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Modification of CD4 Immunoadhesin with Monomethoxypoly(ethylene glycol) Aldehyde via Reductive Alkylation

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CD4 immunoadhesin (CD4-IgG) is a chimeric glycoprotein molecule comprised of the gp120-binding portion of human CD4 fused to the hinge and Fc portions of human IgG. As a candidate for human therapeutic use, CD4-IgG represents an important advance over soluble CD4, insofar as the systemic clearance in humans of CD4-IgG is significantly slower. In an effort to prolong its *in vivo* residence time even further, we have modified CD4-IgG chemically by attaching monomethoxypoly(ethylene glycol) (MePEG) moieties to lysine residues via reductive alkylation. We synthesized MePEG aldehyde and investigated reaction conditions for adding a range of MePEG moieties per protein molecule. At neutral pH in the presence of sodium cyanoborohydride, the reaction was sufficiently slow to allow for significant control over the extent of MePEGylation. Addition of 7.7 or 14.4 MePEG moieties to CD4-IgG resulted in an approximately 4- or 5-fold increase, respectively, in the persistence of the protein in rats, as compared with unmodified CD4-IgG. These results suggest that the therapeutic utility of a human receptor IgG chimera can be improved by MePEGylation technology, provided that the modified immunoadhesin retains its biological activity *in vivo*. Such modification can lead to a significant additional increase in the *in vivo* residence time of the protein.

INTRODUCTION

The value of a recombinant protein as a human therapeutic is determined, in significant part, by how rapidly the molecule is cleared from the body. Recombinant receptors are currently of great interest as candidates for protein therapeutics. One such protein is CD4, the primary receptor for human immunodeficiency virus type 1. CD4 is an integral membrane glycoprotein on the surface of lymphocytes and monocytes. By truncating a full-length cDNA to encode only the ectodomain of the receptor, CD4 was produced in a recombinant, soluble form (Smith et al., 1987). sCD4[3] binds to gp120, the envelope glycoprotein of HIV-1, with high affinity and inhibits infectivity *in vitro*. However, the short *in vivo* half-life and rapid clearance of sCD4 limits its potential for therapeutic use (Schooley et al., 1990).

To increase the *in vivo* residence time of sCD4, a human chimeric molecule, CD4 immunoadhesin, was designed (Capon et al., 1989; Byrn et al., 1990; Chamow et al., 1992). In humans, this hybrid molecule was cleared from the blood approximately 25 times more slowly than sCD4 (Hodges et al., 1991; Kahn et al., 1990). This slower clearance results, in part, from the larger size of the chimeric protein dimer (91 kDa) relative to monomeric sCD4 (41 kDa). The increase in size reduces the rate of clearance of the protein via glomerular filtration in the kidney (Venkatachalam and Rennke, 1978). Although the half-life of CD4-IgG is markedly longer than that of sCD4, it is shorter than the half-life observed generally for human IgG, suggesting that further extension of its half-life by chemical means might be possible. Construction of immunoadhesins of several other soluble receptors has now been reported (reviewed in Ashkenazi et al. (1993)).

An additional means by which the *in vivo* residence time of proteins can be extended is by covalent modification with suitably activated hydrophilic polymers such as monomethoxypoly(ethylene glycol) (MePEG) (for a review, see Francis et al. (1992)). MePEGylation increases the Stokes radius, and thereby can be used to reduce the elimination of relatively small proteins via the kidney. In addition, MePEG can reduce the immunogenicity of modified proteins (Abuchowski et al., 1977a,b). The value of protein MePEGylation as a therapeutic design strategy is demonstrated by the clinical use of MePEG-modified adenosine deaminase for treatment of severe combined immunodeficiency disease caused by adenosine deaminase deficiency (Hershfeld et al., 1987).

Despite the overall structural similarity between CD4-IgG and human IgG, the half-life of the former is significantly shorter, suggesting that additional clearance mechanisms may exist that are relatively more efficient in removing the immunoadhesin versus an IgG antibody. We reasoned that MePEG modification of CD4-IgG might mask potential clearance–receptor binding sites and thus reduce the rate of the protein's clearance. To avoid excessive modification of CD4-IgG, which might compro-

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[3] Abbreviations used: IDA, iminodiacetic acid; MePEG, monomethoxypoly(ethylene glycol); MePEG-CHO, monomethoxypoly(ethylene glycol) aldehyde; CD4-IgG, CD4 immunoadhesin; sCD4, recombinant, soluble form of CD4; HIV-1, human immunodeficiency virus type 1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCA, trichloroacetic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid; AUC, area under curve.

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Table 1. Activated Me-PEG Reagents That Are Reactive with Amines

activated MePEG	structure of activated MePEG	amine linkage	ref
MePEG-OH	RO~OH [a]		
		Alkylation	
cyanurylate[b]		secondary amine	Abuchowski et al., 1977a; Jackson et al., 1987; Koide and Kobayashi, 1983
tresylate		secondary amine	Nilsson and Mosbach, 1984; Delgado et al., 1990
		Acylation	
N-hydroxysuccinimide		amide	Buckmann et al., 1981
succinimidyl succinate		amide	Jopich and Luisi, 1979; Abuchowski et al., 1984
succinimidyl glutarate		amide	Katre et al., 1987; Kitamura et al., 1991
succinimidyl succinamide		amide	Boccu et al., 1983
succinimidyl carbonate		carbamate	Zalipsky et al., 1992
phenylcarbonate		carbamate	Veronese et al., 1985
imidazolyl formate		carbamate	Beauchamp et al., 1983

[a] Boxed structure is derived from MePEG; R = $CH_3(OCH_2CH_2)_n$ as defined in Figure 1. Asterisk indicates reactive center where nucleophilic attack by amine nitrogen occurs. [b] Also reacts with Cys and Tyr.

mise its biological activity, we sought a means of MePEGylation for which the extent of reaction could be controlled.

Several chemistries have been described by which MePEG can be activated for protein modification (reviewed in Zalipsky and Lee, 1992). For this purpose, the hydroxyl group of MePEG has been activated and then coupled, via alkylation or acylation, to nucleophilic sites on proteins. Amino groups are the preferred sites of modification. MePEG cyanurylate has been used most commonly, but a number of other methods have also been developed for coupling MePEG to protein amino groups (Table 1). Our experience with reductive alkylation of proteins led us to seek an alkylating reagent that would be both easy to make and highly selective and one for which the extent of reaction could be controlled, so that partial modification is possible. Building on the initial work of Wirth et al. (1991) who reported modification of horseradish peroxidase with an aldehyde derivative of MePEG, we describe a modified synthesis of this reagent, and we extend the use of MePEG aldehyde to modification of the immunoadhesin CD4-IgG by reductive alkylation.

EXPERIMENTAL PROCEDURES

Recombinant Proteins. CD4-IgG was constructed and expressed in Chinese hamster ovary cells and was purified as described (Byrn et al., 1990). rgp120, a recombinant form of the envelope glycoprotein of HIV-1_{IIIB}, was similarly expressed and purified as described (Leonard et al., 1990).

Synthesis of MePEG Aldehyde. MePEG was oxidized using the Moffatt procedure (Figure 1) essentially as described by Harris et al. (1984) and Shak et al. (1989). Acetic anhydride (3 mL, 30 mmol) was added to dry DMSO (10 mL). In a separate stoppered flask, MePEG (10 g, 2 mmol) and DMSO (10 mL) were warmed to 50–60 °C to give a homogeneous solution and, after cooling to near-room temperature, were added to the acetic anhydride/DMSO solution. After the solution was stirred at ambient temperature for 24 h, further portions of acetic anhydride (3 mL, 30 mmol) and triethylamine (5 mL) were added, and the solution was stirred at room temperature for a further 24 h. Addition of ether (50 mL) and ethyl acetate (50 mL) and cooling the flask in the refrigerator resulted in precipitation of the product. The product was collected by filtration, triturated with ether (5×), and dried under vacuum. Extent of activation of the dried MePEG-CHO was quantitated by a colorimetric assay using alkaline 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Aldrich) to detect aldehydes (Avigad, 1983), with acetaldehyde as the standard. A standard 5 mM solution of acetaldehyde was prepared by periodate oxidation of L-rhamnose, as described (Avigad, 1983). Absorbance was read at 542

nm. This assay method was linear in the range of 0.1–0.6 mM of acetaldehyde.

Conjugation of MePEG to CD4-IgG and Removal of Residual MePEG. Purified CD4-IgG (5 mg/mL; 0.055 mM) was incubated with an approximately 100-fold molar excess of MePEG-CHO (5.2 mM), 20 mM $NaCNBH_3$, in 0.1 M HEPES pH 7.5, for various times at 0 or 23 °C; reactions were stopped by addition of ethanolamine to a final concentration of 50 mM. To recover MePEG-conjugated protein free of residual MePEG or MePEG-CHO, reaction mixtures were applied to IDA-Sepharose loaded with Cu^{2+} and equilibrated in 10 mM Tris pH 8.0, 0.5 M NaCl. After the mixture was washed to remove residual MePEG and MePEG-CHO (Ingham and Ling, 1978), bound MePEG-conjugated protein was recovered by elution with the same pH 8.0 buffer containing 50 mM imidazole.

Characterization of MePEG-Modified CD4-IgG and Quantitation of MePEG. IDA-Sepharose pools were analyzed by SDS–PAGE, and the concentration of MePEG in each sample was determined by NMR (Jackson et al., 1987). Protein concentration was determined by assay using bicinchoninic acid (Smith et al., 1985) with bovine serum albumin as standard. Absorption of the protein at 280 nm ($A_{0.1\%}$ = 1.34) was also used to determine the CD4-IgG concentration. The concentrations determined by both methods agreed. For the NMR analysis, aqueous solutions (0.5 mL) were transferred to NMR tubes, diluted with D_2O (0.2 mL), and placed in the instrument probe for 5 min at 20 °C prior to data acquisition. The intensity of the singlet MePEG signal at δ = 3.7 ppm was measured, and the concentration of MePEG in the sample was determined by comparing the signal intensity to intensities of known MePEG standards that were identically treated. The fraction of total amines in CD4-IgG that are potentially reactive was quantitated by TNBS assay (Fields, 1972).

N-Terminal Sequence Analysis and Peptide Mapping. Desalted aliquots of selected IDA-Sepharose pools were inserted into an Applied Biosystems Model 477A sequencer reaction cartridge atop a preconditioned polybrene-coated filter. N-terminal sequence analysis was performed for five cycles using 500 pmol of CD4-IgG or $MePEG_{5.8}$-modified CD4-IgG. Peptide mapping was performed on trypsin-digested S-carboxymethylated samples as described (Harris et al., 1990).

gp120 Binding. The ability of CD4-IgG and modified CD4-IgG to bind to rgp120 was determined as described previously (Chamow et al., 1990). Briefly, CD4-IgG was immobilized onto microtiter wells coated with anti-IgG antibody. Then, CD4-IgG or MePEG-CD4-IgG conjugates were added simultaneously with ^{125}I-labeled rgp120 (HIV-1_{IIIB}), to determine the ability of added CD4-IgG (or derivatized CD4-IgG) to compete with the immobilized CD4-IgG for binding to labeled rgp120. The assays were done in triplicate; nonspecific binding was determined by omitting the first addition of CD4-IgG.

Determination of in Vivo Clearance. CD4-IgG, $(MePEG)_{7.7}$–CD4-IgG, $(MePEG)_{14.4}$–CD4-IgG, and human IgG (Jackson ImmunoResearch, West Grove, PA) were iodinated with Na[^{125}I] using immobilized lactoperoxidase (Enzymobeads, Bio-Rad). Twelve male Sprague–Dawley-derived rats (295–335 g) received approximately 95 μCi/kg of labeled protein (specific activity 0.3 μCi/μg) as a bolus injection into a femoral vein catheter (three animals per group). Blood samples were collected from a jugular vein catheter at specified times over a 5-day period. The area under the TCA-precipitable radioactivity versus time curve (AUC) from 0 to 120 h was determined for each animal.

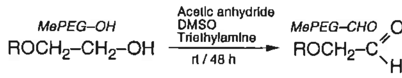

Figure 1. Preparation of MePEG aldehyde (MePEG-CHO) from MePEG (MePEG-OH) via Moffatt oxidation. For MePEG-OH of MW = 5000, $n \approx 110$.

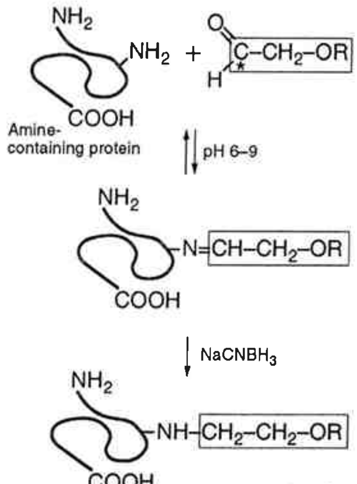

Figure 2. Conjugation of MePEG-CHO to protein amino groups via reductive alkylation. MePEG-CHO incubated with a protein at pH 6–9 results in reversible addition of MePEG to protein amino groups via Schiff's base formation. These linkages are converted to stable secondary amines by reduction with sodium cyanoborohydride. ϵ-Amino groups of lysine residues, as well as the α-amino group at the N-terminus, are targets for modification. The net charge of the modified protein does not change appreciably as a result of MePEGylation by this method, since primary amines are converted to secondary amines. Boxed region represents atoms derived from MePEG. R group and * as defined in Table 1.

RESULTS

Synthesis. MePEG-CHO was synthesized from commercially available monomethoxyPEG (5000 MW) via a Moffatt oxidation (Figure 1). The yield of MePEG-CHO was approximately 52% when assayed against an acetaldehyde standard. Thin-layer chromatography (3:17 CH_3OH/CH_2Cl_2) revealed a single spot with the same R_f as the MePEG-OH, implying that the remaining 48% was unoxidized MePEG-OH[4] (which does not participate in the subsequent alkylation reaction). Therefore, the concentration of MePEG-CHO was assumed to be 5.2 mM in a 10 mM solution of activated MePEG.

Formation of Conjugates. MePEG-CHO was conjugated to CD4-IgG via reductive alkylation to form a Schiff's base which, in the presence of $NaCNBH_3$, was reduced to a stable secondary amine (Figure 2). Using the TNBS assay to quantitate primary amines, we determined that 34% of amino groups in CD4-IgG (26/

[4] Because of the heterogeneous nature of this polymer and the lack of a definitive assay for MePEG-OH (one which does not detect the enol form of the aldehyde or entrapped water), we cannot say with complete certainty that the remaining 48% of MePEG in this preparation is MePEG-OH. However, this conclusion seems only reasonable, considering the predicted course of the Moffatt oxidation, the aldehyde colorimetric analysis, and the thin-layer chromatographic result.

Table 2. Quantitation of MePEG/CD4-IgG Ratio in Conjugates

	CD4-IgG		MePEG		
time[a] (h)	mg/mL[b]	nmol	mM[c]	nmol	MePEG/CD4-IgG
0.5	1.24	9.54	0.040	28.1	2.9
1.0	1.87	14.38	0.073	51.2	3.6
1.5	0.92	7.08	0.051	36.0	5.1
2.0	1.78	13.69	0.114	79.9	5.8
2.5	1.84	14.15	0.132	92.1	6.5
3.0	1.69	13.00	0.137	95.8	7.4

[a] Reaction mixture (22 °C) contained 5 mg/mL of CD4-IgG, 5.2 mM of MePEG-CHO, 20 mM of $NaCNBH_3$, 0.1 M HEPES pH 7.5. [b] Determined by bicinchoninic acid assay (Smith et al., 1985) using bovine serum albumin as standard. CD4-IgG was assumed to have a molecular weight of 91 000, the size of the disulfide-linked dimer. Therefore, the ratio shown is for MePEG per CD4-IgG dimer. [c] Determined by NMR (Jackson et al., 1987). The slope of the line generated by the standard curve (Figure 3) was $y = 0.000489x + 0.0118$, where y = mM of MePEG and x = peak area.

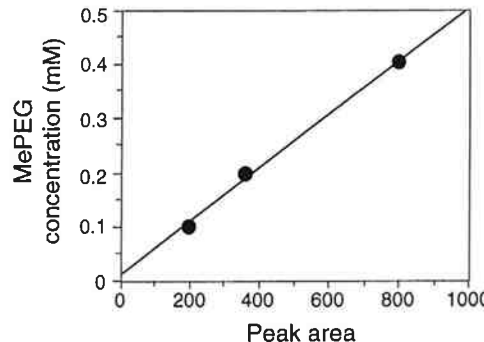

Figure 3. Quantitative assay of MePEG using NMR. The amount of MePEG covalently attached to CD4-IgG was determined by measuring the concentration of MePEG in a modified protein solution, after removal of residual MePEG-CHO from the reaction mixture by metal chelate chromatography. The intensity of the singlet MePEG signal at $\delta = 3.7$ was measured, and peak areas were recorded for a series of MePEG standards of known concentration from 0.1–0.5 mM.

76)[5] are available for reaction (data not shown). Initially, we attempted to quantitate the extent of MePEGylation by this same procedure. However, we found that quantitation by NMR is a superior analytical method for this purpose. Among the reaction parameters that influenced the rate of MePEGylation of CD4-IgG were length of time, temperature of reaction, and molar excess of MePEG-CHO over protein. We found that in 0.5–3.0 h of reaction, at 22 °C and a 100-fold molar excess of MePEG-CHO over CD4-IgG, conjugates were produced with MePEG/CD4-IgG molar ratios ranging from 2.9 to 7.4 (Table 2). This represents reaction of 11–28% of the 26 available amino groups in CD4-IgG. Formation of MePEG–CD4-IgG conjugates generated after 0.5–3 h of reaction could also be followed by SDS-PAGE, a technique that we used to investigate molecular heterogeneity in reaction mixtures of modified CD4-IgG (Figure 4). Electrophoretic analysis indicated that MePEGylated forms of CD4-IgG began to appear after 0.5 h of reaction. At this short time point, a single, predominant, new band was observed at approximately 110 kDa, which presumably represents $MePEG_1$–CD4-IgG. As the reaction time increased, we observed a shift toward the formation of higher molecular weight conjugates. At 3.0 h of reaction, a series of new high molecular weight species that stained intensely in the 130–

[5] Each CD4-IgG polypeptide contains 37 lysine residues (Harris et al., 1990); thus, the total number of amino groups per polypeptide is 38, or 76 amino groups per homodimer. CD4-IgG has a pI of 9.1.

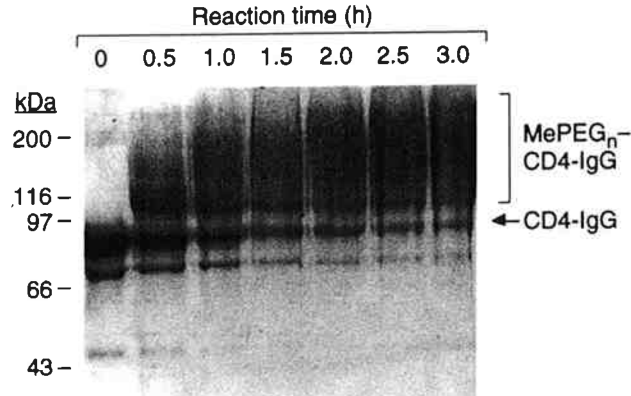

Figure 4. SDS-PAGE analysis of MePEG–CD4-IgG conjugates. CD4-IgG (5 mg/mL; 0.055 mM) samples were incubated with MePEG-CHO (5.2 mM) and $NaCNBH_3$ (20 mM) in 0.1 M HEPES pH 7.5 for 0–3.0 h at 22 °C. Reactions were stopped, and samples were analyzed in 7.5% nonreduced gel, stained with Coomassie blue. Molecular weight standards in kDa are indicated at left. The designation "n" in "$MePEG_n$–CD4-IgG" refers to the number of MePEG molecules incorporated into each polypeptide of CD4-IgG.

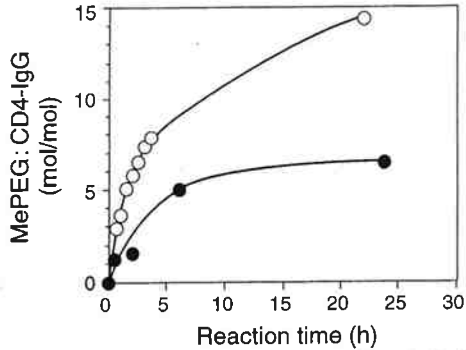

Figure 5. Reaction of MePEG-CHO with CD4-IgG slows with temperature. CD4-IgG (5 mg/mL; 0.055 mM) samples were incubated with MePEG-CHO (5.2 mM) and $NaCNBH_3$ (20 mM) in 0.1 M HEPES pH 7.5 for different lengths of time at 0 °C (●) and 22 °C (○). Reactions were stopped, and samples were recovered by metal chelate chromatography and analyzed to determine the extent of MePEG modification. The molar ratio of MePEG/protein, which is plotted on the y-axis, was determined by measuring the concentration of MePEG using NMR and the concentration of protein using bicinchoninic acid assay, for each sample.

250-kDa size range appeared, with a concomitant decrease in staining intensity of the CD4-IgG and $MePEG_1$–CD4-IgG bands. Thus, electrophoretic analysis indicates that considerable heterogeneity is present in each reaction mixture, and that molecular heterogeneity of MePEG–CD4-IgG conjugates increases with increasing times of reaction. Molar ratios of MePEG/CD4-IgG, which we have derived from NMR and protein assay data (Figure 3 and Table 2), therefore represent an average degree of substitution in a MePEG/CD4-IgG mixture.

We investigated the effect of temperature on the reaction of MePEG-CHO with CD4-IgG and found that we could further reduce the amount of MePEG incorporated into CD4-IgG by lowering the temperature of reaction from 22 to 0 °C (Figure 5). At 22 °C, the amount of MePEG incorporated into CD4-IgG increased steadily (from 0 to 7.7 mol/mol) during the first 3.5 h of reaction, while after 23 h of reaction, the MePEG/CD4-IgG ratio was nearly 15 mol/mol (representing reaction of 55% of the 26 available amino groups). In contrast, a temperature of 0 °C slowed the reaction almost 2-fold. During the first 3.5 h of reaction, a MePEG/CD4-IgG molar ratio of approximately

Table 3. N-Terminal Sequence Analysis of CD4-IgG and MePEG$_{5.8}$–CD4-IgG[a]

		recovery[b] (%)	
cycle no.	residue[c]	CD4-IgG	MePEG$_{5.8}$–CD4-IgG
1	Lys	95	51
2	Lys	114	125
3	Val	104	115
4	Val	102	105
5	Leu	100	100

[a] 500 pmol of CD4-IgG or MePEG$_{5.8}$–CD4-IgG (prepared as described for 2.0 h at 22 °C; see Table 2) was inserted into an Applied Biosystems Model 477A sequencer, and the recoveries of the first five amino acid residues were determined for each. [b] The values shown are relative recoveries of PTH-amino acids. Values are normalized to Leu-5 = 100%. [c] Confirms amino acid sequence reported for CD4-IgG by Harris et al. (1990).

4 was reached, with that ratio increasing to 6.5 after 24 h of reaction. A similar effect of temperature was seen for reductive methylation of albumin (Jentoft and Dearborn, 1979).

Sites of Modification on CD4-IgG. To determine the sites of modification, CD4-IgG containing an average of 5.8 MePEG molecules per CD4-IgG molecule was analyzed (Table 3). N-terminal sequence analysis showed a 50% yield of PTH-Lys at cycle 1, suggesting that 50% of Lys-1 residues were MePEG-modified. We assume that the reactive site in Lys-1 is the α-amino group. Under the reaction conditions used (pH 7.5), the α-amino group of the N-terminus is near its pK_a (~8.0) and thus a significant fraction is unprotonated and available for reaction, while the ϵ-amino groups of Lys-1 and of lysine residues elsewhere in the protein remain almost completely protonated (pK_a = 9.5). No tryptic mapping differences were observed between control CD4-IgG and MePEG$_{5.8}$–CD4-IgG, suggesting that the approximately six MePEG attachment sites were otherwise evenly distributed throughout the molecule. (Modification of the N-terminus would not be detected in the tryptic map, as it is found within a Lys-Lys sequence where cleavage after either of these two residues could generate free amino acids that would elute in the chromatographic void.) Although some reaction with ϵ-amino groups must occur to account for the observed molar ratios, there appear to be no other unique sites that react to an extent approaching that of the α-amino group. ϵ-Amino groups of lysine residues in the protein appear to be indistinguishable in their reactivity with the MePEG-CHO reagent.

Effect of MePEGylation on gp120 Binding. We investigated whether MePEGylation of CD4-IgG affects gp120 binding affinity. Using a solid-phase radioreceptor assay, we observed a decrease in the ability of the modified protein to bind to rgp120 which correlates with the extent of MePEGylation (Figure 6). MePEG–CD4-IgG modified with 3.6, 7.7, and 14.4 mol of MePEG per mol of CD4-IgG displayed gp120 binding ability that was reduced to 42%, 19%, and 5.5%, respectively, of unmodified CD4-IgG. This result was not unexpected; in fact, similar decreases in the activity of other MePEGylated proteins have been reported (see Discussion).

MePEGylation Decreases the Rate of Clearance of CD4-IgG. To test whether the pharmacokinetic properties of CD4-IgG were affected by MePEGylation, the serum concentrations of radioiodinated CD4-IgG, MePEG$_{7.7}$–CD4-IgG, MePEG$_{14.4}$–CD4-IgG, and human IgG were measured for 5 days after intravenous injection into rats (Figure 7). As indicated by comparing the integrated area under the curve (AUC), the clearance rates of MePEG$_{7.7}$–CD4-IgG and MePEG$_{14.4}$–CD4-IgG were approximately

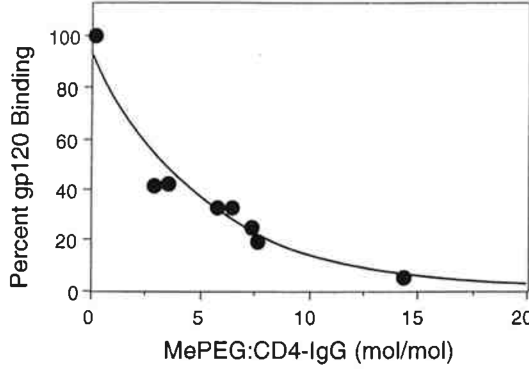

Figure 6. Binding of CD4-IgG conjugates to HIV-1 rgp120. CD4-IgG and its MePEGylated derivatives were tested for their ability to compete with immobilized CD4-IgG for the binding of ^{125}I-labeled rgp120. Plotted are points representing CD4-IgG and CD4-IgG modified with an average of 2.9, 3.6, 5.8, 6.5, 7.4, 7.7, and 14.4 MePEG molecules per CD4-IgG molecule. Percent rgp120 binding reflects the relative amount of competing molecule required to inhibit 50% of the binding to immobilized CD4-IgG.

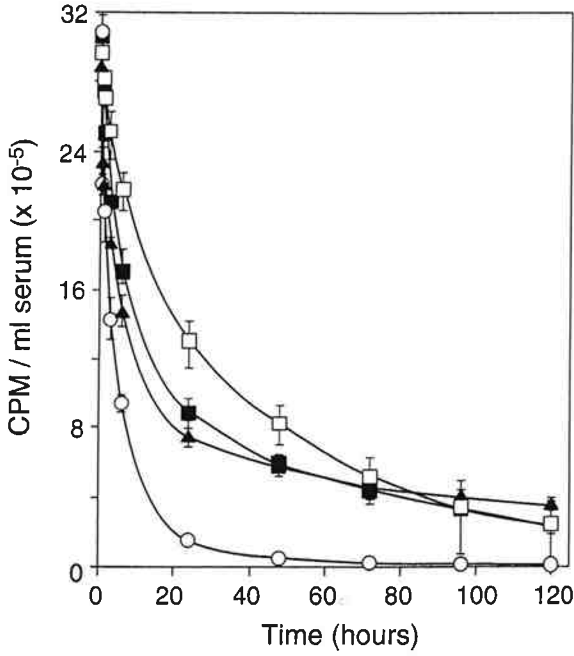

Figure 7. MePEGylation decreases the serum clearance of CD4-IgG. Radioiodinated CD4-IgG (○), human IgG (▲), and CD4-IgG modified with an average number of 7.7 (■) or 14.4 (□) MePEG's per dimer, as determined by NMR quantitation, were injected intravenously into rats. Serum samples from three rats/group were taken over a 5-day period, and TCA-precipitable counts were measured. Approximate AUC values (h-cpm/mL) were 1.96×10^7, 7.90×10^7, 8.18×10^7, and 1.05×10^8, respectively.

4- and 5-fold slower, respectively, than that of CD4-IgG. Indeed, the rates of clearance of MePEGylated CD4-IgG are slightly lower than that of human IgG in the rat.

DISCUSSION

Aldehyde groups react under mild aqueous conditions with aliphatic amines to form a Schiff's base (an imine) which can be reduced selectively by the mild reducing agent sodium cyanoborohydride to give a stable secondary amine (Jentoft and Dearborn, 1979). Although this method of amine modification (an alkylation) is not used in protein conjugations as frequently as the activated ester method (an acylation) (Bragg and Hou, 1975), it is mild, simple, and very effective. For these reasons, we chose to evaluate a MePEG aldehyde reagent for modification of

CD4-IgG. Using this reagent at 100-fold molar excess over protein (4-fold molar excess over amino groups), we were able to achieve modification of 11–55% of the available amino groups in CD4-IgG.

An important goal of adding MePEG groups to a therapeutic protein is an extension of the protein's persistence in circulating blood. Our results show that this indeed is the case for CD4-IgG, since the clearance of MePEGylated CD4-IgG (MePEG:protein = 7.7:1 or 14.4:1) was 4- to 5-fold slower than that of unmodified CD4-IgG. The resulting rate of clearance is, in fact, slightly lower than that observed for human IgG. For comparison, Kitamura et al. (1991) prepared a MePEG-modified monoclonal antibody (MePEG:protein = 5.1:1) and demonstrated less than 2-fold reduced clearance of the modified antibody in mice. The stabilizing effect of MePEGylation may be greater for the immunoadhesin, since its half-life prior to modification is shorter than that of the antibody. Notably, the more excessive modification (MePEG:protein = 14.4:1) resulted in only 20% further reduction in the rate of clearance. This result suggests that, at least for an antibody-like molecule such as CD4-IgG, the additional reduction in clearance that can be achieved by progressive MePEGylation is diminished at high degrees of modification.

By what mechanism might MePEGylation increase the *in vivo* residence time of CD4-IgG? We have determined that metabolic clearance of CD4-IgG occurs predominantly in the liver, and not in the kidney (J.M. and S.M.C., unpublished results). Clearance via glomerular filtration through the kidney is a principal route of elimination for globular proteins less than 60–70 kDa in size (Stokes radius of less than approximately 35 Å) (Venkatachalam and Rennke, 1978). It is believed that MePEGylation of small proteins, such as IL-2 (Knauf et al., 1988), increases their circulatory lifetimes *in vivo* by increasing their effective size, thus reducing filtration through the kidney. This is not the case for CD4-IgG, since its size (91 kDa) is beyond the limit for kidney filtration. However, since CD4-IgG contains a functional immunoglobulin Fc domain, at least some of its clearance may be caused by binding to Fc receptors on reticuloendothelial cells. MePEGylation of CD4-IgG, particularly at sites in the lysine-rich Fc region, may impair the interaction of CD4-IgG with Fc receptors, thus prolonging the serum stability of the modified molecule. Kitamura et al. (1991) suggest that, rather than affecting Fc receptor binding, MePEG may shield the antibody molecule from proteolysis and thereby inhibit catabolism. At least some characteristics of the Fc domain are changed by MePEGylation; for example, we found that while CD4-IgG binds well to *Staphyloccocus aureus* Protein A, MePEGylated CD4-IgG binds only poorly to Protein A (data not shown). In addition, the binding of modified CD4-IgG to other chromatographic supports, such as ion-exchange media, is also adversely affected (data not shown). Similar changes in the physical properties of horseradish peroxidase were noted by Wirth et al. (1991) after modification of the protein with MePEG.

As reviewed in Francis (1992), MePEG-modified proteins have shown a modest to marked reduction in biological or enzymatic activity, almost without exception. Mercuripapain (Boccu et al., 1983), α2-macroglobulin (Beauchamp et al., 1983), L-glutaminase-L-asparaginase (Abuchowski et al., 1981), ribonuclease (Veronese et al., 1985), tissue plasminogen activator (Berger and Pizzo, 1988), and elastase (Koide and Kobayashi, 1983) all lost more than 50% of their activity upon modification with MePEG. MePEG modification of IgG caused complement binding to be reduced but not binding to antigen (Suzuki et al., 1984), while modification of granulocyte-macrophage colony stimulating factor did not affect colony stimulating activity but enhanced neutrophil priming activity (Knusli et al., 1992).

We used an *in vitro* assay based on recombinant, soluble gp120 from the IIIB isolate of HIV-1 to evaluate the effect of MePEGylation on the ligand binding function of CD4-IgG. We observed a decrease in the ability of MePEG-modified CD4-IgG to bind to this form of gp120 that roughly correlates with the extent of MePEG modification. It should be noted, however, that this decrease in binding is not necessarily indicative of a decrease in the ability of modified CD4-IgG to neutralize HIV-1 *in vivo*. For example, the amino acid sequence of gp120 varies dramatically between different strains of HIV-1, and the changes in binding that we observe with gp120 from the IIIB strain may not be representative of other strains (Daar et al., 1990; Ashkenazi et al., 1991; Moore et al., 1992). Moreover, the structure of recombinant, soluble gp120 in solution is monomeric, whereas on the surface of virions it is oligomeric. Thus CD4-IgG, as a homodimer, may bind bivalently to each gp120 oligomer, which might stabilize the binding. Perhaps relevant to this issue is the example of adenosine deaminase, where a decrease in *in vitro* activity did not correlate with a lack of efficacy *in vivo*. MePEG-modified adenosine deaminase displayed a 40% decrease in enzymatic activity *in vitro* (Davis et al., 1981), yet the modified protein has proved to be efficacious *in vivo* (Hershfeld et al., 1987). Therefore, despite an apparent loss of rgp120 binding *in vitro*, the extension in *in vivo* residence time observed for MePEG–CD4-IgG may provide a significant therapeutic benefit.

As to the site(s) that may be responsible for this loss of binding activity, the rgp120 binding activity resides in the N-terminal immunoglobulin-like domain (residues 1–98) of the extracellular portion of CD4. In particular, Lys-46, positioned within the C″ ridge of domain 1, is believed to be involved in such binding (Ashkenazi et al., 1990; Arthos et al., 1989; Brodsky et al., 1990). Since this residue is exposed to solvent (Wang et al., 1990; Ryu et al., 1990), it is possible—although we did not demonstrate this directly—that several of the lysines in this region, including Lys-46, are partially modified such that rgp120 binding is compromised. More generally, for proteins that do not contain active lysine residues, MePEGylation using this chemistry is less likely to compromise biological activity of the modified protein and, thus, may provide a greater therapeutic advantage.

In conclusion, we have applied a method of MePEGylation by reductive alkylation of amino groups to modification of an immunoadhesin. We synthesized an aldehyde derivative of MePEG and identified reaction conditions enabling the incorporation of MePEG into CD4-IgG in the range of approximately 3–15 mol of MePEG per mol of protein. MePEGylation resulted in a significant increase in the residence time of the protein in circulation, rendering it comparable to that of human IgG. While a reduction in *in vitro* binding of MePEG–CD4-IgG to soluble rgp120 was observed, the biological activity against intact virions of clinical strains of HIV remains to be investigated. The more general implication of our results is that MePEGylation by reductive alkylation can be a useful strategy, especially for proteins in which amino groups are not critical for biological activity.

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LITERATURE CITED

Abuchowski, A., McCoy, J. R., Palczuk, N. C., van Es, T., and Davis, F. F. (1977a) Effect of covalent attachment of PEG on immunogenicity and circulating life of bovine liver catalase. *J. Biol. Chem. 252*, 3582–3586.

Abuchowski, A., van Es, T., Palczuk, N. C., and Davis, F. F. (1977b) Alteration of immunological properties of bovine serum albumin by covalent attachment of PEG. *J. Biol. Chem. 252*, 3578–3581.

Abuchowski, A., Davis, F. F., and Davis, S. (1981) Immunosuppresive properties and circulating life of *Achromobacter* glutaminase-asparaginase covalently attached to PEG in man. *Cancer Treat. Rep. 65*, 1077–1081.

Abuchowski, A., Kazo, G. M., Verhoest, C. R., Jr., Van Es, T., Kafkewitz, D., Nucci, M.L., Viau, A. T., and Davis, F. F. (1984) Cancer therapy with chemically modified enzymes: Anti-tumor properties of PEG-asparaginase conjugates. *Cancer Biochem. Biophys. 7*, 175–186.

Arthos, J., Deen, K. C., Chaikin, M. A., Fornwald, J. A., Sathe, G., Sattentau, Q. J., Clapham, P. R., Weiss, R. A., McDougal, J. S., Pietropaolo, C., Axel, R., Truneh, A., Maddon, P. J., and Sweet, R. W. (1989) Identification of the residues in human CD4 critical for binding of HIV. *Cell 57*, 469–481.

Ashkenazi, A., Presta, L. G., Marsters, S. A., Camerato, T. R., Rosenthal, K. A., Fendly, B. M., and Capon, D. J. (1990) Mapping the CD4 binding site for human immunodeficiency virus by alanine-scanning mutagenesis. *Proc. Natl. Acad. Sci. U.S.A. 87*, 7150–7154.

Ashkenazi, A., Smith, D. H., Marsters, S. A., Riddle, L., Gregory, T. J., Ho, D. D., and Capon, D. J. (1991) Resistance of primary isolates of HIV-1 to sCD4 is independent of CD4-rgp120 binding affinity. *Proc. Natl. Acad. Sci. U.S.A. 88*, 7056–7060.

Ashkenazi, A., Capon, D. J., and Ward, R. H. R. (1993) Immunoadhesins. *Intern. Rev. Immunol. 10*, 219–227.

Avigad, G. (1983) A simple spectrophotometric determination of formaldehyde and other aldehydes: Application to periodate-oxidized glycol systems. *Anal. Biochem. 134*, 499–504.

Beauchamp, C. O., Gonias, S. L., Menapace, D. P., and Pizzo, S. V. (1983) A new procedure for the synthesis of MePEG-protein adducts: Effects on function, receptor recognition and clearance of superoxide dismutase, lactoferrin and alpha-2-macroglobulin. *Anal. Biochem. 131*, 25–33.

Berger, H., and Pizzo, S. V. (1988) Preparation of PEG-tissue plasminogen activator adducts that retain functional activity: Characteristics and behavior in three animal species. *Blood 71*, 1641–1647.

Boccu, E., Largajolli, R., and Veronese, F. M. (1983) Coupling of MePEGs to proteins via active esters. *Z. Naturforsch. 38C*, 94–99.

Bragg, P. D., and Hou, C. (1975) Subunit composition, function, and spatial arrangement in the Ca^{2+}- and Mg^{2+}-activated adenosine triphosphatases of *Escherichia coli* and *Salmonella typhimurium*. *Arch. Biochem. Biophys. 167*, 311–321.

Brodsky, M. H., Warton, M., Myers, R. M., and Littman, D. (1990) Analysis of the site in CD4 that binds to the HIV envelope glycoprotein. *J. Immunol. 144*, 3078–3086.

Buckmann, A. F., Morr, M., and Johansson, G. (1981) Functionalization of PEG and monomethoxy-PEG. *Makromol. Chem. 182*, 1379–1384.

Byrn, R. A., Mordenti, J., Lucas, C., Smith, D., Marsters, S. A., Johnson, J. S., Cossum, P., Chamow, S. M., Wurm, F. M., Gregory, T., Groopman, J. E., and Capon, D. J. (1990) Biological properties of a CD4 immunoadhesin. *Nature 344*, 667–670.

Capon, D. J., Chamow, S. M., Mordenti, J., Marsters, S. A., Gregory, T., Mitsuya, H., Byrn, R. A., Lucas, C., Wurm, F. M., Groopman, J. E., Broder, S., and Smith, D. H. (1989) Designing CD4 immunoadhesins for AIDS therapy. *Nature 337*, 525–531.

Chamow, S. M., Peers, D. H., Byrn, R. A., Mulkerrin, M. G., Harris, R. J., Wang, W.C., Bjorkman, P. J., Capon, D. J., and Ashkenazi, A. (1990) Enzymatic cleavage of a CD4 immunoadhesin generates crystallizable, biologically active Fd-like fragments. *Biochemistry 29*, 9885–9891.

Chamow, S. M., Duliege, A. M., Ammann, A., Kahn, J. O., Allen, J. D., Eichberg, J.W., Byrn, R. A., Capon, D. J., Ward, R. H. R., and Ashkenazi, A. (1992) CD4 immunoadhesins in anti-HIV therapy: New developments. *Int. J. Cancer 52*, Suppl. 7, 69–72.

Daar, E. S., Li, X. L., Moudgil, T., and Ho, D. D. (1990) High concentrations of sCD4 are required to neutralize primary HIV-1 isolates. *Proc. Natl. Acad. Sci. U.S.A. 87*, 6574–6578.

Davis, S., Abuchowski, A., Park, Y. K., and Davis, F. F. (1981) Alteration of the circulating life and antigenic properties of bovine ADA in mice by attachment of PEG. *Clin. Exp. Immunol. 46*, 649–652.

Delgado, C., Patel, J. N., Francis, G. E., and Fisher, D. (1990) Coupling of PEG to albumin under very mild conditions by activation with tresyl chloride: characterization of the conjugate by partitioning in aqueous two-phase systems. *Biotechnol. Appl. Biochem. 12*, 119–128.

Fields, R. (1972) The rapid determination of amino groups with TNBS. *Meth. Enzymol. 25*, 464–468.

Francis, G. E., Delgado, C., and Fisher, D. (1992) PEG-modified proteins. In: *Stability of Protein Pharmaceuticals: in vivo Pathways of Degradation and Strategies for Protein Stabilization;* T. J. Ahern, and M. Manning, Eds.), Plenum Press, New York.

Harris, J. M., Struck, E. C., Case, M. G., Paley, M. S., Yalpani, M., van Alstine, J. M., and Brooks, D. E. (1984) Synthesis and characterization of PEG derivatives. *J. Polymer Sci.: Polymer Chem. Ed. 22*, 341–352.

Harris, R. J., Wagner, K. L., and Spellman, M. W. (1990) Structural characterization of a recombinant CD4-IgG hybrid molecule. *Eur. J. Biochem. 194*, 611–620.

Hershfeld, M. S., Buckley, R. H., Greenberg, M. L., Melton, A. L., Schiff, R., Hatem, C., Kurtzberg, J., Markert, M. L., Kobayashi, R. H., Kobayashi, A. L., and Abuchowski, A. (1987) Treatment of adenosine deaminase deficiency with PEG-modified adenosine deaminase. *N. Engl. J. Med. 316*, 589–596.

Hodges, T. L., Kahn, J. O., Kaplan, L. D., Groopman, J. E., Volberding, P. A., Ammann, A. J., Arri, C. J., Bouvier, L. M., Mordenti, J., Izu, A. E. and Allan, J. D. (1991) Phase 1 study of recombinant CD4-IgG therapy of patients with AIDS and AIDS-related complex. *Antimicrob. Agents Chemother. 35*, 2580–2586.

Ingham, K. C., and Ling, R. C. (1978) A quantitative assay for PEG without interference by proteins. *Anal. Biochem. 85*, 139–145.

Jackson, C. J. C., Charlton, J. L., Kuzminski, K., Lang, G. M., and Sehon, A. H. (1987) Synthesis, isolation and characterization of conjugates of ovalbumin with PEG using cyanuric chloride as the coupling agent. *Anal. Biochem. 165*, 114–127.

Jentoft, J. E., and Dearborn, P. G. (1979) Labeling of proteins by reductive methylation using sodium cyanoborohydride. *J. Biol. Chem. 254*, 4359–4365.

Joppich, M., and Luisi, P. L. (1979) Synthesis of glycyl-L-tryptophanylglycine substituted by PEG at both carboxyl and amino end groups. *Makromol. Chem. 180*, 1381–1384.

Kahn, J. O., Allan, J. D., Hodges, T. L., Kaplan, L. D., Arri, C. J., Fitch, H. F., Izu, A.E., Mordenti, J., Sherwin, S. A., Groopman, J. E., and Volberding, P. A. (1990) The safety and pharmacokinetics of sCD4 in subjects with AIDS and AIDS-related complex. *Ann. Int. Med. 112*, 254–261.

Katre, N. V., Knauf, M. J., and Laird, W. J. (1987) Chemical modification of recombinant interleukin 2 by PEG increases its potency in the murine Meth A sarcoma model. *Proc. Natl. Acad. Sci. U.S.A. 84*, 1487–1491.

Kitamura, K., Takahashi, T., Yamaguchi, T., Noguchi, A., Noguchi, A., Takashina, K.I., Tsurumi, H., Inagake, M., Toyokuni, T., and Hakamori, S. (1991) Chemical engineering of the monoclonal antibody A7 by polyethylene glycol for targeting cancer chemotherapy. *Cancer Res. 51*, 4310–4315.

Knauf, M. J., Bell, D. P., Hirtzer, P., Luo, Z. P., Young, J. D., and Katre, N. V. (1988) Relationship of effective molecular size to systemic clearance in rats of recombinant interleukin-2

chemically modified with water-soluble polymers. *J. Biol. Chem. 263,* 15064–15070.

Knusli, C., Delgado, C., Malik, F., Domine, M., Tejedor, M. C., Irvine, A. E., Fisher, D., and Francis, G. E. (1992) PEG modification of GM-CSF enhances neutrophil priming activity but not colony stimulating activity. *Brit. J. Haematol. 82,* 654–663.

Koide, A., and Kobayashi, S. (1983) Modification of amino groups in porcine pancreatic elastase with PEG in relation to binding ability towards anti-serum and to enzymic activity. *Biochem. Biophys. Res. Commun. 111,* 659–667.

Leonard, C. K., Spellman, M. W., Riddle, L., Harris, R. J., Thomas, J. N., and Gregory, T. J. (1990) Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant HIV envelope glycoprotein expressed in Chinese hamster ovary cells. *J. Biol. Chem. 265,* 10373–10382.

Moore, J. P., McKeating, J. A., Huang, Y., Ashkenazi, A., and Ho, D. D. (1992) Virions of primary HIV-1 isolates resistant to sCD4 neutralization differ in sCD4 binding and glycoprotein gp120 retention from sCD4-sensitive isolates. *J. Virol. 66,* 235–243.

Nilsson, K., and Mosbach, K. (1984) Immobilization of ligands with organic sulfonyl chlorides. *Methods Enzymol. 104,* 56–69.

Ryu, S. E., Kwong, P. D., Truneh, A., Porter, T. G., Arthos, J., Rosenberg, M., Dai, X., Xuong, N., Axel, R., Sweet, R. W., and Hendrickson, W. A. (1990) Crystal structure of an HIV-binding recombinant fragment of human CD4. *Nature 348,* 419–426.

Schooley, R. T., Merigan, T. C., Gaut, P., Hirsch, M. S., Holodniy, M., Flynn, T., Liu, S., Byington, R. E., Henochowicz, S., Gubish, E., Spriggs, D., Kufe, D., Schindler, J., Dawson, A., Thomas, D., Hanson, D. G., Letwin, B., Liu, T., Gulinello, J., Kennedy, S., Fisher, R. and Ho, D. D. (1990) Recombinant sCD4 therapy in patients with AIDS and AIDS-related complex. *Ann. Int. Med. 112,* 247–253.

Shak, S., Capon, D. J., and Ward, R. H. R. (1989) CD4 polypeptide derivatives. European Patent Application #EP 0 372 752 A2.

Smith, D. H., Byrn, R. A., Marsters, S. A., Gregory, T., Groopman, J. E., and Capon, D. J. (1987) Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen. *Science 238,* 1704–1707.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem. 150,* 76–85.

Suzuki, T., Kanbara, N., Tomono, T., Hayashi, N., and Shinohara, I. (1984) Physicochemical and biological properties of PEG-coupled immunoglobulin G. *Biochim. Biophys. Acta 788,* 248–255.

Venkatachalam, M. A., and Rennke, H. G. (1978) The structural and molecular basis of glomerular filtration. *Circulation Res. 43,* 337–347.

Veronese, F. M., Largajolli, R., Boccu, E., Benassi, C. A., and Schiavon, O. (1985) Activation of PEG by phenylchloroformates and modification of ribonuclease and superoxide dismutase. *Appl. Biochem. Biotech. 11,* 141–152.

Wang, J., Yan, Y., Garrett, T. P. J., Liu, J., Rodgers, D. W., Garlick, R. L., Tarr, G. E., Husain, Y., Reinherz, E. L., and Harrison, S. C. (1990) *Nature 348,* 411–418.

Wirth, P., Souppe, J., Tritsch, D., and Biellmann J. F. (1991) Chemical modification of horseradish peroxidase with ethanal-MePEG: Solubility in organic solvents, activity and properties. *Bioorganic Chem. 19,* 133–142.

Zalipsky, S., and Lee, C. (1992) Use of functionalized PEGs for modification of polypeptides. In: *PEG Chemistry: Biotechnical and Biomedical Applications* (J. M. Harris, Ed.), pp 347–370, Plenum Press, New York.

Zalipsky, S., Seltzer, R., and Menon-Rudolph, S. (1992) Evaluation of a new reagent for covalent attachment of PEG to proteins. *Biotechnol. Appl. Biochem. 15,* 100–114.