.080 ± 0.01	9.2
Spleen	0.020 ± 0.003	7.4	0.058 ± 0.008	6.7
Small intestine	0.0039 ± 0.001	1.4	0.010 ± 0.001	1.1

[a]Values are the mean for 3 mice; numbers are ± SEM.
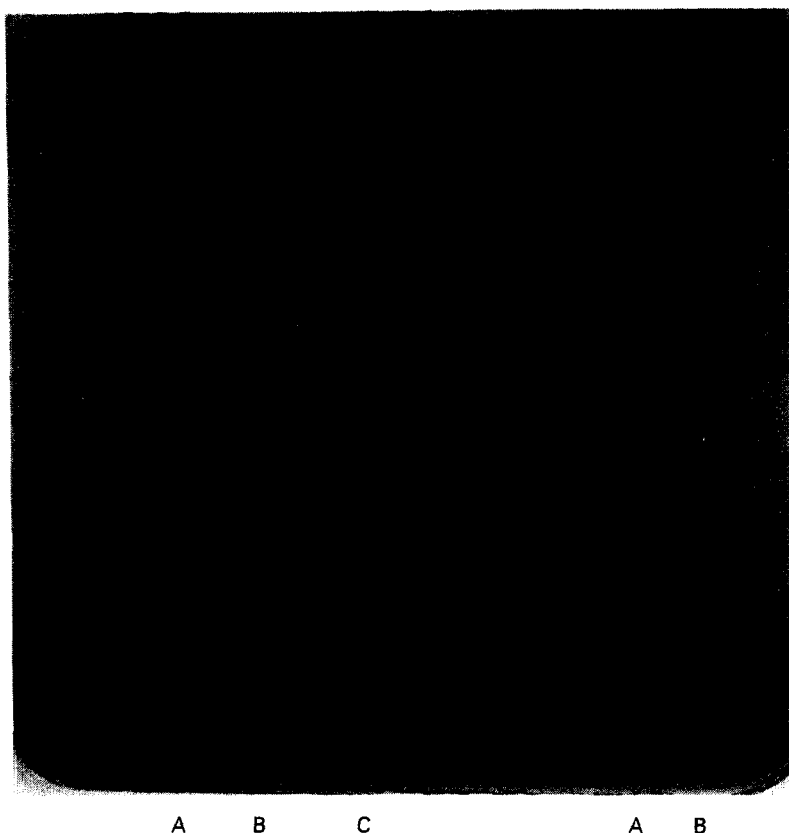[b]Corresponding data for 5 mice.

Fig. 3. Native gel electrophoresis and radiography of 1-alkylated 5G6.4 + [^{125}I]streptavidin (from left to right, first three lanes): (a) molecular weight standards [from top to bottom: 669,000 (thyroglobulin), 440,000 (ferritin), 232,000 (catalase), 140,000 (lactase dehydrogenase), 67,000 (bovine serum albumin)]; (b) [^{125}I]streptavidin and (c) 1-alkylated 5G6.4 + [^{125}I]streptavidin. Longer exposures did not reveal additional bands.

non-specific binding to target cells. Routine labeling of 5G6.4 with $^{125}I/^{131}I$ (iodogen method) in our laboratories typically yield 20–50% specific binding using the same cell binding assays.

The biodistribution data with tumor (s.c.) bearing nude mice after i.p. (Wahl, 1988) injection with the [^{125}I]streptavidin-1-alkylated 5G6.4 complex mixture and [^{125}I]streptavidin ($\approx$ 10–15 μCi/mouse, specific activity $\approx$ 3–4 μCi/μg of 5G6.4) are given in Table 1. The data shows a relatively high tumor/blood (background) ratio ($\approx$ 8) and significant liver and kidney uptake for the labeled antibody. The somewhat elevated uptake seen in the liver is not surprising since the injected mixture contained significant quantities of polymers with size and molecular mass properties that may have prevented adequate whole body circulation and tumor localization (Goodwin, 1987; Leypoldt et al., 1987). In a separate experiment we also found that the kidney uptake was significantly affected by the amounts of free iodine in the labeled streptavidin. Figure 4 shows a graphic comparison between the values given in Table 1. Whereas the tumor uptake for the biotinylated antibody was $\approx$ 6 times higher than with [^{125}I]streptavidin alone, the data also show almost all of the activity ($\approx$ 90%) was localized in the kidney for streptavidin.

While these preliminary experiments demonstrate the viability of utilizing the streptavidin-biotinylated antibody probe in vivo, more work is clearly necessary in order to optimize the many factors that influence favorable tumor localization of these complexes.

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