

The Inhibitory Potencies of Monoclonal Antibodies to the Macrophage Adhesion Molecule Sialoadhesin Are Greatly Increased Following PEGylation

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PEGylation of antibodies is known to increase their half-life in systemic circulation, but nothing is known regarding whether PEGylation can improve the inhibitory potency of antibodies against target receptors. In this paper, we have examined this question using antibodies directed to Sialoadhesin (Sn), a macrophage-restricted adhesion molecule that mediates sialic acid dependent binding to different cells. Anti-Sn monoclonal antibodies (mAbs), SER-4 and 3D6, were conjugated to PEG 5 kDa or and PEG 20 kDa, resulting in the incorporation of up to 3 molecules of PEG per mAb molecule. Following purification of PEGylated mAbs by anion exchange chromatography, it was shown that PEGylation had little or no effect on antigen binding activity but led to a dramatic increase in inhibitory potency that was proportional to both the size of the PEG and the degree of derivatization. Thus, PEGylation of antibodies directed to cell surface receptors could be a powerful approach to improve the therapeutic efficacy of antibodies, not only by increasing their half-life in vivo, but also by increasing their inhibitory potency for blocking receptor–ligand interactions.

INTRODUCTION

The modification of proteins with poly(ethylene glycol) (PEG) is now a well-established technique. At a therapeutic level, a number of benefits of PEGylation have been found, including the prolongation of protein half-life in the body, reduced degradation by metabolic enzymes, and elimination of its immunogenicity (for reviews, see refs 1, 2). PEG is a highly motile polymer which, when conjugated to a protein, can mask antigenic sites and sterically hinder access of proteolytic enzymes. Given the flexibility and large hydrated volume of PEG chains, another potential advantage of coupling PEG to proteins such as antibodies is an enhancement in their inhibitory potency via steric effects. This could be particularly useful for antibodies directed to cell surface proteins designed to disrupt receptor–ligand interactions. For example, blocking antibodies directed to endothelial cell adhesion molecules like VCAM and ICAM are potentially useful for treatment of inflammatory diseases due to their key roles in leukocyte recruitment and activation (3, 4).

Sialoadhesin (Sn, Siglec-1, CD169) is a macrophage-restricted adhesion molecule and a member of the Siglec family of sialic acid binding immunoglobulin (Ig)-like lectins (5). It contains 17 Ig-like domains and mediates sialic acid dependent adhesion to a range of cell types including lymphoid and myeloid cells (6). The sialic acid binding site of Sn is located on the surface of domain 1 and has been characterized at the atomic level by X-ray crystallography (7). Sn was originally characterized in the mouse as a non-phagocytic sialic acid dependent sheep erythrocyte receptor expressed on macrophage subsets (8), especially those in secondary lymphoid organs (9). Sn can be upregulated on monocytes and macrophages during inflamma-

tory responses, including autoimmune diseases such as rheumatoid arthritis (10) and experimental autoimmune encephalomyelitis (11), and on macrophages that infiltrate human breast tumors (12). Studies on Sn-deficient mice have shown that Sn is able to exacerbate T cell dependent inflammatory responses in a model of autoimmune uveoretinitis (13) and in genetically determined demyelination neuropathies of the central and peripheral nervous systems (14). These results suggest an important role for Sn during inflammatory and immune responses (15).

SER-4 and 3D6 are monoclonal rat IgG2a anti-Sn antibodies directed to two distinct epitopes of mouse Sn, domains 2 and/or 3 for SER-4, and domain 1 for 3D6 (6, 16, 17). When used separately, each antibody can partially inhibit Sn-dependent adhesion, and when mixed together, a synergistic effect on inhibition was observed (5, 18). These antibodies therefore provide a useful model system to explore the potential of PEGylation in increasing inhibitory potency of antibodies directed close to the ligand binding site of Sn. The present study describes the preparation and characterization of PEG-mAbs directed to Sn and demonstrates that PEGylation can lead to a dramatic increase in the inhibitory potency of anti-Sn mAbs. This approach could lead to improved therapeutic efficacy for a range of immune and inflammatory disorders, not only for Sn, but also for other adhesion molecules important in cell-to-cell interactions in inflammatory and immune responses and potentially more broadly for other membrane receptors.

EXPERIMENTAL PROCEDURES

PEG. PEG reagents were purchased from Nektar Therapeutics (Huntsville, AL). Two succinimidyl α -methylbutanoate-PEG (abbreviated SMB-PEG) reagents were used: a 5 kDa linear PEG chain and a 20 kDa linear PEG chain. Modification of mAb with PEG molecules is abbreviated as, for example, SER-4–2PEG 5 kDa and denotes a modification of SER-4 mAb with 2 linear PEG chains of 5 kDa.

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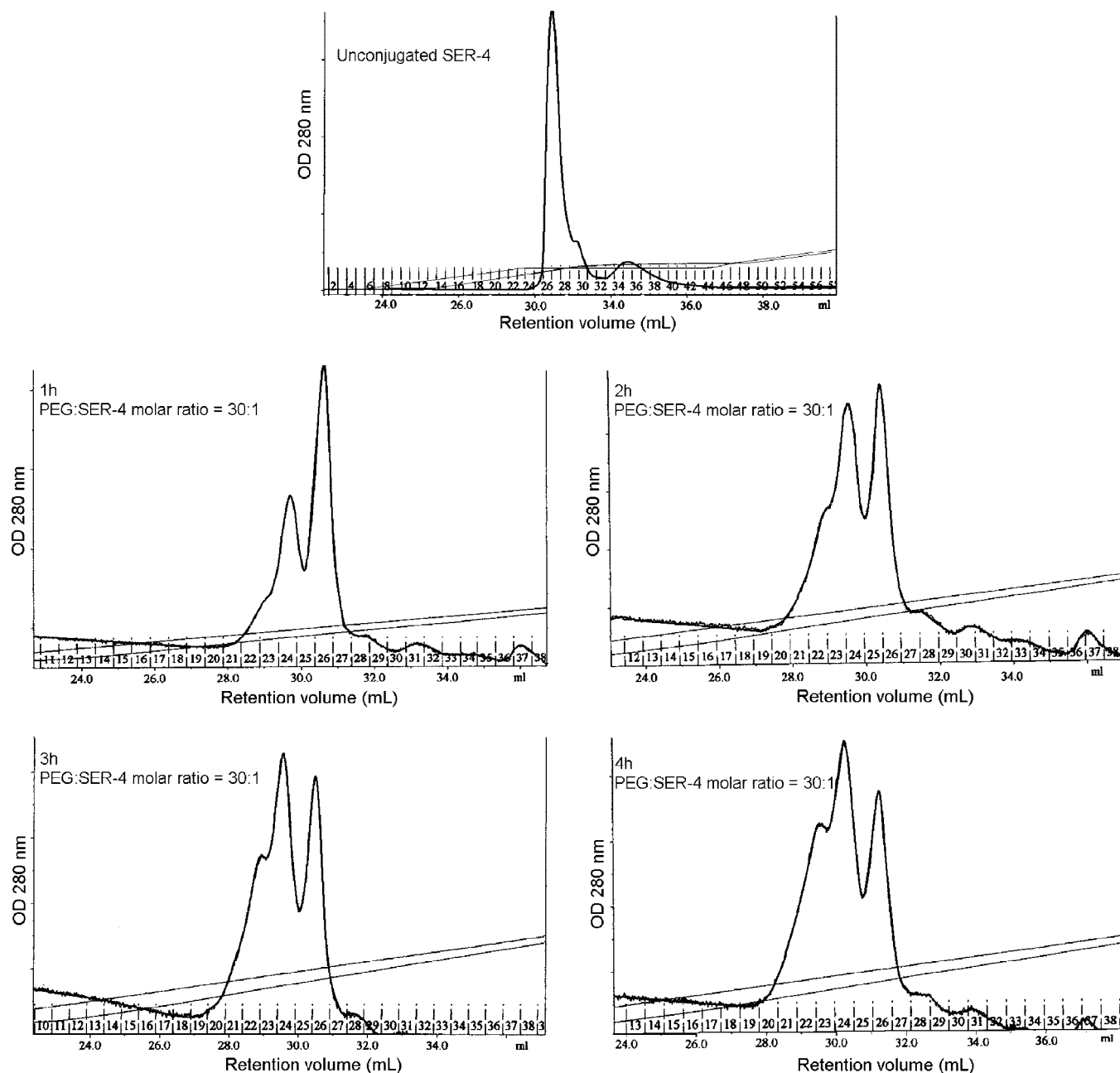


Figure 1. Anion exchange chromatograms of unconjugated SER-4 mAb (top) and modified SER-4 mAb following PEGylation in PBS pH 7.4 with initial PEG 5 kDa/SER-4 molar ratio of 30:1 and incubation for the different times indicated.

Purification of SER-4 and 3D6 Monoclonal Antibodies. Hybridomas producing SER-4 and 3D6 mAbs were expanded in IMDM + L-glutamine + 25 mM HEPES medium (Gibco, Invitrogen Corporation) supplemented with 10% hybridoma serum (FBS Gold from PAA Laboratories GmbH) and 1% penicillin/streptomycin. The conditioned supernatant was passed through a 0.2 μ m filter to remove protein aggregates and cell debris and the SER-4 and 3D6 IgGs were purified from supernatants by affinity chromatography using a Protein-G Sepharose column. Bound IgGs were eluted with 0.1 M glycine (pH 3), and fractions containing protein were neutralized by the addition of 0.1 volume 1 M Tris (pH 8) and dialyzed into PBS. SER-4 and 3D6 mAbs were further purified by anion exchange Mono Q HR 5/5 high-performance liquid chromatography (HPLC) column (Amersham, Biosciences, UK). The flow rate was 0.5 mL/min, and the column was maintained at 4 °C. A salt gradient was used for elution:

Buffer A was 25 mM Tris (pH 8); buffer B was 25 mM Tris, 1 M NaCl (pH 8).

Preparation of mAb-PEG Conjugates. SMB-PEG was allowed to react with free amino groups of SER-4 or 3D6 mAbs (2 mg/mL) at a SMB-PEG:antibody ratio 30:1 in PBS, pH 7.4, for 3 h at room temperature. The reaction was stopped by addition of 0.1 M glycine, and the mixture was then desalted with a PD-10 column (Amersham, Biosciences, UK) equilibrated with 25 mM Tris, pH 8. Anion exchange Mono Q HR 5/5 HPLC column chromatography was used to separate and purify PEG conjugates. Fractions were analyzed by SDS-PAGE using 7.5% and 12% acrylamide gels for nonreducing and reducing conditions, respectively (19), and stained in Brilliant Blue G-Colloidal suspension (Sigma). MALDI TOF-mass spectrometry analysis was also performed on fractions in order to confirm the size and the degree of

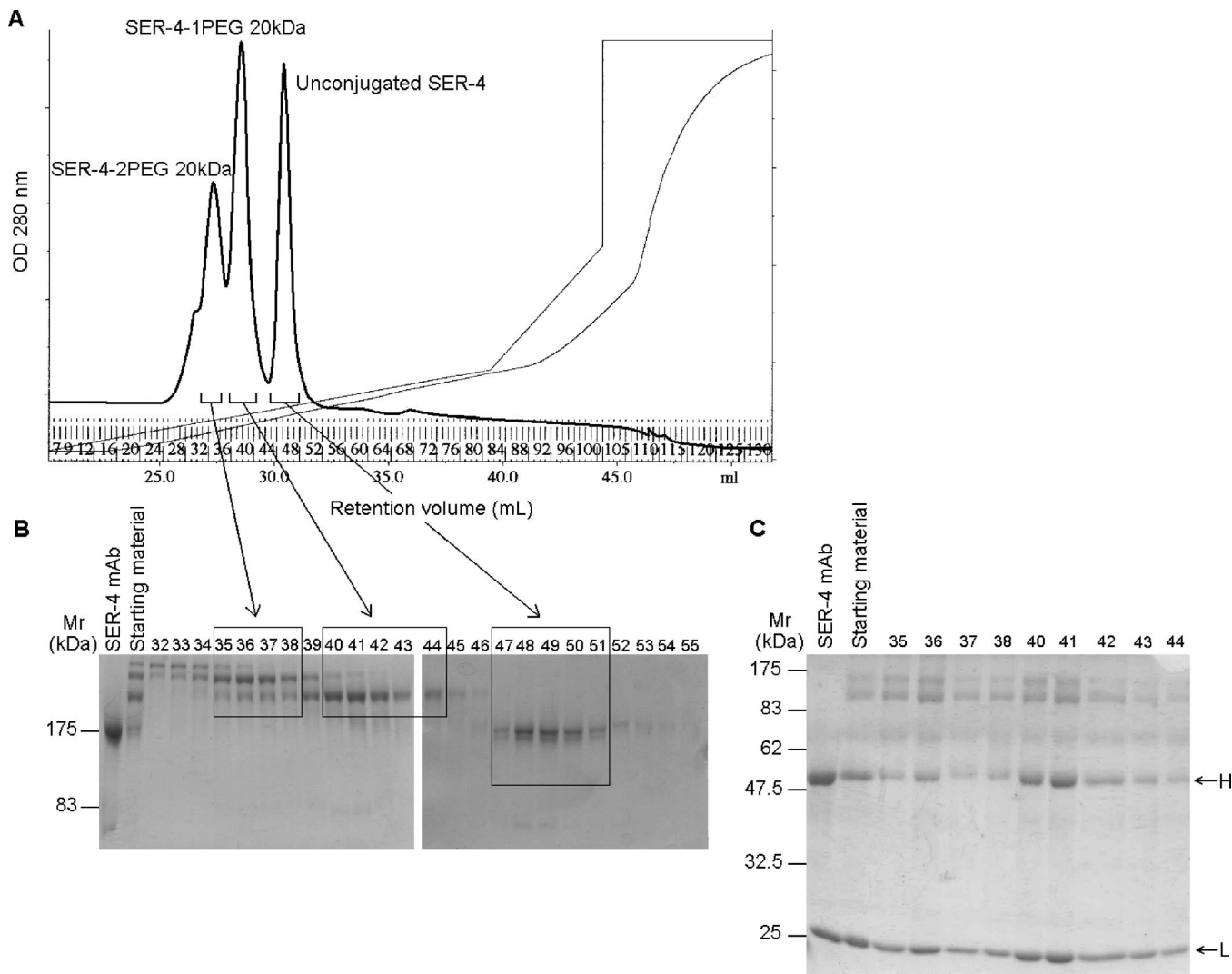


Figure 2. SER-4 mAb was conjugated to PEG 20 kDa (3 h in PBS pH 7.4 at room temperature) and PEG conjugates were purified by anion exchange chromatography (A). Fractions were analyzed by SDS-PAGE under nonreducing and reducing conditions to determine the degree of attachment and location of PEG on heavy and light chains. Sizes of molecular markers are shown. Under nonreducing conditions (B), SDS-PAGE stained with Coomassie blue shows 3 different degree of PEGylation. Under reducing conditions (C), arrows show heavy (H) chains of SER-4 at ~50 kDa and light (L) chains of SER-4 at 25 kDa. The band running between the 62 and 83 kDa markers in all tracks is a contaminant protein that was also present in the buffer-only lane (not shown).

modification of the different PEGylated products (Applied Biosystems 4700 Proteomics Analyzer).

Biotin Labeling of mAbs. Biotin labeling of SER-4 and 3D6 mAbs was performed using EZ-Link NHS-LC-Biotin reagent (Pierce, Rockford, IL) according to the manufacturer's procedure. Briefly, SER-4 or 3D6 mAbs (2 mg/mL) were mixed with 20-fold molar excess of *N*-hydroxysuccinimide (NHS)-activated biotin in PBS, pH 7.4, for 1 h at room temperature. Unreacted NHS-biotin was removed from the reaction mixture using PD-10 columns (Amersham, Biosciences, UK). Concentrations of biotin-labeled SER-4 or 3D6 were measured using a BCA protein assay kit (Pierce, Rockford, IL).

Antibody Binding Activity of PEG-mAb Conjugates. The antibody binding activities of each PEGylated product were determined by a competitive immunoassay using human Fc-chimera Sn (domains 1 to 3) immobilized on microtiter plate. 96 well Immulon-4 plates (Thermo, Milford, MA) were coated with 10 μ g/mL antihuman IgG (Fc specific) mAb (Sigma) diluted in 0.1 M bicarbonate coating buffer (pH 9.6) and blocked with PBS + 5% skimmed milk. After washing,

human Fc-chimera Sn was adsorbed to wells at a concentration of 2 μ g/mL in PBS + 0.1% BSA. The wells were washed and incubated with biotin-labeled SER-4 or 3D6 anti-Sn mAb (0.1 μ g/mL) mixed with serial dilutions of either unlabeled mAbs or mAb-PEG conjugates in PBS + 0.1% BSA. After washing, horseradish peroxidase conjugated streptavidin was added (Caltag). The wells were washed and the peroxidase activity was measured using 0.4 mg/mL *O*-phenylenediamine dihydrochloride (OPD) in 0.05 M phosphate-citrate buffer containing 0.014% H_2O_2 (Sigma) as substrate, and the absorbance at 450 nm was measured. The inhibition of biotin-labeled mAbs binding to Sn by PEG conjugates was compared to the inhibition seen with unlabeled mAbs. The percentage of biotin-labeled mAbs binding was determined by the following formula:

$$\text{Abs}_{\text{with competitor}} \times 100 / \text{Abs}_{\text{without competitor}}$$

where $\text{Abs}_{\text{with competitor}}$ = absorbance when biotin-labeled mAbs are in competition with PEG conjugates or parent mAbs.

$\text{Abs}_{\text{without competitor}}$ = absorbance when biotin-labeled mAbs are used alone (100% of binding).

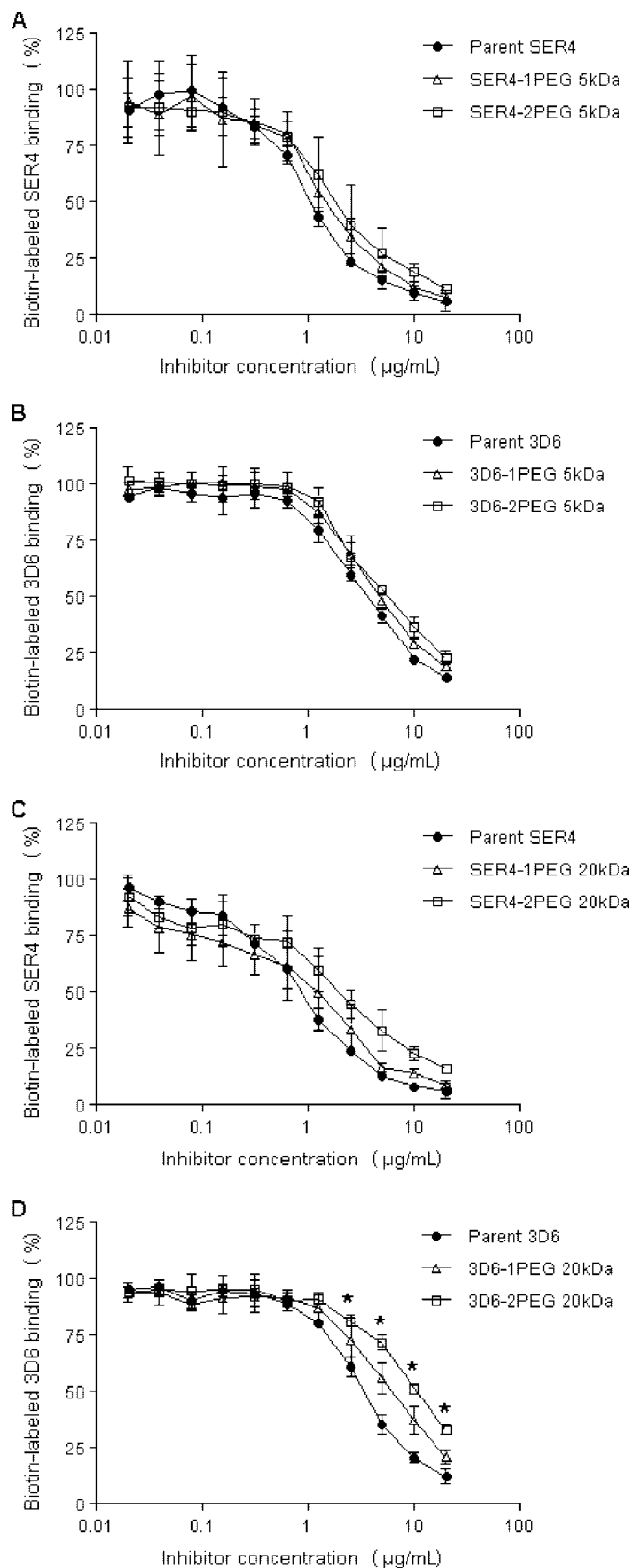


Figure 3. Binding activity of PEGylated mAbs. Conjugates of SER-4 (A,C) and of 3D6 (B,D) were tested at several concentrations for their ability to competitively inhibit the binding of biotin-labeled SER-4 and 3D6, respectively, to Sn-coated microtiter plates. Panels A and B: PEGylation of both mAbs with 1 (open triangles) or 2 (open squares) molecules of PEG 5 kDa does not affect the binding activity of mAbs. Panels C and D: Both SER-4 and 3D6 modified with 2 (open squares) molecules of PEG 20 kDa show a slight reduction of binding activity when compared to unconjugated mAbs (solid circles). * indicates a statistically significant difference in binding inhibition compared to unconjugated mAbs (Tukey test, $P < 0.001$). Each data point represents the mean \pm SD ($n = 3$ in duplicate wells).

Solid-Phase Red Blood Cell (RBC) Binding Assay. To assess the inhibitory potency of PEGylated mAbs, RBC binding assays were carried out. Briefly, 96 well Immulon-4 plates were coated with antihuman IgG (Fc specific) mAb (Sigma) followed by incubation with human Fc-chimera Sn (domains 1 to 3). After washing, the wells were incubated for 1 h with 50 μ L PBS + 0.1% BSA containing serially diluted unconjugated mAbs or PEG conjugates, used alone or in combination. One hundred microliters of freshly prepared human RBC at 0.5% volume/volume were then added directly to the wells, mixed, and incubated for 30 min at room temperature (18). The wells were gently washed with PBS + 0.1% BSA several times. To determine RBC binding quantitatively, the plate was dried, fixed with methanol for 5–10 min, and dried again, and the bound RBC were measured using 0.4 mg/mL *O*-phenylenediamine dihydrochloride (OPD) in 0.05 M phosphate–citrate buffer containing 0.014% H_2O_2 (Sigma). The absorbance at 450 nm was taken using a 96 well plate reader (Roses and Anthos 2001).

The percentage of inhibition of RBC binding was determined by the following formula:

$$\text{Abs}_{\text{with mAbs}} \times 100 / \text{Abs}_{\text{without mAbs}}$$

where $\text{Abs}_{\text{with mAbs}}$ = absorbance when PEG conjugates or parent mAbs were previously incubated with Sn.

$\text{Abs}_{\text{without mAbs}}$ = absorbance when Sn is only incubated with RBC (100% of RBC binding).

Statistics. All results are expressed as mean \pm standard deviation (SD). The one-way analysis of variance (ANOVA) test and Tukey test were performed to demonstrate statistical differences ($P < 0.05$), using the software *GraphPad Prism 5* for Windows.

RESULTS AND DISCUSSION

PEGylation of Anti-mouse Sn mAbs. Following purification by protein G-Sepharose and anion-exchange chromatography, both antibodies were PEGylated using either 5 kDa SMB-PEG, a succinimidyl- α -methylbutanonate derivative of NHS-PEG with a significantly longer half-life in solution (20, 21). PEGylation was carried out at pH 7.4, a condition that favors selective coupling to the N-terminal residues over surface-exposed lysine residues. Since IgG molecules are composed of 2 identical light chains (25 kDa) and 2 identical heavy chains (50–70 kDa), between 1 and 4 PEG molecules can, in principle, be attached per IgG molecule. PEGylation was monitored by anion-exchange chromatography (Figure 1). Under the conditions used, the more strongly PEGylated antibodies eluted first in the increasing salt gradient (Figure 2A), presumably as a result of a charge-shielding effect of PEG that weakens their binding to the anion exchange resin (2). Initial experiments using 5 kDa SMB-PEG showed that a molar ratio of 30:1 PEG/mAb resulted in $\sim 33\%$ of antibody molecules incorporating PEG after 1 h at room temperature (Figure 1). When the time of incubation was increased to 3 h, up to $\sim 70\%$ of antibody molecules were labeled with PEG (Figure 1), but longer incubations did not lead to improved yields. Similar findings were made using SMB-PEG 20 kDa (not shown).

Anion exchange chromatography showed 3 peaks following PEGylation with SMB-PEG 5 kDa (Figure 1, 3 h time) and up to 4 peaks following PEGylation with SMB-PEG 20 kDa (Figure 2A). The 20 kDa-PEGylated mAbs were eluted with a lower salt concentration than 5 kDa-PEGylated mAbs. This may be due to a greater shielding effect of 20 kDa-PEG chains resulting from their increased length and mobility.

Characterization of SER-4-PEG and 3D6-PEG. *PEGylation Analysis.* SDS-PAGE was used under nonreducing and reducing conditions to investigate the attachment and location of PEG to mAbs. With 20 kDa-PEG, the starting material

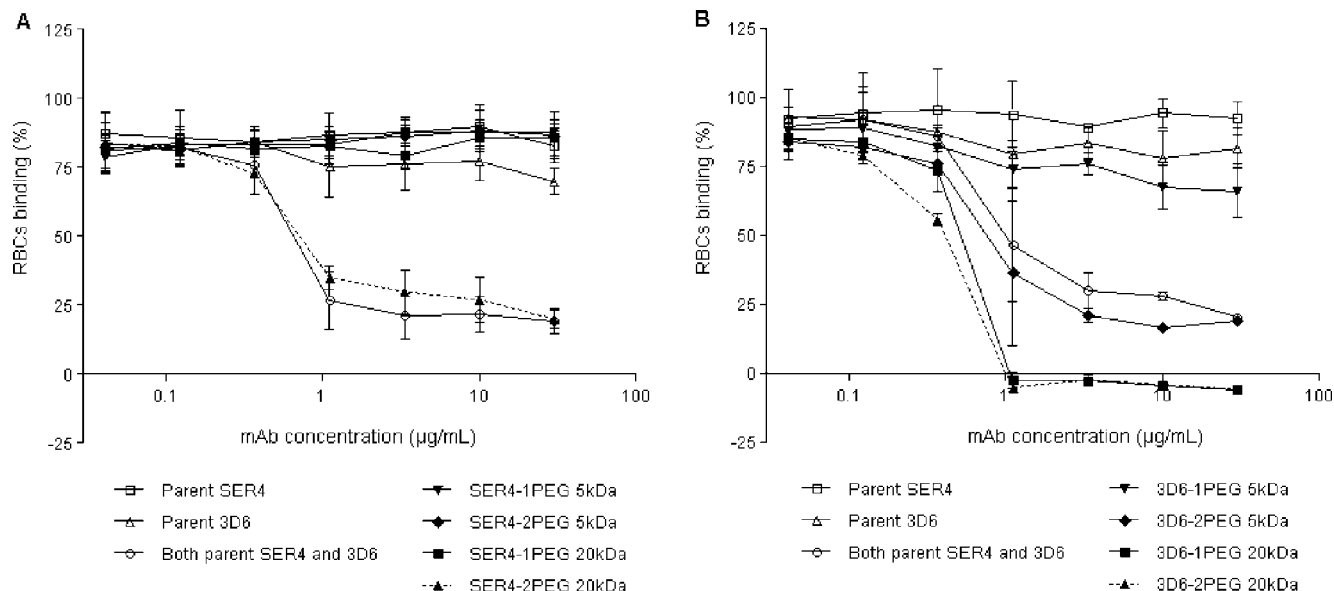


Figure 4. Inhibitory potency of PEGylated SER4 and 3D6 mAbs. A truncated form of Sn (domains 1 to 3) fused to the Fc region of human IgG1 was coated on wells at a concentration of 2 $\mu\text{g/mL}$. Wells were then preincubated with serially diluted unconjugated mAbs or PEG conjugates prior to the addition of human RBC. A preincubation of wells with SER-4 carrying 2 molecules of PEG 20 kDa reduced binding by about 75% (A). In the case of 3D6, a preincubation of wells with 3D6-2PEG 5 kDa resulted in $\sim 75\%$ inhibition, and a complete inhibition of RBC binding was seen with 3D6 carrying either 1 or 2 molecules of PEG 20 kDa (B). Each data point represents the mean \pm SD ($n = 3$ in duplicate wells).

showed the presence of four bands under nonreducing conditions, which corresponded to parent IgG (lower band) and 3 different degrees of PEGylation (Figure 2B). Similar findings were made with both SER-4 and 3D6, and only the data for SER-4 are shown. Following anion-exchange chromatography, the first peak (fractions 32–34) contained doubly and triply derivatized mAbs, the second peak (fractions 35–38) was strongly enriched in doubly derivatized mAb, and the third peak (fractions 40–44) was strongly enriched with singly derivatized mAb (Figure 2B). The fourth peak (fractions 47–51) contained underivatized mAb. These interpretations were supported by analysis of selected fractions by MALDI-TOF mass spectrometry (data not shown).

When these fractions were analyzed under reducing conditions, introduction of PEG was only observed with the heavy chains, suggesting that the N-terminal residues of the light chains were not readily conjugated under the conditions used (Figure 2C). Interestingly, heavy chains contained both one and two PEG molecules per heavy chain, suggesting that both the N-terminal residue and an internal lysine residue were modified on the same chain. These findings indicate that each mAb pool is heterogeneous with respect to the PEG modification, with each heavy chain containing 0, 1, or 2 PEG molecules, although the total number of PEG molecules per antibody molecule rarely exceeded 2.

Binding Activity of PEGylated mAbs. To investigate whether SMB-PEG modification affected the antigen-binding activity, derivatized SER-4 and 3D6 mAbs were tested in a competitive inhibition enzyme-linked assay (Figure 3). For this purpose, SER-4 and 3D6 mAbs were labeled with biotin and the abilities of parent mAbs and PEGylated mAbs (with 1 or 2 molecules of PEG 5 kDa and 20 kDa) to inhibit the binding of biotin-labeled mAb to antigen were compared. We showed that PEGylation of both mAbs with 1 or 2 molecules of PEG 5 kDa did not affect the inhibitory activity of mAbs (Figure 3A,B). However, both the SER-4 and 3D6 mAbs containing 2 molecules of PEG 20 kDa showed a slight reduction in inhibitory activity when compared with unlabeled

mAbs (Figure 3C,D). This difference was statistically significant only in the case of 3D6 mAb ($P < 0.001$). These results showed that PEGylation of N-terminal residues of anti-Sn mAbs had little or no effect on their antigen binding strength.

Effect of PEGylation on Inhibitory Potency of SER-4 and 3D6. Human RBC bind strongly to Sn in a sialic acid dependent manner, and this provides a useful model system to study adhesion mediated by the receptor (17). To investigate whether PEGylation of anti-Sn mAbs could enhance their inhibitory potency, we used a well-defined solid-phase RBC binding assay in which recombinant Sn is coated on a plate and allowed to bind RBC in the presence and absence of anti-Sn mAbs (5). As shown previously (5), we found that neither SER-4 nor 3D6 mAb was able to inhibit binding when added alone, but in combination, they reduced binding by about 75% (Figure 4). When the inhibitory activities of PEGylated mAbs were analyzed, SER-4 carrying 2 molecules of PEG 20 kDa resulted in about 75% inhibition, but incorporation of 1 or 2 molecules of PEG 5 kDa or 1 molecule of PEG 20 kDa had no effect (Figure 4A). As SER-4 is directed to domains 2/3 of Sn, it is likely that the steric effects of 2 molecules of PEG 20 kDa are sufficient to mask the sialic acid binding pocket on domain 1 of Sn and inhibit the binding of RBC.

In the case of 3D6, no increased inhibition was seen with 1 molecule of PEG 5 kDa, but 2 molecules of PEG 5 kDa resulted in $\sim 75\%$ inhibition. Strikingly, complete inhibition of RBC binding was seen with 3D6 carrying either 1 or 2 molecules of PEG 20 kDa with an IC_{50} values of 0.5 and 0.35 $\mu\text{g/mL}$, respectively (Figure 4B).

Finally, we compared inhibitory potency of antibodies when used in combination. A total of sixteen combinations of SER-4-PEG and 3D6-PEG were compared to the parent SER-4 and 3D6 combination in solid-phase RBC binding assays, and the IC_{50} values were calculated (Figure 5). All combinations of PEGylated antibodies were better inhibitors than the mixture of unlabeled SER-4 and 3D6. As expected,

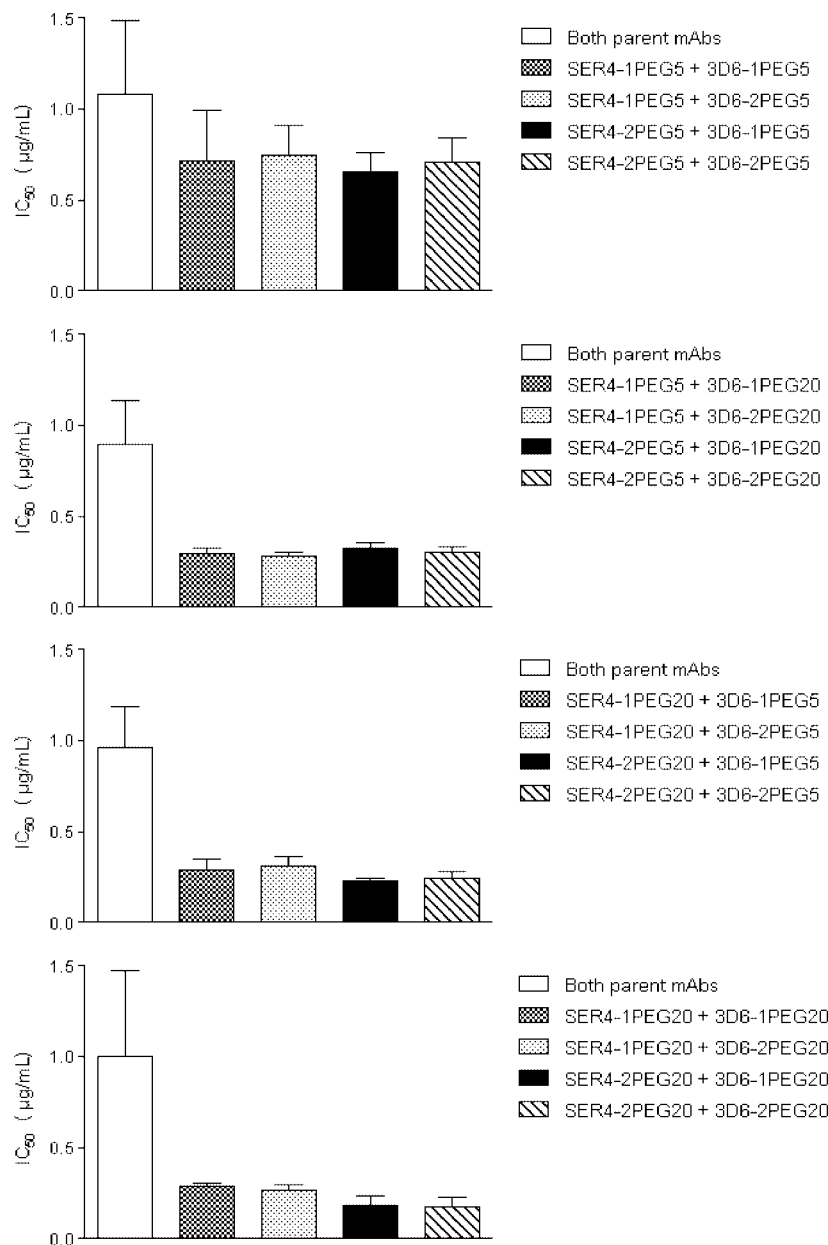


Figure 5. Inhibitory potency of PEGylated SER4 and 3D6 mAbs used in combination. A truncated form of Sn (domains 1 to 3) was coated on wells at a concentration of 2 µg/mL. Wells were then preincubated with serially diluted unconjugated mAbs or PEG conjugates in combination prior to the addition of human RBC. A total of sixteen combinations of SER-4-PEG and 3D6-PEG were compared to the parent SER-4 and 3D6 combination in RBC binding assays and concentrations inhibiting 50% of RBC binding (IC₅₀) were compared. All combinations of PEGylated antibodies were better inhibitors than the mixture of unlabeled SER-4 and 3D6. Bar represents the mean values of IC₅₀ ± SD (*n* = 3 in duplicate wells).

the inhibitory potency further increased in line with the degree of PEGylation, with the most potent combination containing SER-4 and 3D6, each with 2 molecules of PEG 20 kDa, resulting in an IC₅₀ of 0.3 µg/mL compared to an IC₅₀ of ~1.0 µg/mL using the mixture of non-PEGylated SER-4 and 3D6 mAbs (Figure 5).

In conclusion, this study shows that PEGylation of antibodies directed to the macrophage adhesion molecule, Sn, can have a dramatic effect on the inhibitory potency with minimal impact on binding strength. The pattern of results seen using the two antibodies directed to different epitopes, two sizes of PEG and different extents of derivatization fits very well with the concept of PEG-dependent steric hindrance. Thus, PEGylation of antibodies directed to cell surface receptors leads not only to increased circulating half-

life, but also increased inhibitory potency that could be potentially exploited in a therapeutic setting such as in autoimmune or inflammatory diseases.

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