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Monoclonal Antibody-Based Quantitation of Poly(ethylene glycol)-Derivatized Proteins, Liposomes, and Nanoparticles

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Covalent attachment of poly(ethylene glycol) (PEG) molecules to drugs, proteins, and liposomes is a proven technology for improving their bioavailability, safety, and efficacy. Qualitative and quantitative analysis of PEG-derivatized molecules is important for both drug development and clinical applications. We previously reported the development of a monoclonal IgM antibody (AGP3) to PEG. We now describe a new IgG_1 monoclonal antibody (E11) to PEG and show that it can be used in combination with AGP3 to detect and quantify PEG-derivatized molecules. Both antibodies bound the repeating subunits of the PEG backbone and could detect free PEG and PEG-modified proteins by ELISA, immunoblotting, and flow cytometry. Detection sensitivity increased with the length and the number of PEG chains on pegylated molecules. Both antibodies also efficiently accelerated the clearance of a PEG-modified enzyme in vivo. A sandwich ELISA in which E11/AGP3 were employed as the capture/detection antibodies was developed to detect PEG-modified proteins at concentrations as low as 1.2 ng/mL. In addition, the ELISA could also quantify, in the presence of 10% fetal bovine serum, free methoxy-$PEG_{20,000}$, $PEG_{2,000}$-quantum dots, and $PEG_{2,000}$-liposomes at concentrations as low as 20 ng/mL (1.0 nM), 1.4 ng/mL (3.1 pM), and 2.4 ng/mL (3.13 nM phospholipids), respectively. Finally, we show that the sandwich ELISA could accurately measured the in vivo half-life of a PEG-modified enzyme. These antibodies should be generally applicable to the qualitative and quantitative analysis of all PEG-derivatized molecules.

INTRODUCTION

PEG[1] is a water-soluble, nontoxic, nonantigenic, biocompatible polymer that has been approved by the Food and Drug Administration (FDA) for human intravenous, oral, and dermal applications (*1*). Attachment of PEG chains to proteins can reduce their immunogenicity (*2*, *3*), minimize proteolytic cleavage (*4*, *5*), and increase their serum half-life (*6*, *7*). PEG-interferon α (*8*, *9*), PEG-filgrastim (*10*, *11*), and PEG-visomant (*12*) have been approved for clinical use by the FDA. PEG-modified liposomes are being developed for controlled-release and selective-delivery of drugs (*13*) and genes (*14*). PEG has also been attached to small molecules such as camptothecin (CPT) to produce greater tumor accumulation as compared to unmodified CPT (*15*). PEG-derivatization of superparamagnetic iron oxide (*16*) and quantum dots (*17*, *18*) can improve their biocompatibility and reduce receptor-mediated uptake by the reticuloendothelial system. It is anticipated that PEG will be increasingly employed to improve the properties of therapeutic agents as more proteins, macromolecular drugs, and imaging agents enter clinical trials.

Clinical development of PEG-modified molecules requires measurement of pharmacokinetic parameters in animals and patients. Ideally, the concentration of intact PEG-modified molecules should be measured in vivo. Until recently, however, simple and sensitive methods to measure intact PEG−protein conjugates were unavailable. We helped alleviate this problem by developing a monoclonal IgM antibody (AGP3) with specificity for PEG (*19*). AGP3 can be employed to measure PEG-modified protein by ELISA (*20*). We now report the development of a new monoclonal IgG antibody (E11) that specifically binds to PEG. We compare AGP3 and E11 for the detection of free PEG and PEG-modified proteins in immunoblots, ELISA, and flow cytometry. Because the specificity of two-step targeting strategies depends on the efficient clearance of the first-step targeting agent from the circulation (*21*), we also investigated the effectiveness of AGP3 and E11 for the in vivo clearance of PEG−enzyme conjugates. Finally, a sandwich ELISA using E11

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[1] Abbreviations: PEG, poly(ethylene glycol); ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; βG, *Escherichia coli* β-glucuronidase; DNS, dansyl (5-(dimethylamino)-1-naphthalenesulfononic acid), DNS scFv, anti-dansyl single chain antibody; DNS−PEG, PEG modified with a dansyl moiety; PBS-T, phosphate-buffered saline containing 0.05% Tween-20; HRP, horseradish peroxidase.

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as the capture antibody and AGP3 as the detection antibody was examined for the quantitation of free methoxy PEG, PEG−enzymes, PEG−quantum dots, and PEG−liposomes.

EXPERIMENTAL PROCEDURES

Reagents. Recombinant *E. coli*-derived β-glucuronidase (βG) was produced as described (*6*). Methoxy-PEG_{750}-NH_2 (750 Da) was obtained from Fluka Chemie (Buchs, Switzerland). PEG_{3400}-disuccinimidyl succinamide (3400 Da) and methoxy-PEG succinimidyl propionate (2000, 5000, and 20 000 Da) were purchased from Nektar Therapeutics (Birmingham, AL). Methoxy-PEG_{5000}-NH_2 (5000 Da), NH_2-PEG_{3400}-NH_2 (3400 kDa), methoxy-PEG_{2000}-OH (2000 Da), NH_2-PEG_{1500}-NH_2 (1500 Da), dansyl choride, dansylcadaverine, fluorescamine, and *p*-nitrophenyl-β-D-glucuronide were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Amino-PEG_{2000}-qdot nanoparticles (10∼20 nm, 440 kDa) were purchased from Quantum Dot Corporation (Hayward, CA). $PEG_{2,000}$-liposomes were composed of hydrogenated soy phosphatidylcholine, cholesterol, and poly(ethylene glycol)-distearoylphosphatidylethanolamine with a mean size of 107 nm (polydispersity = 0.097) as described (*22*). Sephacryl S-200 HR gel was from Amersham Pharmacia Biotech Asia Pacific Ltd. (Taiwan). Specific pathogen-free BALB/c mice were obtained from the National Laboratory Animal Center, Taipei, Taiwan.

Antibodies and Cells. 1E8 (IgG_1 mAb against βG) and AGP3 (IgM mAb against PEG) have been described (*19*). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM μ-chain antibody and HRP-conjugated goat anti-mouse IgG Fc were from Jackson ImmunoResearch Laboratories (Westgrove, PA). B16/DNS melanoma cells that stably express a functional anti-dansyl single-chain antibody (DNS scFv) on their surface have been described (*23*). To generate mAb E11 (IgG_1 mAb against PEG), female BALB/c mice were first iv injected with 200 μg of RH1−βG−PEG, a conjugate formed between a murine antibody and PEG-derivatized βG (*19*). The mice were ip injected with 100 μg RH1−βG−PEG one week later and then sc injected at weekly intervals with 50 μg of βG−PEG in complete Freund's adjuvant, 30 μg of βG−PEG in incomplete adjuvant, 30 μg of BSA−PEG in incomplete adjuvant, and 10 μg of BSA−PEG in incomplete adjuvant. Three days before fusion with FO myeloma cells, the mice were ip injected with 30 μg of BSA−PEG in PBS. Subsequent steps were the same as employed to generate AGP3 (*19*).

Synthesis of DNS−PEG Derivatives. NH_2-PEG_{1500}-NH_2 and NH_2-PEG_{3400}-NH_2 were reacted with dansyl chloride at a molar ratio of 5:1 in dichloromethane containing 0.1% triethylamine at room temperature for 1 h. The reaction mixture was separated on silica gel with dichloromethane-methanol (8:2 vol:vol) to produce DNS−PEG_{1500}-NH_2 and DNS−PEG_{3400}-NH_2. The presence of the dansyl group was verified by UV detection whereas primary amine groups were detected by reaction with nihydrin/2% solution (Sigma). PEG_{3400}-disuccinimidyl succinamide was reacted with dansylcadaverine at a molar ratio of 2:1 in dry dichloromethane containing 0.1% triethylamine at room temperature for 1 h. The resulting DNS−PEG_{3400}-succinimidyl succinamide (DNS−PEG_{3400}−SSA) was detected under UV illumination, eluted from silica gel with dichloromethane-methanol (9:1 vol:vol) and then immediately reacted with βG.

PEG Modification of *β*G. βG was passed through a Sephadex G-25 column equilibrated with 0.1 M $NaHCO_3$, pH 8.0, and then concentrated by ultrafiltration to 2 mg/mL. DNS−PEG_{3400}−SSA (4 mg per mg βG) was added for 2 h at room temperature. One-tenth volume of a saturated solution of glycine (pH 8.0) was added to stop the reaction. Unreacted PEG was removed by gel filtration on a Sephacryl S-200 HR column. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL) with bovine serum albumin used as the reference protein. The number of PEG molecules introduced in βG was estimated by measuring amine groups before and after PEG modification with fluorescamine (*23*). An average of 3.5 DNS_{3400}−PEG chains was attached to each 70-kDa subunit of the βG tetramer to form a 3.5DNS−PEG_{3400}−βG conjugate. Methoxypoly-(ethylene glycol) succinimidyl propionate with MW of 20,000, 5000, and 2000 Da was reacted with βG (20 mg per mg βG) as previously described (*20*). An average of 5.1, 7.1, and 11.4 PEG chains were introduced per subunit of the βG tetramer to form 5.1$PEG_{20,000}$−βG, 7.1PEG_{5000}−βG, 11.4PEG_{2000}−βG with retention of 70%, 50%, and 43% of original βG enzymatic activity, respectively.

Immunoblotting of Free PEG and PEG−*β*G. Samples of βG (5 μg), 3.5DNS−PEG_{3400}−βG (5 μg), NH_2-PEG_{1500}-NH_2 (30 μg), and CH_3-PEG_{2000}-OH (30 μg) were electrophoresed in an 8% sodium-dodecyl sulfate polyacrylamide gel under reducing conditions before overnight transfer to nitrocellulose paper by capillary transfer in blotting buffer (50 mM NaCl, 2 mM EDTA, 0.5 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7.5). Blots were blocked for 2 h with 5% skim milk and incubated for 1 h at 37 °C with 1 μg/mL 1E8, AGP3 or E11 in PBS containing 0.05% BSA. The blots were washed 3 times with PBS-T (0.05% Tween 20 in PBS) and twice with PBS before incubation with goat anti-mouse Ig-HRP for 40 min at 37°C. The blots were then washed three times with PBS-T and twice with PBS before specific bands were visualized by ECL detection according to the manufacturer's instructions (Pierce, Rockford, IL).

Antibody ELISA. Maxisorp 96-well microplates (Nalge Nunc International, Roskilde, Denmark) were coated with 10 μg/well CH_3-PEG_{750}-NH_2, NH_2-PEG_{1500}-NH_2, NH_2-PEG_{3400}-NH_2, or 3.5DNS−PEG_{3400}−βG in 0.1 M $NaHCO_3$, pH 8.0, for 3 h at 37 °C and then blocked with 2% skim milk. Graded concentrations of AGP3 and E11 were added to the plates at 37 °C for 1 h. The plates were washed three times with PBS-T and two times with PBS and then stained with HRP-labeled goat anti-mouse IgM μ chain (2 μg/mL) or HRP-labeled goat anti-mouse IgG Fc (2 μg/mL) for 1 h at 37 °C, respectively. The plates were washed and bound peroxidase activity was measured by adding 100 μL/well ABTS solution (0.4 mg/mL 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid), 0.003% H_2O_2, 100 mM phosphate-citrate, pH 4.0) for 30 min at room temperature. Absorbance (405 nm) of wells was measured in a microplate reader (Molecular Device, Menlo Park, CA). Binding of CH_3-PEG_{750}-NH_2, NH_2-PEG_{1500}-NH_2, NH_2-PEG_{3400}-NH_2, and 3.5DNS−PEG_{3400}−βG to the microtiter plates was determined by assaying for the presence of amine groups with fluorescamine (*24*).

Flow Cytometer Analysis. B16/DNS cells (5×10^5) were incubated with 200 μL of DNS−PEG_{1500}-NH_2 (10 μg/mL), DNS−PEG_{3400}-NH_2 (10 μg/mL), 3.5DNS−PEG_{3400}−βG (10 μg/mL), or PBS at 4 °C for 1 h. The cells were washed twice with cold PBS and sequentially stained with AGP3 (5 μg/mL) and FITC-labeled goat anti-mouse IgM μ chain (5 μg/mL) or E11 (5 μg/mL) and FITC-labeled goat anti-mouse IgG Fc (5 μg/mL). The surface immun-

ofluorescence of 10 000 viable cells was measured with a flow cytometry (Beckman-Coulter, Miami, FL).

Clearance of PEG−βG Conjugates. Groups of 3 BALB/c mice were iv injected with100 μg of 5.1PEG$_{20,000}$−βG, 7.1PEG$_{5000}$−βG, or 11.4PEG$_{2000}$−βG at time zero. Blood samples (30 μL) were taken at 0.5, 3.5, and 9 h before iv injection of PBS, 150 μg of E11, or 180 μg of AGP3 at 9 h. Additional blood samples were taken at subsequent times (11, 14, and 28 h), and the serum concentration of βG−PEG was measured by a βG enzyme microassay as described (*19*). Sample concentrations were calculated by comparison of absorbance values with a standard curve constructed from known concentrations of 5.1PEG$_{20000}$−βG, 7.1PEG$_{5000}$−βG, and 11.4PEG$_{2000}$−βG. For meaningful comparison of clearance results, the measured concentrations of PEG−βG conjugates in mice injected with clearing antibodies were normalized to the mean concentration of PEG−βG conjugates in control (uncleared) mice just before clearance as previously described (*19*).

Sandwich ELISA. Maxisorp 96-well microplates were coated with 50 μL/well of E11 (20 μg/mL) or AGP3 (20 μg/mL) in 0.1 M $NaHCO_3$, pH 8.0, for 3 h at 37 °C. The plates were blocked with 2% skim milk in PBS overnight and then washed twice with PBS before graded concentrations of 3.5DNS−PEG$_{3400}$−βG, methoxy-PEG$_{20000}$ succinimidyl propionate (inactivated with 0.1 M Tris, pH 8.0), and PEG$_{2000}$-quantum dots or PEG$_{2000}$-liposomes in PBS containing 10% FBS were added for 1 h at 37 °C. The plates were washed three times with PBS-T and twice with PBS and were then sequentially stained with 50 μL/well AGP3 (20 μg/mL) and HRP-labeled goat anti-mouse IgM μ chain (5 μg/mL) or E11 (20 μg/mL) and HRP-labeled goat anti-mouse IgG Fc (5 μg/mL). The plates were washed and 100 μL/well ABTS substrate was added for 30 min at room temperature. The absorbance (405 nm) of the wells was measured in a microplate reader.

Determination of 3.5DNS−PEG$_{3400}$−βG Pharmacokinetics. BALB/c mice were iv injected with 150 μg of 3.5DNS−PEG$_{3400}$−βG and serum was isolated from blood samples (30 μL) that were periodically removed from the tail vein of the mice (0.17, 1.5, 4, 7, 11, 24, and 48 h). The concentration of 3.5DNS−PEG$_{3400}$−βG was measured by the new sandwich ELISA and by a βG enzyme microassay (*19*). βG concentrations were calculated by comparison of absorbance values with a standard curve constructed from known concentrations of 3.5DNS−PEG$_{3400}$−βG.

Data Analysis. Statistical significance of differences between mean values was estimated with Excel (Microsoft, Redmond, WA) using the independent *t*-test for unequal variances.

RESULTS

Detection of Free PEG and PEG-Modified Molecules. The specificity of the anti-PEG antibodies AGP3 and E11 was first examined in immunoblots. Figure 1a shows that the anti-βG antibody 1E8 detected βG and 3.5DNS−PEG$_{3400}$−βG, but not free NH_2-PEG$_{1500}$-NH_2 or CH_3-PEG$_{2000}$-OH. AGP3 and E11, in contrast, did not bind βG but detected 3.5DNS−PEG$_{3400}$−βG as well as free NH_2-PEG$_{1500}$-NH_2 and CH_3-PEG$_{2000}$-OH (Figures 1b and 1c). The ability of AGP3 and E11 to bind both NH_2-PEG$_{1500}$-NH_2 and CH_3-PEG$_{2000}$-OH demonstrates that these antibodies bind to the repeating OCH_2CH_2 backbone of PEG rather than to terminal CH_3, NH_2, or OH groups.

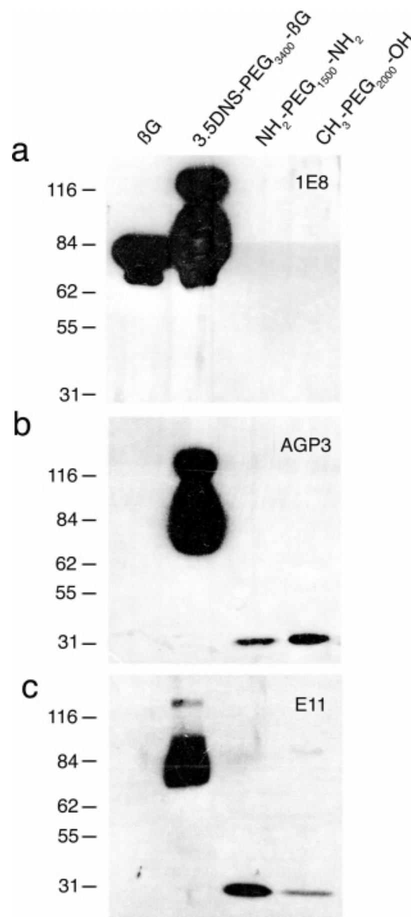

Figure 1. Immunoblot of free PEG and a PEG-modified enzyme. Samples were electrophoresed on a 8% reduced SDS polyacrylamide gel, transferred to nitrocellulose paper, and probed with mAb 1E8 (a), AGP3 (b) or E11 (c) as described in Experimental Procedures. Lane 1, βG; lane 2, 3.5DNS−PEG$_{3400}$−βG; lane 3, NH_2-PEG$_{1500}$-NH_2; lane 4, CH_3-PEG$_{2000}$-OH. kDa, molecular mass in thousands.

The binding of AGP3 and E11 to different lengths of free PEG (MW 750, 1500, 3400, and 5000 Da) was examined by first coating NH_2-PEG in the wells of microtiter plates and then performing ELISA. Coating of NH_2-PEG in the wells was verified by detecting the presence of the amine groups of NH_2-PEG with fluorescamine. Figure 2a shows that NH_2-PEG and 3.5DNS−PEG$_{3400}$−βG were successfully coated in the wells as demonstrated by increased fluorescence as compared with uncoated wells. Measurement of AGP3 (Figure 2b) and E11 (Figure 2c) binding to NH_2-PEG revealed that both antibodies bound NH_2-PEG$_{1500}$-NH_2, NH_2-PEG$_{3400}$-NH_2, and CH_3-PEG$_{5000}$-NH_2 but not CH_3-PEG$_{750}$-NH_2. Furthermore, the assay sensitivity was proportional to the length of the PEG chains, especially for E11. These results suggest that the binding epitope of both antibodies consists of at least 16 OCH_2CH_2 subunits (the number of subunits in PEG$_{750}$).

Adsorption of PEG chains to the plastic surface of microtiter plates could affect antibody binding. We therefore employed flow cytometry to measure AGP3 and E11 binding to PEG attached to the surface of mammalian cells. This was accomplished by introducing a dansyl moiety to the terminus of PEG to create DNS−PEG$_{1500}$-NH_2, DNS−PEG$_{3400}$-NH_2, and 3.5DNS−PEG$_{3400}$−βG. These conjugates were allowed to specifically bind to DNS scFv present on the surface B16/DNS cells. The cells were then sequentially stained with AGP3 and

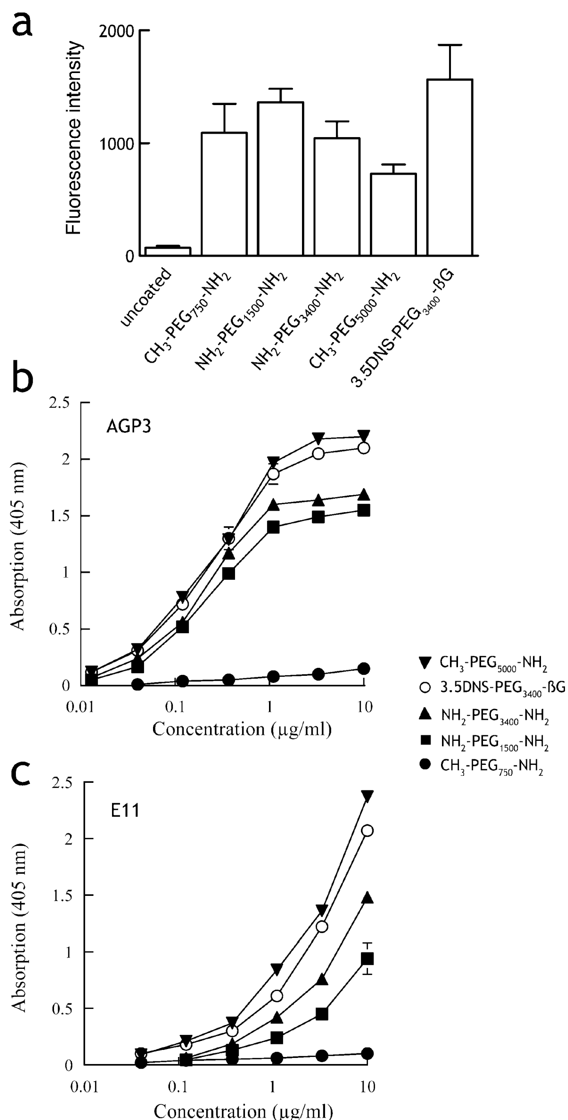

Figure 2. Qualitative analysis of PEG-derivatized molecules. (a) Coating of PEG and PEG-modified βG in microplates was confirmed by measuring the fluorescence intensity ($n = 3$) after reaction of amine groups with fluorescamine. Bars, SD. The binding of AGP3 (b) and E11 (c) to PEG and PEG-modified βG coated in microplates was detected by ELISA as described in the Experimental Procedures. The mean absorbance values (405 nm) of triplicate determinations are shown. Bars, SD.

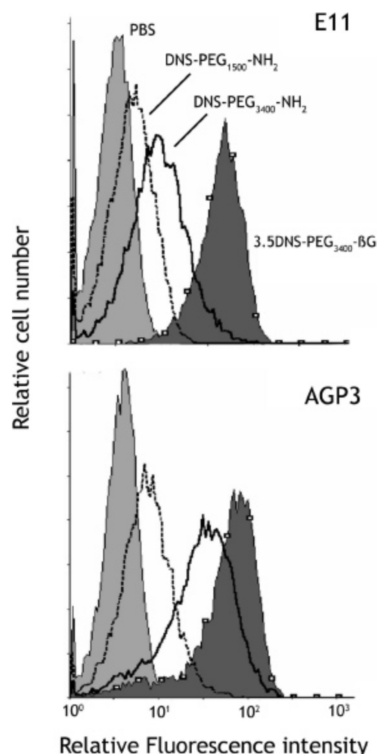

Figure 3. Flow cytometry analysis of PEG-derivatized molecules. B16/DNS cells were treated with PBS, DNS−PEG_{1500}-NH_2, DNS−PEG_{3400}-NH_2 or 3.5DNS−PEG_{3400}−βG at 4 °C for 1 h. The cells were washed and sequentially stained with E11 and FITC-labeled goat anti-mouse IgG Fc (upper panel) or AGP3 and FITC-labeled goat anti-mouse IgM μ chain (lower panel). The surface immunofluorescence of 10,000 viable cells was measured with a FACScaliber flow cytometer.

FITC-labeled goat anti-mouse IgM μ chain or E11 and FITC-labeled goat anti-mouse IgG Fc. Figure 3 shows that AGP3 and E11 could detect DNS−PEG_{1500}-NH_2, DNS−PEG_{3400}-NH_2, and 3.5DNS−PEG_{3400}−βG on B16/DNS cells. Consistent with the ELISA results, longer PEG chains resulted in increased sensitivity of the assay.

In Vivo Clearance of PEG−βG. To investigate the ability of AGP3 and E11 to clear PEG-modified proteins from the circulation, BALB/c mice were iv injected with 100 μg of 11.4PEG_{2000}−βG, 7.1PEG_{5000}−βG, or 5.1PEG_{20000}−βG, followed 9 h later by iv injection of AGP3 or E11 (2 nmoles of antigen-binding sites). Figure 4 shows that both AGP3 and E11 accelerated the clearance of PEG−βG conjugates from the circulation. E11 cleared significantly more PEG−βG conjugate than did AGP3. E11 was particularly effective at clearing βG modified with long-chained PEG; the serum concentration of 5.1$PEG_{20,000}$−βG was reduced by 200-fold in 2 h and 580-fold within 19 h after E11 administration as compared to control mice. These results suggest that AGP3 and E11 may be useful for accelerating the clearance of PEG-modified proteins in vivo.

Quantitative Sandwich ELISA. A sandwich ELISA was developed in which E11/AGP3 or AGP3/E11 was used for the capture/detection antibodies to measure free PEG and PEG-modified substances. Figure 5a shows that the assay sensitivity was about 4-fold greater when using E11 for capture and AGP3 for detection of 3.5DNS−PEG_{3400}−βG as compared with the opposite format. This assay could detect 3.5DNS−PEG_{3400}−βG at concentrations as low as 1.2 ng/mL (14.5 pM). In addition, the sandwich ELISA assay could also detect free methoxy-PEG_{20000}, PEG_{2000}-quantum dots, and PEG_{2000}-liposomes at concentrations as low as 20 ng/mL (1.0 nM), 1.4 ng/mL (3.1 pM) and 2.4 ng/mL (3.13 nM phospholipids), respectively (Figure 5b). The presence of 10% bovine serum in the samples did not affect assay sensitivity. To determine whether the sandwich ELISA assay could detect PEG-modified proteins in vivo, BALB/c mice were iv injected with 150 μg of 3.5DNS−PEG_{3400}−βG, and serum samples were periodically assayed for the concentration of the conjugate by the new sandwich ELISA or by measuring βG enzyme activity. Figure 5c shows that the measurement of 3.5DNS−PEG_{3400}−βG concentration by the sandwich ELISA and βG enzyme microassay gave similar results with estimated terminal half-lives of 6.3 and 5.5 h, respectively.

DISCUSSION

An increasing number of PEG conjugates are in late stage clinical trials or already approved for human use by the FDA including PEG-Intron for hepatitis C (*25, 26*), PEG-Neupogen for neutropenia associated with cancer

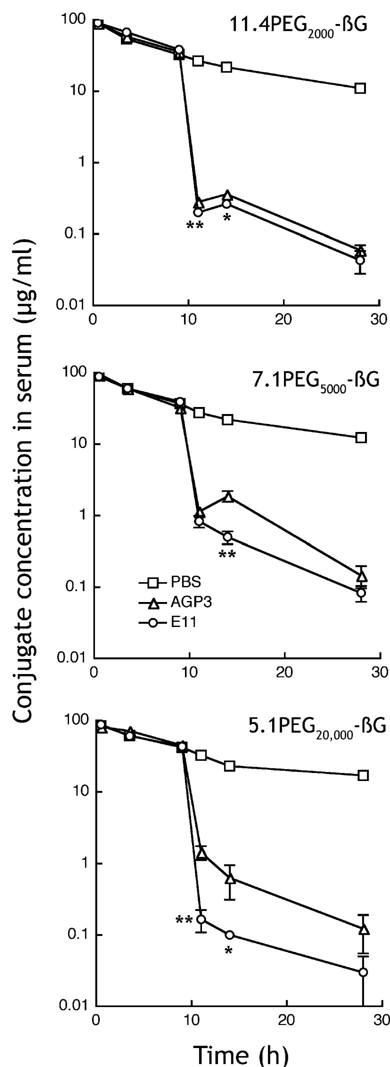

Figure 4. In vivo clearance of PEG−βG conjugates. Groups of 3 BALB/c mice were iv injected with 100 μm 11.4PEG$_{2000}$−βG (upper panel), 7.1PEG$_{5000}$−βG (middle panel) or 5.1PEG$_{20,000}$−βG (lower panel) at time zero. The mice received an iv injection of PBS (□), AGP3 (△) or E11 (○) at 9 h. The concentrations of the βG−PEG conjugates in serum at the indicated times were determined by assaying βG enzymatic activity. Bars, SD. Significant differences between βG−PEG concentrations after E11 and AGP3 treatment are indicated: *, $p \leq 0.05$; **, $p \leq 0.005$.

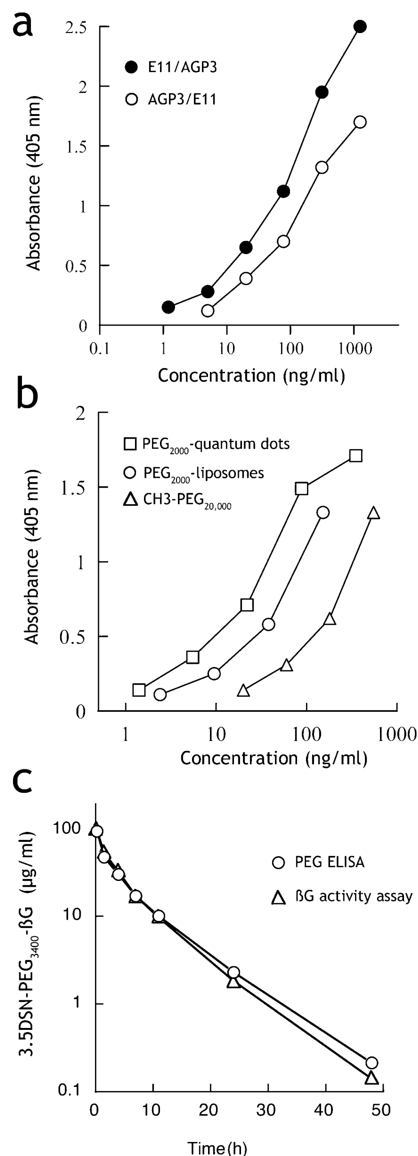

Figure 5. Quantitative analysis of PEG-derivatized molecules by ELISA. (a) Graded concentrations of 3.5DNS−PEG$_{3400}$−βG were assayed with a sandwich ELISA in which E11/AGP3 (●) or AGP3/E11 (○) were used for the capture/detection antibodies, respectively. The mean absorbance values of triplicate determinations are shown. Bars, SE. (b) A sandwich ELISA with E11 and AGP3 employed as the capture and detection antibodies, respectively, was used to measured the concentrations of PEG$_{2000}$-quantum dots (□), PEG$_{2000}$-liposomes (○) or free methoxy-PEG$_{20,000}$ (△) in PBS containing 10% serum. (c) Groups of three mice were iv injected with 3.5DNS−PEG$_{3400}$−βG at time zero. The concentration of 3.5DNS−PEG$_{3400}$−βG is serum was measured by the E11/AGP3 sandwich ELISA (O) or by the βG enzyme microassay (△). Bars, SD.

chemotherapy (*27*), PEG-Visomant for acromegaly (*28*), and PEG-coated liposomal doxorubicin (Caelyx) for cancer therapy (*29*). The ability to effectively control these diseases and minimize the injection frequency can result in better compliance and improved patient quality of life. For noninvasive imaging, PEG−quantum dots and PEG−superparamagnetic iron oxide have been extensively used in cell and animal biology for cell trafficking studies (*30*, *31*), tumor targeting (*18*, *32*), and diagnostics (*33*, *34*). Qualitative and quantitative analysis of poly(ethylene glycol)-derivatized molecules is important to determine their pharmacodynamic and pharmacokinetic parameters in animals and patients. In this report, we found that AGP3 and E11 recognize the repeating (OCH_2CH_2) subunits of the PEG backbone and could effectively clear PEG−βG from the circulation of mice. Furthermore, we developed a sandwich ELISA for the sensitive quantitation of PEG-modified molecules.

PEG can be linked to biological molecules by terminal group derivatization (*35*). First generation, low molecular weight PEG chains were found to poorly cover the surface of macromolecules and often drastically decreased the biological or pharmacological potency of proteins (*36*−*38*). To solve these problems, high molecular weight PEG can be used to maximize protection with better preservation of the bioactivity of pegylated molecules, allowing improved and prolonged performance (*39*). We found that AGP3 and E11 bind to the PEG backbone so that antibody binding did not depend on the identity of the pegylated molecule. These antibodies could therefore detect pegylated proteins, liposomes, and nanoparticles. The detection sensitivity increased with the length of the PEG chains on pegylated molecules. These results indicate that multiple AGP3 and E11 antibodies can bind to a single PEG chain, allowing amplification of the signal

as PEG chain length increases. These antibodies are therefore particularly suitable for the qualitative and quantitative analysis of high molecular weight PEG-modified molecules.

Given the well-known ability of PEG to decrease the immunogenicity of proteins (*2*, *3*), it is interesting that we have successfully generated two monoclonal antibodies against PEG. Antibodies can theoretically be generated against any molecule if it is first attached to a carrier protein to allow activation of cognate T helper cells. Indeed, antibodies have been generated against polymers such as dextran (*40*) and double-stranded synthetic polyribonucleotide complexes (*41*). PEG-modified proteins have been known to induce anti-PEG antibodies in rabbits (*42*) and repeated injections of rabbits with liposomes containing a PEG derivative of phosphatidyl ethanolamine induced anti-PEG antibodies (*43*). It should be appreciated, however, that we generated a reasonable anti-PEG antibody response in mice only after repeated immunizations with PEG−protein conjugates, indicating the difficulty in inducing a robust immune response against this polymer.

We have previously shown that AGP3 can accelerate the clearance of PEG_{5000}-modified proteins and improve the tumor/blood ratios of antibody−βG−PEG_{5000} conjugates for glucuronide prodrug therapy of cancer (*21*). In the present study, we found that both AGP3 and E11 reduced the serum concentration of 7.1PEG_{5000}−βG as well as 11.4PEG_{2000}−βG and 5.1$PEG_{20,000}$−βG from the blood. Thus, effective clearance could be achieved for conjugates containing different lengths of PEG chains. AGP3 induced rapid uptake of radiolabeled antibody−βG−PEG conjugates in the liver of mice (*21*). Although in the present study we did not follow the distribution of the conjugates in tissues after clearance with E11, the liver is a likely route of elimination. These results indicate that AGP3 and E11 can be used to clear PEG-modified conjugates from the circulation and control the serum half-life to regulate the bioactivity of PEG-modified molecules in vivo.

Measurement of PEG-modified molecules by ELISA and flow cytometry revealed that AGP3 allowed greater assay sensitivity as compared to E11 (Figures 2 and 3). This is likely due to the higher avidity of AGP3 (10 antigen-binding sites versus 2 for E11) as well as the presence of 5 μ chain domains in AGP3, providing more binding sites for secondary detection antibodies. Increased binding of secondary detection antibodies may also explain why the combination of E11/AGP3 was more sensitive than AGP3/E11 as the capture/detection antibodies in the sandwich ELISA.

The newly developed sandwich ELISA can measure the concentrations of PEG-modified proteins, liposomes, quantum dots, and free PEG. The assay sensitivity ranged from the low nM to low pM level, several orders of magnitude more sensitive than previous methods (*24*, *44*). Assay sensitivity can likely be further increased by employing more sensitive colorimetric or fluorometric substrates (*45*, *46*). The sandwich ELISA did not require prior separation of conjugates from complex mixtures and allowed the detection of PEG−βG in serum samples. These characteristics should simplify measurement of PEG-modified molecules in serum samples for pharmacological and pharmacokinetic studies in vivo.

In summary, AGP3 and E11 bind to the repeating ethylene oxide subunits of PEG and therefore can detect PEG independently of the identity of the pegylated compound or chemistry of the linker. The sensitivity of detection depends on the length of the PEG chains; longer PEG chains result in increased sensitivity. These two antibodies should be generally applicable to quantify a wide-range of PEG-derivatized molecules. The newly developed sandwich ELISA may also be useful to measure PEG-modified molecules in patients for preclinical and clinical studies.

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LITERATURE CITED

(1) Fu, J., Fiegel, J., Krauland, E., and Hanes, J. (2002) New polymeric carriers for controlled drug delivery following inhalation or injection. *Biomaterials 23*, 4425−33.

(2) Chaffee, S., Mary, A., Stiehm, E. R., Girault, D., Fischer, A., and Hershfield, M. S. (1992) IgG antibody response to polyethylene glycol-modified adenosine deaminase in patients with adenosine deaminase deficiency. *J. Clin. Invest 89*, 1643−51.

(3) Yang, Z., Wang, J., Lu, Q., Xu, J., Kobayashi, Y., Takakura, T., Takimoto, A., Yoshioka, T., Lian, C., Chen, C., Zhang, D., Zhang, Y., Li, S., Sun, X., Tan, Y., Yagi, S., Frenkel, E. P., and Hoffman, R. M. (2004) PEGylation confers greatly extended half-life and attenuated immunogenicity to recombinant methioninase in primates. *Cancer Res. 64*, 6673−8.

(4) Brinckerhoff, L. H., Kalashnikov, V. V., Thompson, L. W., Yamshchikov, G. V., Pierce, R. A., Galavotti, H. S., Engelhard, V. H., and Slingluff, C. L., Jr. (1999) Terminal modifications inhibit proteolytic degradation of an immunogenic MART-1(27−35) peptide: implications for peptide vaccines. *Int. J. Cancer 83*, 326−34.

(5) Roseng, L., Tolleshaug, H., and Berg, T. (1992) Uptake, intracellular transport, and degradation of polyethylene glycol-modified asialofetuin in hepatocytes. *J. Biol. Chem. 267*, 22987−93.

(6) Cheng, T. L., Chen, B. M., Chan, L. Y., Wu, P. Y., Chern, J. W., and Roffler, S. R. (1997) Poly(ethylene glycol) modification of beta-glucuronidase-antibody conjugates for solid-tumor therapy by targeted activation of glucuronide prodrugs. *Cancer Immunol. Immunother. 44*, 305−15.

(7) Cantin, A. M., Woods, D. E., Cloutier, D., Dufour, E. K., and Leduc, R. (2002) Polyethylene glycol conjugation at Cys232 prolongs the half-life of alpha1 proteinase inhibitor. *Am. J. Respir. Cell Mol. Biol. 27*, 659−65.

(8) Heathcote, E. J., Shiffman, M. L., Cooksley, W. G., Dusheiko, G. M., Lee, S. S., Balart, L., Reindollar, R., Reddy, R. K., Wright, T. L., Lin, A., Hoffman, J., and De Pamphilis, J. (2000) Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis. *N. Engl. J. Med. 343*, 1673−80.

(9) Reddy, K. R., Wright, T. L., Pockros, P. J., Shiffman, M., Everson, G., Reindollar, R., Fried, M. W., Purdum, P. P., 3rd, Jensen, D., Smith, C., Lee, W. M., Boyer, T. D., Lin, A., Pedder, S., and DePamphilis, J. (2001) Efficacy and safety of pegylated (40-kd) interferon alpha-2a compared with interferon alpha-2a in noncirrhotic patients with chronic hepatitis C. *Hepatology 33*, 433−8.

(10) van Der Auwera, P., Platzer, E., Xu, Z. X., Schulz, R., Feugeas, O., Capdeville, R., and Edwards, D. J. (2001) Pharmacodynamics and pharmacokinetics of single doses of subcutaneous pegylated human G-CSF mutant (Ro 25−8315) in healthy volunteers: comparison with single and multiple daily doses of filgrastim. *Am. J. Hematol*. *66*, 245−51.

(11) Morstyn, G., Foote, M. A., Walker, T., and Molineux, G. (2001) Filgrastim (r-metHuG-CSF) in the 21st century: SD/01. *Acta Haematol 105*, 151−5.

(12) Goffin, V., and Touraine, P. (2002) Pegvisomant. Pharmacia. *Curr Opin Investig Drugs 3*, 752−7.
(13) Sharpe, M., Easthope, S. E., Keating, G. M., and Lamb, H. M. (2002) Polyethylene glycol-liposomal doxorubicin: a review of its use in the management of solid and haematological malignancies and AIDS-related Kaposi's sarcoma. *Drugs 62*, 2089−126.
(14) Kim, J. K., Choi, S. H., Kim, C. O., Park, J. S., Ahn, W. S., and Kim, C. K. (2003) Enhancement of polyethylene glycol (PEG)-modified cationic liposome-mediated gene deliveries: effects on serum stability and transfection efficiency. *J. Pharm Pharmacol. 55*, 453−60.
(15) Conover, C. D., Greenwald, R. B., Pendri, A., Gilbert, C. W., and Shum, K. L. (1998) Camptothecin delivery systems: enhanced efficacy and tumor accumulation of camptothecin following its conjugation to polyethylene glycol via a glycine linker. *Cancer Chemother Pharmacol*. *42*, 407−14.
(16) Zhang, Y., Sun, C., Kohler, N., and Zhang, M. (2004) Self-assembled coatings on individual monodisperse magnetite nanoparticles for efficient intracellular uptake. *Biomed Microdevices 6*, 33−40.
(17) Ballou, B., Lagerholm, B. C., Ernst, L. A., Bruchez, M. P., and Waggoner, A. S. (2004) Noninvasive imaging of quantum dots in mice. *Bioconjug Chem. 15*, 79−86.
(18) Kim, S., Lim, Y. T., Soltesz, E. G., De Grand, A. M., Lee, J., Nakayama, A., Parker, J. A., Mihaljevic, T., Laurence, R. G., Dor, D. M., Cohn, L. H., Bawendi, M. G., and Frangioni, J. V. (2004) Near-infrared fluorescent type II quantum dots for sentinel lymph node mapping. *Nat. Biotechnol. 22*, 93−7.
(19) Cheng, T. L., Wu, P. Y., Wu, M. F., Chern, J. W., and Roffler, S. R. (1999) Accelerated clearance of poly(ethylene glycol)-modified proteins by anti-poly(ethylene glycol) IgM. *Bioconjugate Chem. 10*, 520−8.
(20) Tsai, N. M., Cheng, T. L., and Roffler, S. R. (2001) Sensitive measurement of polyethylene glycol-modified proteins. *Biotechniques 30*, 396−402.
(21) Cheng, T. L., Chen, B. M., Chern, J. W., Wu, M. F., and Roffler, S. R. (2000) Efficient clearance of poly(ethylene glycol)-modified immunoenzyme with anti-PEG monoclonal antibody for prodrug cancer therapy. *Bioconjug Chem 11*, 258−66.
(22) Vaage, J., Mayhew, E., Lasic, D., and Martin, F. (1992) Therapy of primary and metastatic mouse mammary carcinomas with doxorubicin encapsulated in long circulating liposomes. *Int. J. Cancer 51*, 942−8.
(23) Cheng, T. L., Liao, K. W., Tzou, S. C., Cheng, C. M., Chen, B. M., and Roffler, S. R. (2004) Hapten-directed targeting to single-chain antibody receptors. *Cancer Gene Ther*. *11*, 380−8.
(24) Stocks, S. J., Jones, A. J., Ramey, C. W., and Brooks, D. E. (1986) A fluorometric assay of the degree of modification of protein primary amines with polyethylene glycol. *Anal. Biochem 154*, 232−4.
(25) Keating, G. M., and Plosker, G. L. (2005) Peginterferon alpha-2a (40KD) Plus Ribavirin: A Review of its Use in the Management of Patients with Chronic Hepatitis C and Persistently 'Normal' ALT Levels. *Drugs 65*, 521−36.
(26) Siebert, U., and Sroczynski, G. (2005) Effectiveness and cost-effectiveness of initial combination therapy with interferon/peginterferon plus ribavirin in patients with chronic hepatitis C in Germany: a health technology assessment commissioned by the German Federal Ministry of Health and Social Security. *Int J Technol Assess Health Care 21*, 55−65.
(27) Yang, B. B., Lum, P. K., Hayashi, M. M., and Roskos, L. K. (2004) Polyethylene glycol modification of filgrastim results in decreased renal clearance of the protein in rats. *J. Pharm Sci. 93*, 1367−73.
(28) Ross, R. J., Leung, K. C., Maamra, M., Bennett, W., Doyle, N., Waters, M. J., and Ho, K. K. (2001) Binding and functional studies with the growth hormone receptor antagonist, B2036−PEG (pegvisomant), reveal effects of pegylation and evidence that it binds to a receptor dimer. *J. Clin. Endocrinol. Metab. 86*, 1716−23.
(29) Hong, R. L., and Tseng, Y. L. (2001) Phase I and pharmacokinetic study of a stable, polyethylene-glycolated liposomal doxorubicin in patients with solid tumors: the relation between pharmacokinetic property and toxicity. *Cancer 91*, 1826−33.
(30) Tkachenko, A. G., Xie, H., Liu, Y., Coleman, D., Ryan, J., Glomm, W. R., Shipton, M. K., Franzen, S., and Feldheim, D. L. (2004) Cellular trajectories of peptide-modified gold particle complexes: comparison of nuclear localization signals and peptide transduction domains. *Bioconjugate Chem 15*, 482−90.
(31) Kircher, M. F., Allport, J. R., Graves, E. E., Love, V., Josephson, L., Lichtman, A. H., and Weissleder, R. (2003) In vivo high-resolution three-dimensional imaging of antigen-specific cytotoxic T-lymphocyte trafficking to tumors. *Cancer Res*. *63*, 6838−46.
(32) Gao, X., Cui, Y., Levenson, R. M., Chung, L. W., and Nie, S. (2004) In vivo cancer targeting and imaging with semiconductor quantum dots. *Nat. Biotechnol*. *22*, 969−76.
(33) Denis, M. C., Mahmood, U., Benoist, C., Mathis, D., and Weissleder, R. (2004) Imaging inflammation of the pancreatic islets in type 1 diabetes. *Proc. Natl. Acad. Sci. U.S.A. 101*, 12634−9.
(34) Kloepfer, J. A., Mielke, R. E., Wong, M. S., Nealson, K. H., Stucky, G., and Nadeau, J. L. (2003) Quantum dots as strain- and metabolism-specific microbiological labels. *Appl. Environ. Microbiol. 69*, 4205−13.
(35) Li, J., and Kao, W. J. (2003) Synthesis of poly(ethylene glycol) (PEG) derivatives and PEGylated-peptide biopolymer conjugates. *Biomacromolecules 4*, 1055−67.
(36) Veronese, F. M. (2001) Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials 22*, 405−17.
(37) Photos, P. J., Bacakova, L., Discher, B., Bates, F. S., and Discher, D. E. (2003) Polymer vesicles in vivo: correlations with PEG molecular weight. *J Controlled Release 90*, 323−34.
(38) Gref, R., Luck, M., Quellec, P., Marchand, M., Dellacherie, E., Harnisch, S., Blunk, T., and Muller, R. H. (2000) 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. *Colloids Surf. B Biointerfaces 18*, 301−313.
(39) Roberts, M. J., Bentley, M. D., and Harris, J. M. (2002) Chemistry for peptide and protein PEGylation. *Adv. Drug Delivery Rev. 54*, 459−76.
(40) Newman, B. A., and Kabat, E. A. (1985) An immunochemical study of the combining site specificities of C57BL/6J monoclonal antibodies to alpha (1- - - -6)-linked dextran B512. *J. Immunol. 135*, 1220−31.
(41) Nahon-Merlin, E., Michelson, A. M., Lacour, F., and Camus, G. (1980) Immunological recognition of three polynucleotide structures associating adenylic and uridylic acids. *Ann Immunol. (Paris) 131C*, 279−87.
(42) Richter, A. W., and Akerblom, E. (1983) Antibodies against polyethylene glycol produced in animals by immunization with monomethoxy polyethylene glycol modified proteins. *Int. Arch. Allergy Appl. Immunol. 70*, 124−31.
(43) Srroda, K., Rydlewski, J., Langner, M., Kozubek, A., Grzybek, M., and Sikorski, A. F. (2005) Repeated injections of PEG-PE liposomes generate anti-PEG antibodies. *Cell Mol. Biol. Lett. 10*, 37−47.
(44) Habeeb, A. F. (1966) Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem 14*, 328−36.
(45) Porstmann, T., and Kiessig, S. T. (1992) Enzyme immunoassay techniques. An overview. *J. Immunol. Methods 150*, 5−21.
(46) Huang, Z., Olson, N. A., You, W., and Haugland, R. P. (1992) A sensitive competitive ELISA for 2,4-dinitrophenol using 3,6-fluorescein diphosphate as a fluorogenic substrate. *J. Immunol. Methods 149*, 261−6.

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