

Site-specific photobiotinylation of immunoglobulins, fragments and light chain dimers

Gabriela Pavlinkova^a, Krishnan Rajagopalan^b, Sybille Muller^{c,d}, Ashok Chavan^e, Gail Sievert^e, Dingyuan Lou^d, Carol O'Toole^d, Boyd Haley^{b,d}, Heinz Kohler^{a,d,*}

^a Department of Microbiology and Immunology, University of Kentucky, Lexington, KY 40436, USA

^b Division of Medicinal Chemistry and Pharmaceutics, University of Kentucky, Lexington, KY 40436, USA

^c Department of Medicine College of Pharmacy, University of Kentucky, Lexington, KY 40436, USA

^d Markey Cancer Center, University of Kentucky, Lexington, KY 40436, USA

^e Immpheron Inc., Lexington, KY 40436, USA

Received 6 May 1996; revised 29 September 1996; accepted 2 October 1996

Abstract

Herein we report a new method to rapidly photoinsert biotin into a specific and highly conserved site on the Ig structure using a mild photochemical activation step. This site resides in the Fv fragment and involves invariant residues which provide base stacking interactions to the purine ring of ATP (Rajagopalan et al. (1996) Proc. Natl. Acad. Sci. USA 93, 6019–6024). Biotin was coupled to either the phosphate or the ribose of the 8-azidopurine nucleotide or nucleoside photoaffinity probe and shown to insert into the affinity site efficiently. Several monoclonal and polyclonal antibodies, as well as enzymatic and recombinant antibody fragments and light chain dimers were photoaffinity biotinylated and used in ELISA, FACS and Western blots. The selectivity of this site-specific biotinylation method also allows for biotinylation of antibodies in culture supernatants and immune sera without prior purification. Because the biotinylation takes place under physiological conditions and within a short time period, photobiotinylation would be the preferred method for antibodies which are easily damaged by classical non-site specific random biotinylation chemistry.

Keywords: Photoaffinity labeling; Antibody; Site-specific biotinylation

1. Introduction

The avidin-biotin complex represents the underlying mechanism in many antibody based assay systems because of its high affinity and the ease of attaching biotin to antibodies and reporter molecules to avidin. Biotinylated antibodies are used in ELISA, Western blots, FACS and in vivo antibody imaging and therapy delivery methods. Typically, ϵ -amino groups and other reactive side chains of amino acids

Abbreviations: AP, alkaline phosphatase; BSM, bovine submaxillary gland mucin; HRPO, horseradish peroxidase; HSA, human serum albumin; HRPO, horseradish peroxidase; BPB, Bromphenol Blue; Fv, immunoglobulin fragment variable; V_H, variable heavy chain; V_L, variable light chain; MES, 2[*N*-morpholine]ethanolsulfuric acid; FCS, fetal calf serum.

* Corresponding author. At: Markey Cancer Center, 205 Combs Building, University of Kentucky, 800 Rose Street, Lexington, KY 40536, USA. Tel.: (606) 257-6485; Fax: (606) 257-8940.

are utilized to react with the chemically reactive groups of the biotin derivatives. Depending on the number and location of the reactive groups on antibodies, biotinylated immunoglobulins represent heterogeneous molecular species because standard protein conjugation methods modify amino acid side chains in an uncontrollable and random fashion (Wilcheck and Bayer, 1990). While this does not present problems for most applications of biotin-avidin based assays, the randomly biotinylated Igs can cause non-specific binding to non-targeted antigens and tissues. Furthermore, randomly conjugated antibodies are recognized by scavenger cells in liver and lung leading to undesired and non-specific uptake of biotinylated antibodies impeding their use as diagnostic imaging agents (Paganelli et al., 1992; Slavin-Chiorini et al., 1995). Finally, the non-site-restricted nature of the biotin conjugation can lead to modification of residues in the antigen binding site or residues in the vicinity of the binding site in some antibodies which reduces or abolishes antigen binding. A site-specific conjugation method is available based on reduction of S-S bonds; however, this carries the risk of subunit fragmentation and loss of avidity or even total binding (Wong, 1991).

In the present series of experiments we took advantage of the newly discovered site in the Fv region of antibodies, to attach biotinylated derivatives of 8-azidopurine nucleotide or nucleoside without losing antigen binding (Rajagopalan et al., 1996). Photoaffinity labeling is known to be an effective method for detecting peptide regions involved in the nucleotide base-binding domain of proteins (Salvucci et al., 1972). Nucleotide photoaffinity probes bind with high affinity into a hydrophobic pocket in the V_H - V_L interface and, on photolysis form covalent bonds with side chains in the surrounding protein fold. A three-dimensional model showing the inserted ADP into the binding pocket on the Fv fragment was constructed based on the conserved nature of crystallographic Fab structures (Rajagopalan et al., 1996). In particular, the invariant TRP at the position H103 in the heavy chain framework FR4 and the TYR at position L36 in the framework FR2 of the light chain contact the purine of the nucleotide photoaffinity probe. This model illustrates how the purine ring binds in the pocket via stacking and that the phosphate groups

and the ribose ring are accessible from the surface of the molecule.

In this study, we synthesized two biotin derivatives containing azidopurine and demonstrated that these compounds can be used as site-specific photobiotinylation reagents for antibodies of different classes, specificity and species origin. We also present data on the site-specific biotinylation of light chain dimers, Fab fragments, and single chain fragments.

2. Materials and methods

2.1. Cell lines and corresponding antibodies

The carcinogen-induced mouse B cell lymphoma, 38C13 and monoclonal anti-idiotypic antibody S1C5 (Maloney et al., 1985) and the diffuse histiocytic human lymphoma cell line SU-DHL-4 and monoclonal anti-idiotypic antibody DHL-4 (Levy et al., 1985) were kindly provided by S. Levy, Stanford. Antibody CC49 specific for TAG-72 (Murano et al., 1983) and Fab fragments were generated as described (Johnson et al., 1988). Recombinant scFv constructs and scFv fragments were grown in *E. coli* and purified on S-Sepharose, Mono Q and Mono S columns (Pharmacia, Piscataway, NJ). Monoclonal antibodies were purified from ascites or cell-culture supernatant by protein A or protein G affinity chromatography. κ light chain dimers Lay and B6 were kindly provided by Alan Solomon, University of Tennessee.

2.2. Human sera

Sera from HIV-1 infected individuals i.e. patients with confirmed AIDS, were kindly provided by Richard Greenberg, University of Kentucky, Lexington. Additional specimens of plasma from HIV-1 infected individuals and seronegative control sera were purchased from NABI, Miami, FL. An HIV-1 seropositive serum containing a myeloma protein with specificity for HIV-1 p17 was kindly provided by Leslie Silberstein, University of Pennsylvania, Philadelphia, PA.

2.3. Recombinant proteins

Recombinant p24 (HIV-1 IIIB) was purchased from Intracel, Cambridge, MA. An HIV-1 HXB2 recombinant p17 and p24 were expressed in a GEX system as glutathione *S*-transferase (GST) fusion protein in *E. coli*.

2.4. Synthesis of azido-ATP- γ -biotin

8-N₃ATP was synthesized using the previously published procedures (Czarnecky et al., 1979). A methanolic solution of 8-N₃ATP (5 μ l) was concentrated by vacuum and dissolved in 400 ml of 100 mM MES (pH 5.1) (Rajagopalan et al., 1993). 14.4 mg of EDC (75 μ mol, 15 equiv.) were added to this solution together with 16.4 mg (50 μ mol, 10 equiv.) of 5-(biotinamido)pentylamine (Pierce, Rockford, IL). This solution was stirred at room temperature for 3 h. Deionized water (1 ml) was added and the solution was loaded on a benzyl DEAE cellulose column (HCO₃⁻ form) and washed with 50 ml of water. Elution was carried out with a linear gradient of 125 ml of water and 125 ml of 500 mM triethylammonium bicarbonate buffer and monitored at 278 nm. Fractions 42–47 (4 ml each) were pooled, concentrated under vacuum at 30°C, and lyophilized four times with MeOH. 2.7 mol of 8-azido-ATP- γ -biotin were recovered, a 54% yield as determined spectrophotometrically. The compound was characterized by TLC with a $R_f = 0.29$ on PEI cellulose using 400 mM NH₄HCO₃ and a $R_f = 0.44$ on silica using a mixture of isobutyric acid:NH₄OH:H₂O (66:1:33).

2.5. Synthesis of azidoadenosine-biotin

8-N₃-adenosine (16 μ mol) was suspended in 2 ml of 10 mM sodium phosphate pH 7.5 and reacted with 4.0 mg of sodium *m*-periodate. The reaction was complete in 45 min as determined by TLC. The dialdehyde was purified by passage through C18 Sep-Pak cartridge. The recovery was 68%. The dialdehyde (5 μ mol) was resuspended in 0.5 ml of 10 mM sodium phosphate pH 8.5 and reacted with 2.0 mg of biotin hydrazide (Pierce, IL). The reaction was complete in 1 h at room temp. as determined by TLC. The product was purified by C18 Sep-Pak

cartridge and was found to be > 95% pure when checked by TLC in two different solvent systems. The recovery was 80%. The UV spectra before and after photolysis with a hand-held UV lamp showed the characteristic photo decomposition similar to the UV decomposition of azido-ATP. The product has been found to be stable in water at room temperature up to 72 h with no decomposition observed on TLC. Although instability of dialdehyde nucleosides in the presence of reacting amine has been reported (Potter and Haley, 1983), we have not observed any loss of the dialdehyde ribose portion by maintaining a pH greater than 8 during synthesis.

2.6. Photobiotinylation of antibodies

Optimization experiments for photobiotinylation were performed as described before (Khatoun et al., 1989). A protein concentration of 0.5 mg/ml and a 20–50 μ l reaction volume yielded excellent results. For saturation experiments, the antibodies and light chain dimers were incubated with different concentrations of the photobiotinylation reagents in a final volume of 20 μ l at 4°C for 10 min. Photolysis was performed in a Fisherbiotech FB-UVXL-1000 microprocessor-controlled UV crosslinker using 1200 \times 100 mJ/cm². The photobiotinylated antibodies were stored at 4°C without losing binding as determined in ELISA and FACS analysis.

Protection experiments were performed by incubating the antibodies with unlabeled nucleotide for 5 min at 4°C followed by incubation with the photobiotinylation agents for 10 min at 4°C. Photolysis was performed as above.

2.7. Antisera and antibodies

Goat anti-mouse Ig and goat anti-human Ig sera were purchased from a commercial supplier (Sigma, MO). Biotinylated goat anti-mouse IgM were also obtained from Sigma. Goat anti-mouse Fc specific antibodies were obtained from Jackson Lab. CC49 MoAb (IgG1,12) and its fragments were kindly provided by David Colcher, Omaha, NB.

2.8. ELISA

A standard ELISA was used in order to determine the specific activity of photobiotinylated antibodies.

Costar vinyl assay plates (Costar, Cambridge, USA) were coated with antigen at concentration 2–5 mg/ml in 0.05 M carbonate-bicarbonate buffer (pH 9.6). After overnight incubation at 4°C, the coating solution was removed, and the wells were washed three times with PBS containing 0.1% Tween 20, then were blocked with a 3% BSA in PBS for 2 h and were washed again. Test antibodies were titrated into these plates. After 2 h, plates were washed and 100 μ l of streptavidin-HRPO conjugates (Southern Biotechnology Associates, AL) at a dilution of 1/4000 were incubated for 1.5 h at room temperature. After washing, the *p*-phenylenediamine dihydrochloride substrate was added for 10 min. The reaction was stopped with 4 N H₂SO₄. Plates were read at 490 nm on the Bio Kinetics EL 312e microplate reader set.

2.9. ELISA for photobiotinylated sera

To measure anti-p17 and anti-p24 antibodies in sera, plates were coated with 100 ng (100 μ l of 1 μ g/ml) of recombinant proteins. After blocking with 1% BSA solution plates were incubated for 2 h with photobiotinylated serum, diluted in PBS. After washing with PBS containing 0.2% Tween 20 streptavidin-HRPO was added. ELISA color was developed with *o*-phenylenediamine and read at 490 nm.

2.10. ELISA of CC49 and its fragments

IgG, F(ab')₂, Fab' and sFv of CC49 were added with 8-N₃-adenosine-biotin as described above. Plates were coated with 50 ng bovine submaxillary gland mucin (BSM, Sigma, St. Louis, MO) in PBS, dried overnight at 37°C and blocked with 5% BSA at 37°C for 1 h. 4 μ g of photobiotinylated antibody protein was added in two-fold serial dilutions for 2 h. After washing, the plates were incubated with streptavidin-alkaline-phosphatase (Sigma, MO) for 1.5 h and ELISA was developed with *p*-nitrophenyl phosphate substrate (Sigma, MO). The plates were read at 405 nm (BioRad, CA). For comparison of commercial and affinity labeled anti-mouse antibodies 50 μ l of MoAb CC49 (10 μ g/ml in PBS) were added to BSM coated plates overnight at 4°C. The unbound Ig was removed by washing with 1% BSA in PBS. Serial dilutions (1/2) of commercially biotinylated (Jackson ImmunoResearch Laboratory, West Grove,

PA) and affinity biotinylated goat anti-Fc specific antibody were added and incubated for 2 h at RT. Plates were washed with 0.025% Tween 20 in PBS and incubated with a 1/2000 dilution of streptavidin-AP (Jackson ImmunoResearch Laboratory) for 1.5 h at RT. After washing, plates were developed with *p*-nitrophenol phosphate in diethanolamine buffer. Color was read at Dynatech MR5000 microplate reader at 410–630 nm. As control plates were coated with BSA and developed as described.

2.11. Western blot of photobiotinylated CC49 fragments

Proteins were separated by 5–20% gradient SDS-PAGE and transferred by electrophoretic elution to Immobilon-NC membrane (Millipore, MA). After overnight blocking in blocking solution (3% BSA in 0.02 M Tris, 1 M NaCl, 0.3% Tween 20), the streptavidin-alkaline phosphatase in a dilution of 1/1000 was added and incubated at room temperature for 2 h. The membrane was washed with Tris-buffered saline and developed with bromochloroindolyl phosphate-nitro blue tetrazolium (BCIP/NBT) substrate (BioRad, CA).

2.12. Western blot of light chain dimers

After photobiotinylation the light chain dimers (5 μ g) were treated with 2% SDS, 100 mM DTT, 13% sucrose and 0.04% BPB (final concentrations), boiled 1 min and run on a 6–12% SDS-PAGE. The gel was transblotted onto nitrocellulose membrane (BioRad, CA), treated with avidin-AP (Southern Biotechnology, AL) 1.5 h at room temperature and developed with substrate for alkaline phosphatase (Promega CA).

2.13. Western HIV-1 immunoblot

The HIV-1 immunoblot membranes (BioRad, CA) were incubated with photobiotinylated or unbiotinylated primary human antisera at a dilution of 1/100 for 1 h at the room temperature. Free sites were blocked and subsequently washed with buffer containing 0.02 M Tris, 0.5% nonfat dry milk, 1 M NaCl, 0.3% Tween 20, antifoam, and 0.01% sodium azide. After washing, strips containing photobiotinylated human sera were incubated with streptavidin-

HRPO conjugates (1:2000). As positive control secondary goat anti-human IgG-HRPO (1:500) was added to a human serum sample that was not photobiotinylated. After 1 h the color was developed with 3,3'-diamidobenzidine substrate (Sigma, MO).

2.14. Immunofluorescent staining

Cells were centrifuged for 5 min at 1000 rpm, then were resuspended and washed with cold PBS. After discarding the supernatant, cells were resuspended at 2×10^7 /ml in PBS. 50 ml of cell suspension (1×10^6 cells) and 50 ml of photobiotinylated antibody in PBS or photobiotinylated culture supernatant was added per each tube. After 1 h of incubation at 4°C, cells were centrifuged with 1 ml FCS and washed twice with PBS (wash media). Cells were then incubated with a 1/50 dilution of fluorescein isothiocyanate (FITC)-conjugated streptavidin (Southern Biotechnology, AL). Finally, cells were washed twice and fixed in 1% paraformaldehyde in PBS and analyzed on the fluorescence-activated cell sorter (FACS). FACS data were generated from a minimum of 9900 cells per sample.

3. Results

3.1. Synthesis of 8-N₃ATP- γ -biotin and 2,3-biotinylated 8-N₃-adenosine and photoinserterion into monoclonal antibodies

Two new photoaffinity probes containing the biotin molecule were synthesized as described in Sec-

tion 2. Basically, in reaction scheme A, the biotin moiety is attached to the γ -phosphate of 8-N₃ATP, and in scheme B biotin is covalently linked to the oxidized ribose (see Fig. 1). Reaction conditions and molar ratios for photobiotinylation were established and found similar to the conditions for the photoinserterion of 8-N₃[³²P]ATP (Rajagopalan et al., 1996; Czarnecky et al., 1979).

3.2. Assays using photobiotinylated purified antibodies

To determine the affinity of the newly synthesized photobiotinylation reagents for antibodies we used increasing concentrations of 8-N₃-adenosine-biotin to label a range of different antibodies. We compared the immunoreactivity of one antibody, goat anti-mouse IgG, photobiotinylated with either 8-N₃-adenosine biotin or 8-N₃ATP- γ -biotin in ELISA. Fig. 2a depicts the ELISA of polyclonal goat anti-mouse antibody photobiotinylated with increasing concentration of 8-N₃-adenosine-biotin and 8-N₃ATP- γ -biotin. Saturation of photobiotinylation is achieved between 100–200 μ M concentration with both reagents, which is similar to what was observed with 8-N₃[γ -³²P]ATP on other antibodies (Rajagopalan et al., 1996). The efficiency in ELISA with both photobiotinylated antibody preparations is equivalent. The data in Fig. 2b demonstrate that saturation of photobiotinylation as measured by ELISA is not affected by antibody concentration over a 30-fold range.

To determine the binding affinity of photobiotinylated antibody we used unlabeled antibody as a

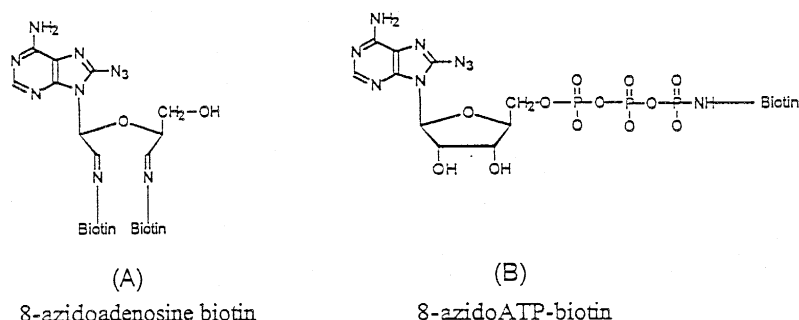


Fig. 1. Synthesis scheme of photobiotinylation compounds. Scheme A depicts the 8-azidoadenosine photobiotinylation reagent which has biotin molecule attached via the ribose ring. Scheme B shows the 8-azido-ATP-biotin reagent where biotin is linked to the γ phosphate. For details of synthesis see Section 2.

competitor in ELISA tests. As seen in Fig. 3 the amount of unlabeled antibody needed to give 50% inhibition of binding is close to amount of photobiotinylated antibody used (150 ng). This indicates that the photobiotinylated antibody is as reactive in ELISA as the unmodified antibody.

To compare the photoaffinity biotinylation with conventional biotinylation techniques two goat anti-mouse Ig antibodies were affinity biotinylation. First, a goat anti-mouse IgM antibody (Sigma) was biotinylation with 8-N₃ATP- γ -biotin. Dilutions of the

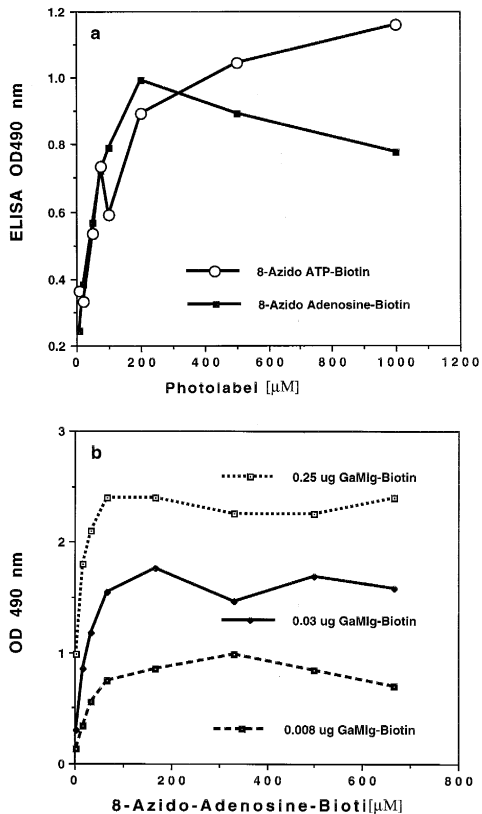


Fig. 2. *a*: comparison of binding of goat anti-mouse Ig photobiotinylated with azido-adenosine and azido-ATP. Goat antisera were photobiotinylated using increasing amounts of 8-azido-adenosine-biotin or 8-azido-ATP-biotin. Photobiotinylated sera were used in ELISA plates coated with mouse S1C5 (IG2b, κ). ELISA was developed using streptavidin- HRPO as described in Section 2. *b*: photobiotinylation using increasing concentrations of 8-azido-adenosine-biotin to label goat anti-mouse antibody. Three different amounts of antibody were photobiotinylated and tested in ELISA.

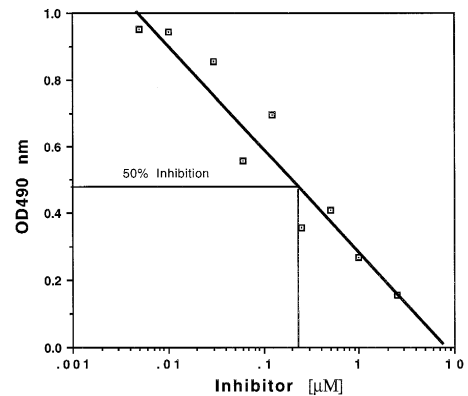


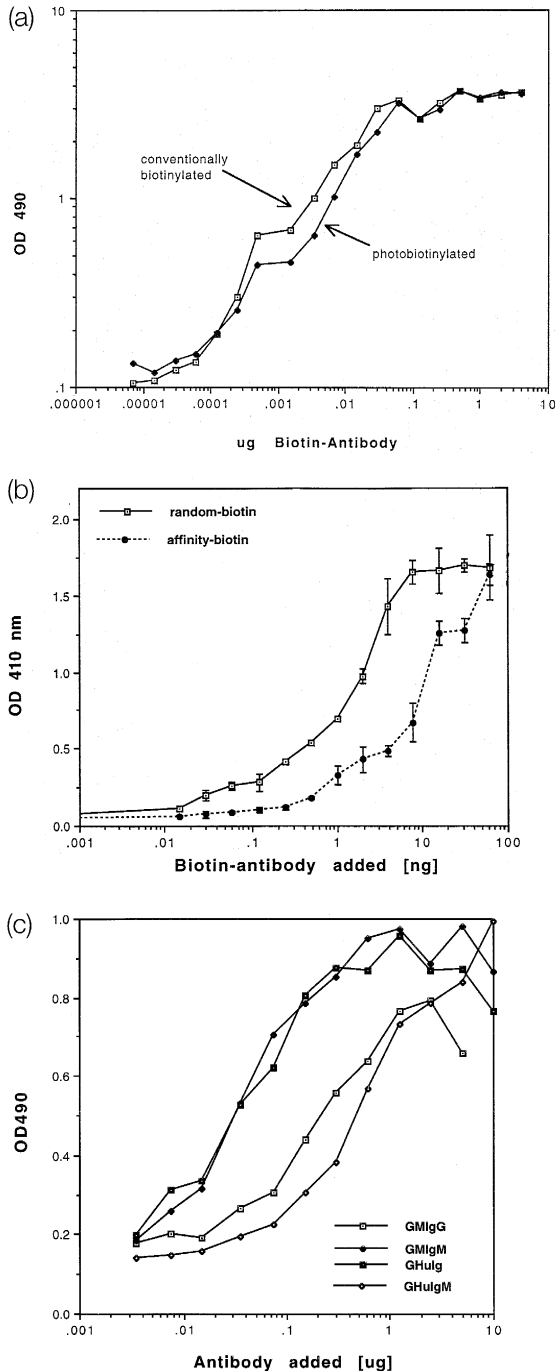
Fig. 3. Competition ELISA of photobiotinylated goat anti-mouse IgG using unlabeled antibody. Increasing amounts of unlabeled goat anti-mouse Ig were coincubated with a constant amount of (150 ng) of photobiotinylated antibody. The 50% inhibition value is extrapolated to the amount of competitor (*x* axis) which indicates about 150–200 ng of unlabeled antibody.

corresponding commercial biotin goat anti-mouse IgM from the same supplier and the photo-biotinylated antibody were used in ELISA to react with a monoclonal mouse IgM. As seen in Fig. 4a, titration of both biotinylation antibodies showed almost identical ELISA reactivity. Second, a goat anti-mouse Fc-specific was also affinity biotinylation and compared with the corresponding commercial biotin Fc-specific goat anti-mouse in an antigen capture assay. Both biotin antisera were used in serial dilutions. The binding of the mouse monoclonal CC49 to BSM coated plates was detected with both biotin goat anti-mouse antibodies. As seen in Fig. 4b, the ELISA reaction with the dilutions of the conventional biotin antisera is stronger than with the affinity biotinylation antibody. This was expected since the number of biotin molecules per Ig is higher in conventional biotin antisera than with affinity biotinylation antibodies: 8–10 biotin molecules/Ig on average with conventional methods versus two with the affinity labeling method (Rajagopalan et al., 1996).

Furthermore, four polyclonal goat antibodies against mouse and human Igs were photo-biotinylation with 8-N₃ATP- γ -biotin and used in ELISA (Fig. 4c). Goat anti-human IgG and goat anti-mouse IgM showed antigen-specific binding in criss-cross experiments (data not shown).

3.3. Photobiotinylated antibodies

Since the label reacts with high affinity to a unique site on the Ig, it seemed conceivable that



antibodies could be labeled without prior purification in the mixtures such as serum, ascites or tissue culture supernatants. Thus, we labeled several immune sera, ascites fluids and culture supernatants from hybridoma cells. These photobiotinylated samples were tested by different methods.

3.3.1. ELISA with HIV⁺ sera

First, human sera from HIV-1 infected individuals were photo-biotinylated and used in ELISA on p24 and p17 recombinant viral antigen coated plates. As shown in Fig. 5, anti-p17 human antibodies could be easily detected using immune sera directly photobiotinylated. Similar results were obtained with biotinylated HIV-1 sera on gp120/160 coated ELISA plates. Compared to the indirect ELISA, using as secondary antibody a conventionally biotinylated goat anti-human Ig, the direct ELISA based on photobiotinylated antisera offered slight reduction in sensitivity and an increase of background (data not shown).

3.3.2. Western blot with HIV⁺ sera

We used the sera from HIV-1 infected donors in HIV-1 antigen specific immunoblots again. HIV strips (BioRad immunoblot membranes) were incubated with biotinylated HIV positive and negative sera. The photobiotinylated sera were compared with a commercial biotinylated secondary antibody (biotin goat anti-human IgG) in Western blot. As seen in Fig. 6, the Western blots developed with the direct and indirect methods are nearly identical in staining the HIV-1 antigens. As a control, we used directly

Fig. 4. *a*: comparison of conventional and photoaffinity biotinylated antisera. ELISA plates were coated with mouse IgM MOPC104E (2 μg /well). Increasing amounts of photobiotinylated goat anti-mouse IgM (Sigma) and commercial goat biotin anti-mouse IgM (Sigma) were added and ELISA was developed using avidin-HRPO. *b*: comparison of conventional and photoaffinity biotinylated antisera. Plates were coated with BSM and incubated with CC49 as first antibody. Serial dilutions of either commercial biotinylated Fc-specific anti-mouse Ig or of affinity biotinylated Fc-specific anti-mouse Ig were added as secondary and ELISA was developed as described. *c*: binding of various photobiotinylated goat antisera in ELISA. Polyclonal anti-mouse and anti-human Ig antibodies were photobiotinylated and tested in their corresponding assays using avidin-HRPO.

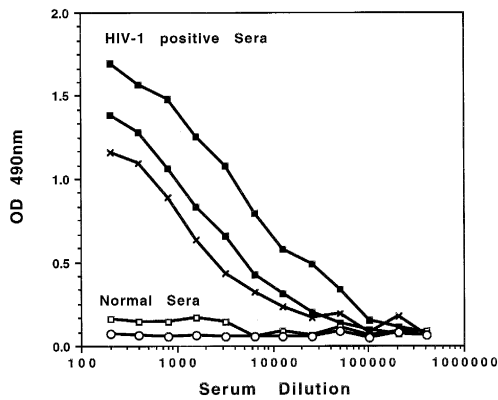


Fig. 5. ELISA using photobiotinylated immune sera. Sera from normal and HIV-1 infected individuals were photobiotinylated using 8-azidoadenosine-biotin. ELISA plates were coated with recombinant p17, dilutions of photobiotinylated sera were added and ELISA was developed as described in Section 2.

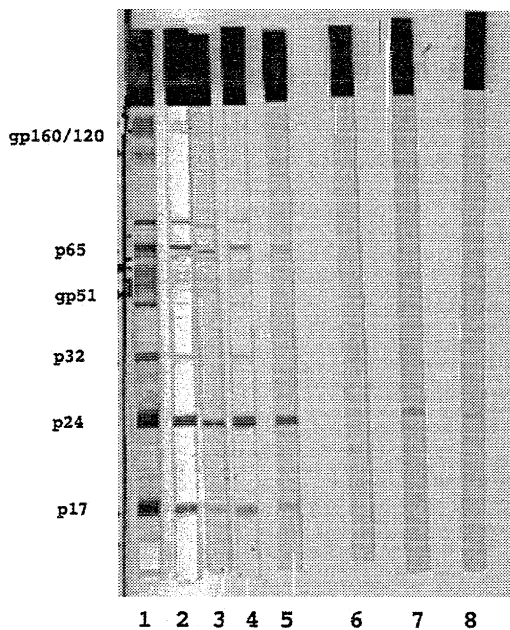


Fig. 6. Western blot with HIV-1 antigens. Sera from normal and three HIV-1 infected individuals were photobiotinylated. Photobiotinylated sera were incubated on BioRad HIV-1 immunoblot membranes. For comparison same sera were developed using commercial biotinylated antiserum. All samples were developed with streptavidin-HRPO. Lane 1: control HIV-1 proteins; lanes 2, 4 and 7: HIV-1⁺ sera developed with commercial biotinylated anti-human antiserum; lanes 3, 5 and 8: photobiotinylated HIV-1⁺ serum; lane 6, photobiotinylated normal serum.

photobiotinylated HIV negative serum which did not react with HIV-1 antigens.

3.3.3. FACS analysis using photobiotinylated hybridoma ascites fluid and culture supernatants

A mouse lymphoma cell line was used to test photobiotinylated antibodies in FACS analysis. The S1C5 antibody recognizes a tumor idiotype on 38C13 B cell murine lymphoma (Maloney et al., 1985). S1C5 hybridoma culture supernatant was biotinylated and compared with photobiotinylated purified S1C5 antibody in FACS on 38C13 cells. The control in Fig. 7 shows the FACS analysis of 38C13 tumor cells with FITC-avidin only; Fig. 7 also shows positive cell staining using photobiotinylated culture supernatant from S1C5 hybridoma producing 20 ng/ml antibody. For comparison, purified S1C5 antibody was photobiotinylated and used in FACS on 38C13 cells. These FACS analysis data show that supernatants from hybridoma cell lines can be directly photobiotinylated without prior purification or concentration.

3.4. Photobiotinylation of sensitive IgM MoAb

Monoclonal antibodies of the IgM class are known to be easily damaged by chemical or enzymatic iodination and biotininylation. 1F7, a κ IgM MoAb,

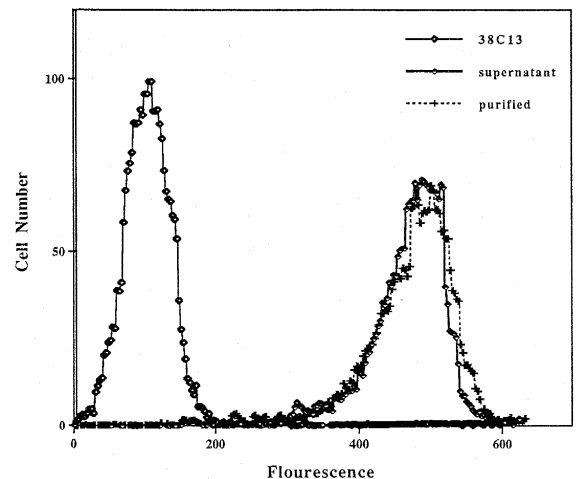


Fig. 7. FACS analysis on 38C13 tumor cells using as control FITC-streptavidin, photobiotinylated supernatant from S1C5 cultures and photobiotinylated purified S1C5 antibody.

recognizes a shared idotype on anti-HIV-1 antibodies (Müller et al., 1991). In our experience, within 24 h 1F7 lost binding activity after conventional labeling techniques. Photobiotinylated 1F7 retained binding for weeks, as measured by ELISA (data not shown) on human anti-p24 antibodies. As a control, TEPC183 (IgM, κ) was also photobiotinylated and tested in ELISA.

3.5. Photobiotinylation of light chain dimers and Fab fragments

Light chain dimers are considered as a model for the folds of Fv fragments because of their crystal structure and their ability to bind haptens (Schiffer et al., 1973). Thus, we considered that light chain dimers could also be photobiotinylated using the biotin reagent. Two naturally existing κ light chain dimers, Lay and B6, were labeled with increasing concentrations of N_3 -adenosine-biotin or N_3 ATP- γ -biotin and separated on SDS gels. The gels were then developed as Western blots using avidin-AP. As shown in Fig. 8 the light chain dimer is readily

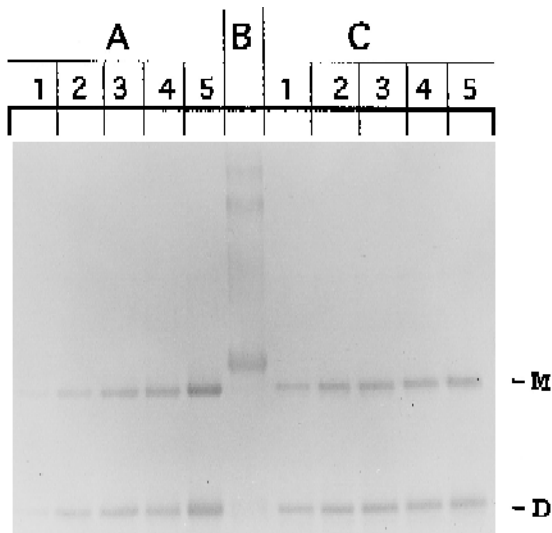


Fig. 8. Western blot analysis of photobiotinylated light chain dimers. A1–5 and C1–5: Lay light chain dimers were photobiotinylated with increasing concentrations of 8- N_3 -adenosine-biotin (10, 25, 50, 75, 100 μ mol) and developed as Western using streptavidin-HRPO. B: monoclonal S1C5 IgG photobiotinylated. The banding pattern shows dimers (D) and monomeric (M) light chains.

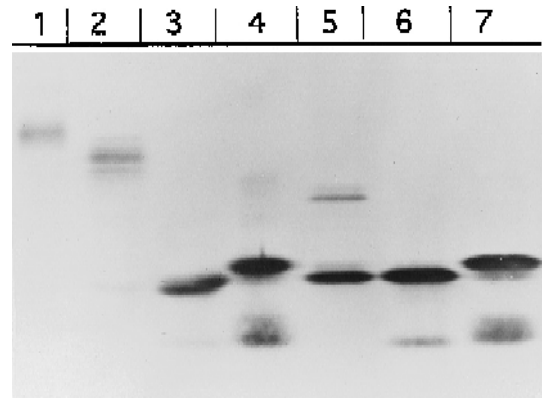


Fig. 9. Western blot analysis of photobiotinylated Fab' fragments and their CC49 parental Ig. Blots were developed as described in Section 2. Lanes 1–4: Western blotting under non-reducing conditions. 1: CC49 Ig; 2: F(ab')₂; 3: Fab'; 4: scFv. Lanes 5–7: blotting under reducing conditions; 5: CC49 Ig; 6: F(ab')₂; 7: scFv.

photobiotinylated. Since the antigen binding of these light chains is not known, antigen binding assays could not be used here.

Since enzymatic Ig fragments could be photolabeled efficiently it was of interest to label genetically engineered fragments and to compare them with enzymatic fragments. F(ab')₂ and Fab' were generated from the CC49 anti-TAG-72 MoAb and photobiotinylated. In addition, a recombinant scFv expressed in *E. coli* was purified and photobiotinylated. These fragments were analyzed by Western blot. As seen in Fig. 9 these photobiotinylated fragments are visualized with streptavidin-AP as well as the parental CC49 Ig. Antigen binding of photobiotinylated fragments were tested in ELISA. As seen in Fig. 10 intact CC49, F(ab')₂ and scFv bind identically to TAG-72, while the monomeric Fab bound significantly less. This could be due to the intrinsically lower stability of the monomeric fragment in the absence of the interchain disulfide bond.

3.6. Stability of photobiotinylated antibodies

The shelf life of photobiotinylated antibodies was tested by storing several of these antibodies at 4°C in PBS for up to 4 months. Aliquot samples were periodically tested in biotin-avidin ELISA and no loss of reactivity was detected. Furthermore, photobiotinylated sera and culture supernatants were also

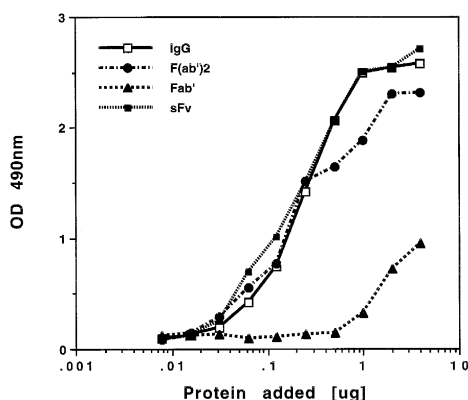


Fig. 10. ELISA using photobiotinylated CC49 Ig and fragments. Plates coated with BSM were incubated for 2 h with increasing amounts of photobiotinylated antibody or Ig fragments. ELISA was developed as described in Section 2.

used in FACS after two and four months storage at -20°C without decrease of staining.

4. Discussion

Biotin-avidin based immunoassays are commonly used because of their ease of preparation, high sensitivity and flexibility to adapt to different assay configurations. Typically the biotin moiety is linked to the antibody via chemically reactive groups which form covalent bonds with the antibody molecule. The chemical nature of the bond depends on the conjugation chemistry and the availability of reactive side chains. While this random biotinylation, in most instances, does not degrade the assay, in certain applications the random substitutions can limit the use of the biotinylated antibody.

In this study, we have used a recently discovered site in the immunoglobulin molecule which has affinity for purine containing nucleotides (Rajagopalan et al., 1996). This site can be efficiently photolabeled with $8\text{-N}_3\text{ATP}$ at low micromolar concentrations. The quantified saturation kinetics indicate the incorporation of two ATP molecules into one Ig molecule. Monoclonal and polyclonal Igs of different Ig classes and species origin are labeled with the $8\text{-N}_3\text{ATP}$ photoprobes with similar saturation. Tryptic peptides derived from affinity radiolabeled mono-

clonal antibodies were used to identify the sequence location of the affinity site. The radiolabeled peptides are derived from the Fv domains of H and L chains and contain variant framework residues. A computer model was constructed placing the ADP into a hydrophobic pocket at the $V_{\text{H}}\text{-}V_{\text{L}}$ interface away from the CDR loops which constitute the antigen binding surface of the Ig molecule (Rajagopalan et al., 1996). Antigen binding studies with several photolabeled antibodies confirmed this prediction.

We used the nucleotide affinity site to photochemically link biotin to antibodies for use in avidin-biotin immunoassays. Biotin was attached to the azido-ATP via the phosphate group and to azidoadenosine via the ribose. Both biotin reagents photoinserted into the affinity site with almost identical kinetics. Several antibodies were photobiotinylated and tested in their respective immunoassays, showing intact antigen binding.

The novel photobiotinylation method based on affinity site conjugation has several advantages over currently used chemical procedures which biotinylate antibodies at randomly distributed sites based on availability of amino acids side chains reactive in the conjugation chemistry: (i) the concentration of the photoaffinity reagent is adjusted to saturate the affinity site which is below the concentration where significant non-site specific modification would occur. This produces a defined modified antibody product which is reproducible without the typical batch variation seen with non-site specific modification; (ii) the reaction conditions are mild and physiological for antibodies and the reaction is completed within a short time; (iii) there are no long-lived secondary products which could continue reacting with the protein during storage or handling. This makes the purification steps after photolysis unnecessary for many applications. The strict control of the photoreaction by UV light exposure together with no need for purification after the photoreaction, makes this procedure very suitable for extremely small-scale and quick biotinylation protocols. The fact that the Ig molecule has only two affinity sites for photoinsertion of biotin (Rajagopalan et al., 1996) may at first appear as disadvantage over the random chemical conjugation methods which allow higher substitution ratios. While in a side-by-side compari-

son the ELISA color yield is lower with the affinity-site biotinylated antisera the color can be easily increased by increasing the amount of biotin antibody in the ELISA protocol without changing the signal to background ratio (unpublished data). Taken these properties of the affinity biotinylation method into consideration affinity biotinylation will be the method of choice for antibodies which are easily damaged by any random chemical modification and for biotinylation of small samples.

The computer model of the inserted photoprobe into the affinity site (Rajagopalan et al., 1996) shows that the ribose and the phosphate are protruding from the surface of the Ig molecule. This mode of insertion into the site makes the ribose and the phosphate groups available for attaching reporter molecules which are need to be surface exposed, such as biotin. The biotin in the N₃ATP or N₃-adenosine therefore is also available for binding to avidin. Because of the location of the affinity site which is distant from the antigen binding surface, the binding of the avidin proteins does not interfere with antigen binding.

The affinity site based photobiotinylation occurs at a precisely defined site which is distant from the antigen binding surface. Random biotinylation can modify the antigen binding site and impair or destroy antigen binding and can also modify sites which affect biodistribution and half life in vivo. While polyclonal antibody preparations are usually not affected by random biotinylation methods, some monoclonal antibodies are quite sensitive and can lose much of their antigen binding capacity. As an example of a sensitive antibody, we studied the photobiotinylation effect on a monoclonal IgM and demonstrated that affinity site used here for photobiotinylation is a naturally occurring site in the Ig structure. Attaching reporter molecules to this site is not expected to alter or change its overall or local conformation thereby preserving the structural and functional integrity of antibodies.

This photobiotinylation method can be used to biotinylate the smaller Fab and Fv fragments because of the location of the affinity site in the Fv arm. Photobiotinylation of a single chain Fv fragment was possible without loss of antigen binding. The photobiotinylation could also become useful in the screening of Fab-phage libraries based on biotin-avidin detection method.

Photobiotinylation can be applied to non-purified antibody preparations for use in ELISA and Western blots as shown with HIV⁺ sera and with culture supernatants. Effective photobiotinylation of the high affinity site on Ig takes place at concentrations of the biotin reagents where non-site specific biotinylation is avoided. Thus, antibodies are preferentially photobiotinylated under the low saturation concentration over many other proteins which do not have similar purine affinity sites. However, this selectivity for antibodies in serum is relative, since various other proteins in serum do have ATP binding sites. Operationally, this implies that antibodies can be directly photobiotinylated in high titer sera and used in immunoassays. Similar consideration apply to culture supernatants produced by hybridoma cells.

The successful use of a photoaffinity site-specific conjugation method to obtain biotinylated antibodies with full structural and functional integrity suggests that this site can also be used to tether other molecules of biological and medical interest to antibodies. Future application of this affinity conjugation strategy are directed towards coupling drugs, oligonucleotides and cytokines to antibodies and their smaller recombinant fragments.

Acknowledgements

This work was supported by a grant from the Tobacco Research Council grant 3954, a SBRI grant R43 CA65299 to Immpheron and an NIH grant R01 GM-35766, and by the McDowell Foundation. We thank Drs. David Colcher and Guy Beresford, University of Nebraska for the CC49 antibodies and fragments, Dr. Alan Solomon, University of Tennessee, for the light chain dimers, and Dr. Soshona Levy, Stanford, for the 38C13 tumor cells and the S1C5 antibody.

References

- Czarnecky, J., Geahlen, R. and Haley, B. (1979) Synthesis and use of azido photoaffinity analogs of adenine and guanine nucleotides. *Methods Enzymol.* 56, 642.
- Johnson, V.G., Schlom, J. Paterson, A.J., Bennett, J., Magnani, J. and Colcher, D. (1988) Analysis of a human tumor-associated glycoprotein TAG-72, identified by a monoclonal antibody B72.3. *Cancer Res.* 46, 850.

- Khatoun, S., Cambell, S.R., Haley, B. and Slevin, J.J. (1989) Aberrant GTP b-tubulin interactions in Alzheimer's disease. *Ann. Neurol.* 26, 215.
- Levy, S., Medel, E. and Kon, S. (1985) A rapid method for cloning and sequencing variable-region genes of expressed immunoglobulins. *Gene* 54, 167.
- Maloney, D.G., Kaminski, M.S., Burowski, D., Haimovich, J. and Levy, R. (1985) Monoclonal anti-idiotypic antibodies against the murine B cell lymphoma 38C13: characterization and use of probes for the tumor in vivo and in vitro. *Hybridoma* 4, 4479.
- Müller, S., Wang, H.-T., Kaveri, S., Chattopadhyay, S. and Köhler, H. (1991) Generation and specificity of monoclonal anti-idiotypic antibodies against human HIV-specific antibodies. *J. Immunol.* 147, 933.
- Murano, R., Kuroki, M., Wunderlich, D., Poole, D.J., Colcher, D., Thur, A., Greiner, J.W., Simpson, J.F., Molinolo, A., Noguchi, P. and Schlom, J. (1983) Generation and characterization of B.73.3 second generation monoclonal antibodies reactive with the tumor-associated glycoprotein p 72 antigen. *Cancer Res.* 43, 736.
- Paganelli, G., Belloni, C., Magnani, P., Zito, F., Pasini, A., Sassi, I., Meroni, M., Mariani, M., Vignali, M., Siccardi, A.G. and Fazio, F. (1992) Two-step tumor targeting in ovarian cancer patients using biotinylated monoclonal antibodies and radioactive streptavidin. *Eur. J. Nucl. Med.* 19, 322.
- Potter, R.L. and Haley, B.E. (1983) Photoaffinity labeling of nucleotide binding sites with 8-azidopurine analogs: techniques and applications. *Methods Enzymol.* 91, 13.
- Rajagopalan, K., Chavan, A.J., Haley, B. and Watt, D.S. (1993) Synthesis and application of bidentate photoaffinity cross-linking reagents: Nucleotide photoaffinity probes with two photoreactive groups. *J. Biol. Chem.* 268, 14230.
- Rajagopalan, K., Pavlinkova, G., Levy, S., Pokkuluri, P., Schiffer, M., Haley, B.E. and Köhler, H. (1996) Novel unconventional binding site in the variable region of immunoglobulins. *Proc. Natl. Acad. Sci. USA* 93, 6019.
- Salvucci, M., Chavan, A. and Haley, B., E. (1972) Identification of peptides for the adenine binding domains of ATP and AMP in adenylate kinase: Isolation of photoaffinity labeled peptides by metal chelate chromatography. *Biochemistry* 31, 4479.
- Schiffer, M., Girling, R.L., Ely, K.R. and Edmundson, A.B. (1973) Structure of a lambda-type Bence-Jones protein at 3.5-Å resolution. *Biochemistry* 12, 4620.
- Slavin-Chiorini, D.C., Kahmiri, S.V., Schlom, J. Calvo, B., Shu, L.M., Schott, M.E., Milenic, D.E., Snoy, P., Carrasquillo, J. and Anderson, K. (1995) Biological properties of chimeric domain-deleted anticarcinoma immunoglobulins. *Cancer Res.* 55, 5957.
- Wilcheck, M. and Bayer, E.A. (1990) *Avidin-Biotin Technology. Methods in Enzymology*, Vol. 84. Academic Press, New York.
- Wong, S.S. (1991) *Chemistry of Protein Conjugation and Crosslinking*. CRC Press, Boca Raton, FL, 1.