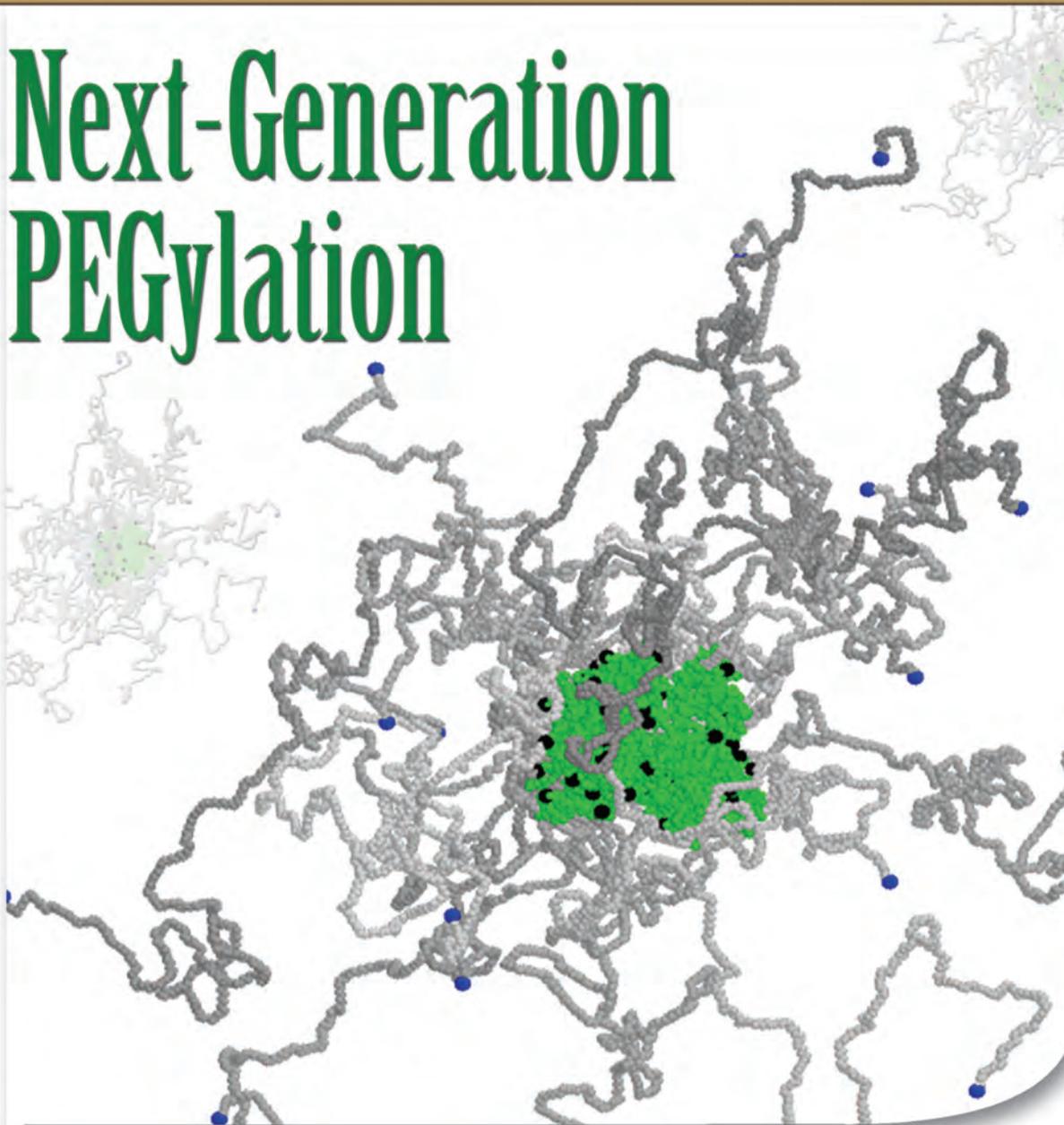


Drug Development[®] & Delivery

June 2012 Vol 12 No 5

www.drug-dev.com

Next-Generation PEGylation



The science & business of drug development in specialty pharma, biotechnology, and drug delivery

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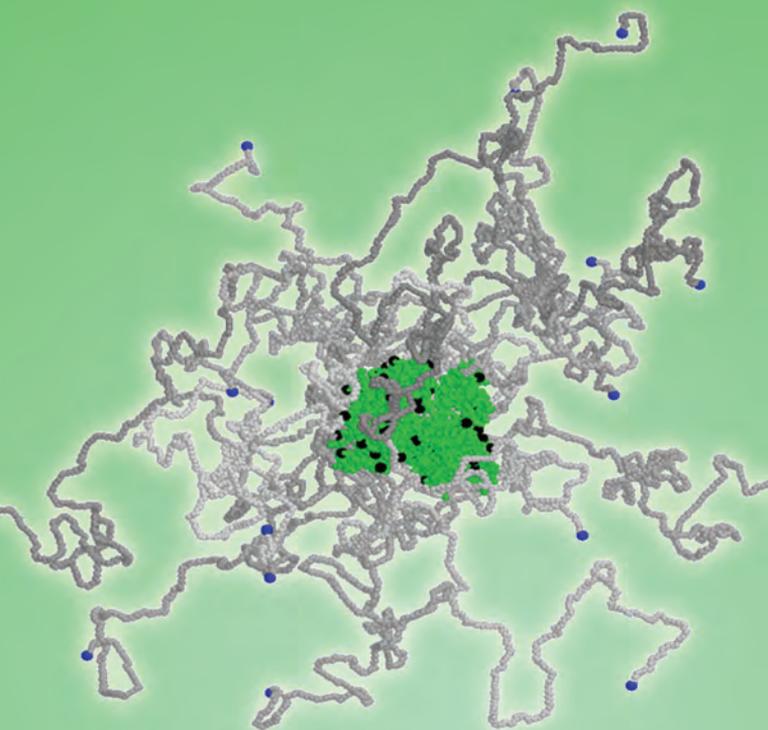
Next-Generation
PEGylation Enables
Reduced
Immunoreactivity of
PEG-Protein
Conjugates



**Shunji Haruta,
PhD**

Meeting the Needs
of Nasal Delivery
Devices for Powder
Formulations

Next- Generation PEGylation



“The ensemble of data presented here and in our previous publications suggests that clinically important decreases in the immunoreactivity of a next generation of PEGylated drugs (PharmaPEG® conjugates), including proteins, liposomes, nucleic acids, viruses, and red blood cells, may be attainable by the use of monofunctionally activated hydroxyPEGs instead of methoxyPEGs in their synthesis.”

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NEXT-GENERATION PEGYLATION

Next-Generation PEGylation Enables Reduced Immunoreactivity of PEG-Protein Conjugates

By: Merry R. Sherman, PhD; Mark G.P. Saifer, PhD; L. David Williams, PhD; Shawnya J. Michaels, MS; and Monika A. Sobczyk, MS

INTRODUCTION

The covalent attachment of therapeutic proteins to poly(ethylene glycol) (PEGylation) is intended to decrease their immunoreactivity, improve their solubility and stability, prolong their duration of action, decrease the frequency of dosing, and enhance their safety. Observations that repeated injections of animals with methoxyPEG (mPEG) conjugates of various proteins provoke the formation of antibodies directed against mPEG, however, are important because all currently approved PEGylated therapeutic products contain mPEG.¹⁻⁴ In the case of the mPEG-uricase (pegloticase) that was recently approved for treatment of refractory chronic gout (RCG), most of the anti-pegloticase antibodies detected by enzyme-linked immunosorbent assays (ELISAs) of sera from patients in the Phase I clinical studies, who had received only one subcutaneous or intravenous dose of the drug, were shown to be directed against the mPEG.⁵⁻⁷

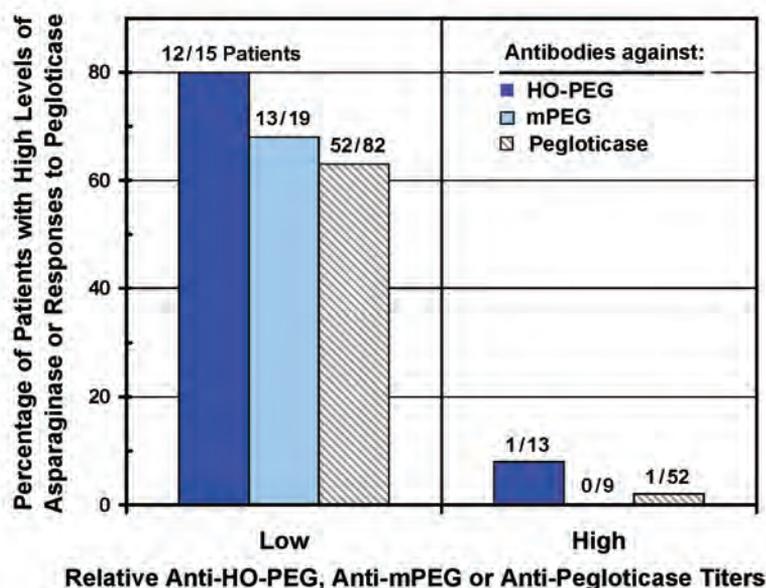
The potential impact of anti-PEG antibodies on the safety, pharmacokinetics, and pharmacodynamics of many PEGylated therapeutic proteins and other PEGylated therapeutic agents is supported by these observations: 1) high levels of anti-PEG antibodies in the sera of patients with acute lymphoblastic leukemia (ALL) treated with mPEG-asparaginase

(Oncaspar[®]) are associated with an accelerated loss of circulating enzyme; 2) high titers of anti-PEG antibodies in the sera of RCG patients treated with mPEG-uricase are associated with a loss of responsiveness to the drug; 3) clearance of mPEG-modified red blood cells is accelerated after repeated administration; 4) clearance of mPEG-modified liposomes is accelerated after repeated administration; and 5) monoclonal anti-PEG antibodies can be used to accelerate the clearance of

several PEGylated proteins.⁶⁻¹⁵ Detailed analyses of the anti-PEG antibodies detected in our research have led to the recognition that much of the immunogenicity and antigenicity of the polymers in mPEG-protein conjugates is attributable to the methoxy group at the terminus of the polymer that is remote from the protein.^{2,16}

In this report, we illustrate the close parallels between the results of Armstrong et al with respect to anti-PEG antibodies

FIGURE 1



The percentages of acute lymphoblastic leukemia patients with high serum asparaginase after treatment with mPEG-asparaginase (Oncaspar[®]) correlate inversely with the titers of anti-PEG antibodies measured with a hydroxyPEG ligand (TentaGel[®] OH) (■) or with mPEG-modified erythrocytes (■).^{8,10,11} The percentage of refractory chronic gout patients whose plasma uric acid responds to biweekly infusions of pegloticase (mPEG-uricase; KRYSTEXXA[®]) correlates inversely with the titers of anti-pegloticase antibodies (hatched bars), which are directed primarily against mPEG.⁵⁻⁷

detected in sera of ALL patients treated with mPEG-asparaginase and the results reported by Sundry et al based on the Phase III clinical trials of mPEG-uricase in patients with RCG.^{5,8} We compare the immunogenicity in rabbits and the antigenicity measured in rabbit sera of multi-PEGylated conjugates of human serum albumin (HSA) synthesized using similarly activated mPEG or hydroxyPEG (HO-PEG). The HSA conjugates used in these studies had a similar mass ratio of PEG-to-protein (3-to-1) to that of pegloticase.^{1,5} The results of these comparisons suggest there may be advantages to using HO-PEG instead of mPEG in the development of next-generation PEGylated therapeutic agents, including proteins, liposomes, nucleic acids, viruses, and red blood cells.

ANTI-PEG ANTIBODIES & SERUM ASPARAGINASE

Armstrong et al reported that the concentrations of asparaginase in previously frozen sera from ALL patients who were treated with mPEG-asparaginase (Oncaspar) were correlated inversely with the relative titers of anti-PEG antibodies in the same sera assayed by two methods.⁸ Some of their data are summarized in Figure 1. When the relative antibody titers (Low versus High, based on a cut-off selected by Armstrong et al) are measured by binding to TentaGel® OH, they reflect antibodies against the PEG backbone and possibly the terminal hydroxy group.⁸ They are referred to in this report as “anti-HO-PEG titers,” and the dark blue bars in Figure 1 indicate the corresponding data. When the relative antibody titers are measured by the binding to mPEG-modified erythrocytes, they reflect binding to the methoxy group of mPEG, as well as to the PEG backbone. They are referred to in this report as “anti-mPEG titers,” and the corresponding data are indicated by the light blue bar(s) in Figure 1.

The results represented by the blue bars in Figure 1 demonstrate a clear inverse correlation between the serum asparaginase concentrations and anti-PEG antibody titers, regardless of the method of antibody measurement. Among 28

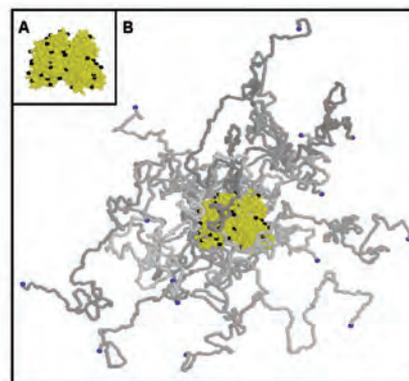
serum samples analyzed by Armstrong et al, high asparaginase was detected in 80% of those with low titers of anti-HO-PEG antibodies and only 8% of those with high titers.⁸ Among the same 28 samples, high asparaginase was detected in 68% of those with low titers of anti-mPEG antibodies, and none of those with high titers. Analyses of a larger number of samples by a common method of measurement of both anti-mPEG and anti-HO-PEG antibodies would be needed to determine whether high anti-mPEG or anti-HO-PEG titers are more closely correlated with low serum asparaginase levels in such patients.

ANTI-PEGLOTICASE ANTIBODIES REDUCE RESPONDERS

Sundry et al summarized the results of two Phase III clinical trials of an mPEG-uricase (pegloticase, KRYSTEXXA®) for the treatment of refractory chronic gout (RCG).⁵ Their report includes correlations of the primary endpoint of the trials - the persistent lowering of plasma uric acid below a specified level - with the relative titers of antibodies detected against the PEGylated enzyme as a whole. From the Phase I clinical studies of patients who received a single subcutaneous or intravenous dose of this drug, however, the results of ELISAs of sera from most of the patients with measurable anti-pegloticase antibodies suggested the antibodies were specific for the polymer (mPEG), rather than the enzyme (uricase).^{6,7}

Some of the data from the Phase III trials of pegloticase, for patients who received biweekly intravenous infusions of the drug, are summarized in Figure 1 (hatched bars). The inverse correlation of the percentage of gout patients who responded to pegloticase with the relative titers of anti-pegloticase antibodies (Low versus High, based on a cut-off selected by Sundry et al) resembles the inverse correlations of the percentages of leukemia patients who had high serum asparaginase with the relative titers of either anti-HO-PEG or anti-mPEG antibodies after treatment with Oncaspar (blue bars in Figure 1).^{5,8} This similarity is consistent with the hypothesis that

FIGURE 2



Space-filling molecular models of human serum albumin (A) and a conjugate of albumin with 17 molecules of 10 kDa PEG (B) were generated as described previously for uricase and PEG-uricase.¹ In both models, albumin is shown in yellow, with the nitrogen atoms of the epsilon amino groups of lysine residues in black. In B, the PEG molecules are shown in shades of gray, with the terminal oxygen atom of each PEG enlarged slightly and shown in blue. This is the site of the methoxy group at the remote terminus of each polymer molecule in mPEG.

the anti-pegloticase antibodies detected by Sundry et al in sera from the gout patients in the Phase III clinical trials of pegloticase are directed primarily against the many mPEG moieties in that drug, as reported for the Phase I studies.^{1,5-7}

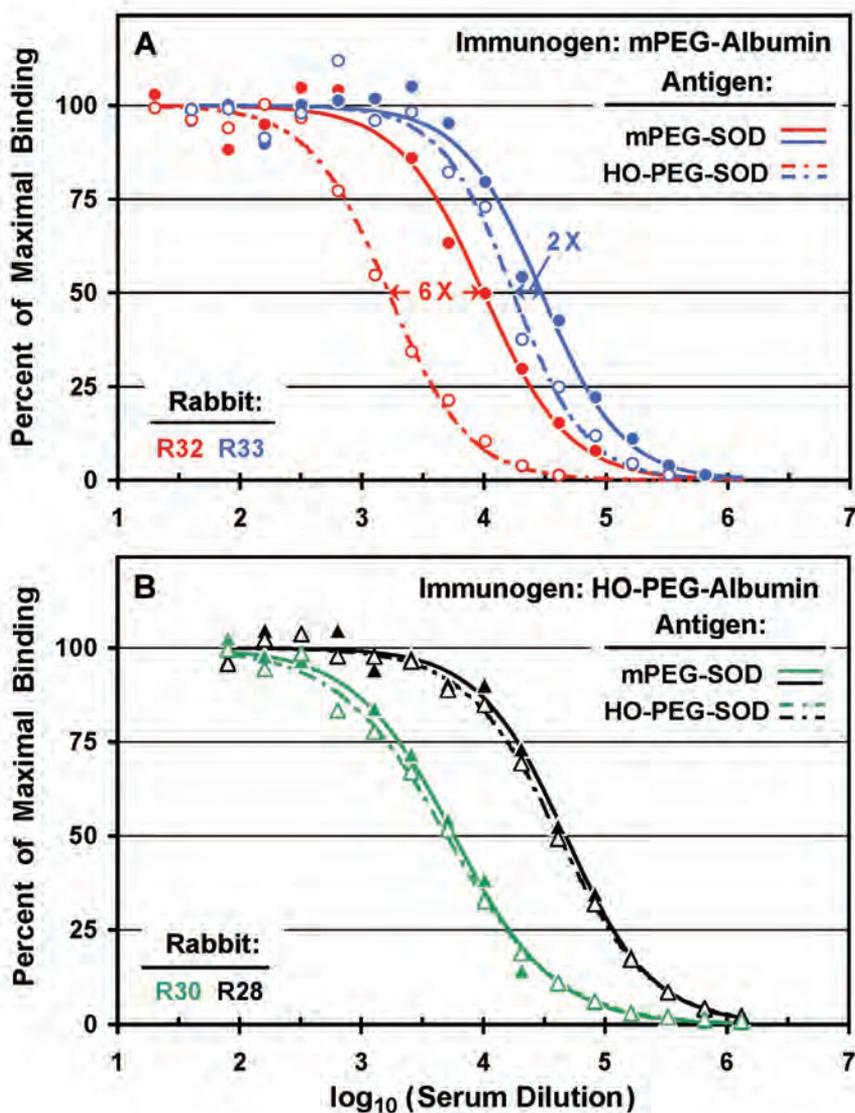
MODELS FOR MULTI-PEGYLATED THERAPEUTIC PROTEINS

The PEGylated therapeutic proteins currently approved for sale in the US and/or Europe are reported to contain between one and an average of 40 molecules of mPEG.^{3,4} This report is based on data from rabbits immunized with HSA coupled to between 17 and 20 molecules of either 10-kDa mPEG (n=7) or HO-PEG (n=7) or recombinant human interferon-alpha coupled to one or two molecules of 20-kDa mPEG (n=5) or HO-PEG (n=6). Some of the data for 17 of these rabbits were published previously.² Data from eight additional rabbits, four each immunized with mPEG-HSA or HO-PEG-HSA, are included in this report.

Space-filling molecular models of unPEGylated HSA and HSA conjugated to 17

FIGURE 3

HO-PEG LESS IMMUNOGENIC THAN MPEG



Anti-mPEG and anti-HO-PEG antibodies were assayed in sera from four rabbits that had been immunized with human serum albumin conjugates of either mPEG (A) or HO-PEG (B). Direct ELISAs were performed on assay plates coated with porcine Cu-Zn superoxide dismutase (SOD) conjugated to either mPEG (filled symbols, solid curves) or HO-PEG (open symbols, dashed curves). Data for rabbits R28, R30, R32, and R33 are shown in black, green, red, and blue, respectively.

molecules of 10-kDa PEG are shown in Figures 2A and 2B, respectively. These models were developed using the methods and computer programs described previously for analogous models of uricase and PEG-uricase.¹ A comparison of the models of “naked” albumin and multi-PEGylated albumin reveals a marked difference in size, even though PEG is not coupled to all of the solvent-accessible lysine residues, which are identified in Figure 2 by the black-colored nitrogen atoms of their epsilon amino groups. This difference in size between albumin and multi-PEGylated albumin

has been demonstrated in size-exclusion chromatographic analyses of the naked and PEGylated protein (see supporting information of Sherman et al).² In the model of PEG₁₇-albumin in Figure 2B, the terminal oxygen atom of each strand of PEG is enlarged and colored blue. This is intended to emphasize that the terminal methoxy groups of conjugates synthesized with mPEG are predicted to be accessible in vitro to anti-mPEG antibodies in the sera of laboratory animals or patients previously immunized with mPEG-protein conjugates and to the immune system in vivo.

In the direct ELISAs shown in Figure 3, the wells of the assay plates were coated with mPEG or HO-PEG conjugates of porcine superoxide dismutase (SOD) that were PEGylated to the same extent. Serial dilutions of sera from rabbits immunized with either mPEG-HSA or HO-PEG-HSA were added to the wells, followed by the addition of an enzyme-linked secondary antibody, as described previously.² Goat anti-rabbit IgG (H and L chain specific) conjugated to horseradish peroxidase was used to enable colorimetric quantification of the binding of the rabbit primary antibodies to PEG-SOD in the wells. No Tween® or other PEG-containing detergent was used in washing the assay plates.²

The assays of sera from two rabbits in Figure 3A illustrate that 1) the absolute titers of anti-PEG antibodies (reflected by the serum dilution at which 50% of maximal binding is attained) varied between the rabbits, and 2) the ratio of the titer detected with mPEG-SOD to the titer detected with HO-PEG-SOD (the “relative titer”) also varied between the rabbits, from 2X to 6X in these examples. The consistent observation was that when rabbits were immunized with mPEG conjugates of HSA or two other proteins, the titers detected with mPEG-SOD exceeded the titers detected with HO-PEG-SOD, with only one exception among 15 rabbits tested to date.² In contrast, as illustrated in Figure 3B, when rabbits were immunized with HO-PEG conjugates of HSA or other proteins, the titers detected with mPEG-SOD and with HO-PEG-SOD did not differ appreciably in sera from any of 16 rabbits tested to date. From these results and related data published elsewhere, we infer that the methoxy groups of mPEG-proteins are important contributors to the immunogenicity of the conjugates.^{2,16} In contrast, our assays have revealed no contribution of the hydroxy groups of the HO-PEG moieties to the immunogenicity of HO-PEG-protein conjugates.

ANTI-MPEG VERSUS ANTI-HO-PEG AFFINITY DIFFERENCES

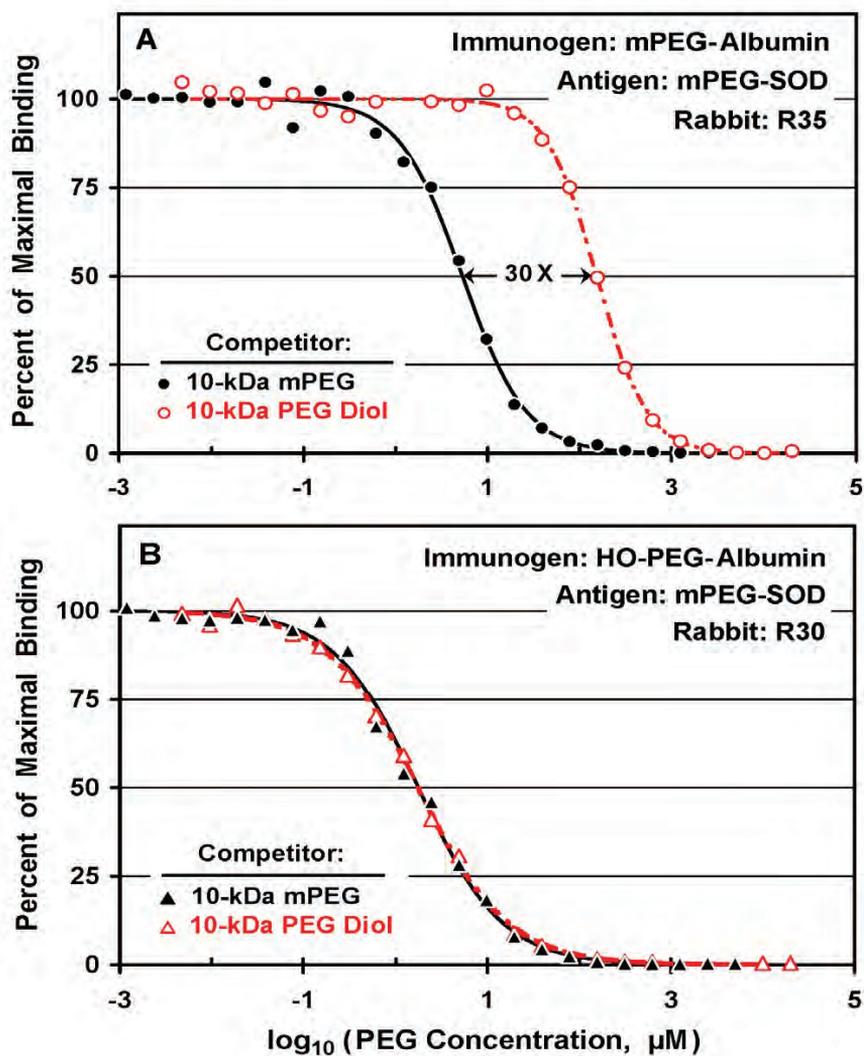
In the competitive ELISAs shown in Figures 4 and 5, the independent variable is the PEG concentration in the competitor, rather than the dilution of serum (as in the direct ELISAs shown in Figure 3). Figure 4A demonstrates that in serum from the particular rabbit tested (rabbit R35), the anti-PEG antibodies have 30 times higher affinity for 10-kDa mPEG than for 10-kDa PEG diol (HO-PEG-OH). The ratio of the affinity of this rabbit's antibodies for mPEG to that of PEG diol is much higher than the relative titers determined by direct ELISAs (30 fold versus 3 fold; data not shown for the direct ELISAs of rabbit R35). The high relative affinities suggest that if an animal or a patient were previously immunized with an mPEG-protein conjugate, subsequent treatment with a HO-PEG conjugate of the same protein would be less susceptible to accelerated clearance because of its much lower affinity for the circulating antibodies.

In contrast with the results in Figure 4A, the data in Figure 4B show that the affinities of antibodies elicited by HO-PEG-albumin for mPEG and PEG diol are indistinguishable. This has been a consistent observation for sera from all rabbits immunized with HO-PEG conjugates of each of three proteins: human serum albumin ($n=7$), recombinant human interferon-alpha ($n=6$) and porcine uricase ($n=3$).² These results imply that the anti-PEG antibodies formed against HO-PEG-protein conjugates are directed primarily against the polymer backbone and that competition for their binding to a PEG-protein antigen (such as PEG-SOD) is neither enhanced nor inhibited by the presence of a methoxy group in a competitor.

MULTI-PEGYLATION AMPLIFIES AFFINITY DIFFERENCES

The differences between the affinities for mPEG and HO-PEG of antibodies elicited by mPEG-protein conjugates were amplified when

FIGURE 4



Competitive ELISAs show that antibodies elicited by mPEG-albumin bind 10-kDa mPEG with 30-fold higher affinity than 10-kDa PEG diol (A). In contrast, antibodies raised against HO-PEG-albumin have the same affinities for mPEG and PEG diol (B).

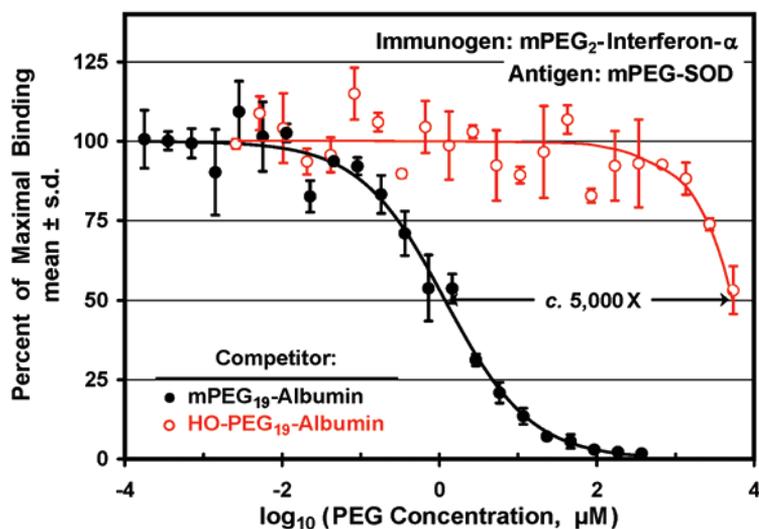
competitive ELISAs were performed with multi-PEGylated competitors, such as HSA conjugates containing an average of 19 molecules of 10-kDa PEG (PEG₁₉-albumin), as shown in Figure 5. The illustrated data were obtained with serum from a rabbit immunized with a conjugate of recombinant human interferon-alpha containing two molecules of 20-kDa mPEG (mPEG₂-IFN-alpha), synthesized as described previously.² In this serum, the ratio of the affinity for mPEG-HSA to that for HO-PEG-HSA was so high (more than three orders of magnitude) that the highest available concentration of HO-PEG-HSA was able to inhibit only half of the binding of the antibodies to mPEG-SOD in the wells of the assay plate. In analogous experiments with sera

from rabbits immunized with mPEG conjugates of porcine uricase ($n=3$), recombinant human interferon-alpha ($n=5$) or HSA ($n=7$), the ratios of affinities for mPEG-HSA versus HO-PEG-HSA, each containing 17 to 22 molecules of 10-kDa PEG per molecule of HSA, exceeded 1,000 in all except one of the sera tested.²

CONCLUSIONS

Despite the underlying assumption of countless scientific reports on the use of PEGylation to enhance the solubility and stability, prolong the duration of action and/or suppress the immunoreactivity of therapeutic agents ranging from low-molecular-weight

FIGURE 5



Competitive ELISAs show that rabbit anti-PEG antibodies raised against a diPEGylated conjugate of human interferon-alpha (mPEG₂-IFN-alpha) have c. 5,000 fold higher affinity for conjugates of human serum albumin with 19 molecules of 10-kDa mPEG than for analogous conjugates with 10-kDa HO-PEG.

drugs to whole cells, a growing body of evidence supports the conclusion that mPEG is both antigenic and immunogenic when conjugated to a protein or larger carrier, such as a liposome.^{2,12,13,16} This report includes our analysis of data published by others showing strong inverse correlations between the circulating levels of an oncolytic enzyme (asparaginase), following administration of mPEG-asparaginase, and the relative titers of anti-PEG antibodies in the same human sera.⁸ We have demonstrated the analogies between the latter data of Armstrong et al, based on studies of mPEG conjugates of an enzyme used for treatment of ALL, and the data of Sundry et al, based on studies of mPEG conjugates of recombinant porcine-like uricase used for the treatment of refractory chronic gout (Figure 1).^{1,5,8}

A comparison of the molecular models of human serum albumin and multi-PEGylated HSA provides a visual rationale for the expected (and observed) decrease in the vulnerability of the protein to proteolysis, as well as its prolonged circulation time, when administered as a PEG conjugate (Figure 2). The model of PEG₁₇-HSA also provides a visual image of the expected accessibility of

the methoxy group at the remote terminus of each mPEG molecule to the immune system in vivo and to antibodies present in the sera of immunized laboratory animals or humans.

The results of direct ELISAs provide compelling evidence that immunization of rabbits with mPEG conjugates of HSA elicits the production of higher titers of antibodies that bind to mPEG conjugates of an unrelated protein, SOD, than of antibodies that bind to HO-PEG-SOD (Figure 3A). In contrast, antibodies formed against HO-PEG-HSA have indistinguishable titers measured on mPEG-SOD or HO-PEG-SOD (Figure 3B). The results of competitive ELISAs provide compelling evidence that antibodies elicited in rabbits by HSA conjugates with mPEG, but not HO-PEG, have higher affinity for mPEG than for PEG diol (Figure 4), and that antibodies elicited by mPEG conjugates of recombinant human interferon-alpha have higher affinity for mPEG-HSA than for HO-PEG-HSA (Figure 5). While the relative titers of antibodies raised against mPEG-HSA and measured by binding to mPEG-SOD versus HO-PEG-SOD differ by factors of 2 or 6 in two rabbits, respectively (Figure 3A), the affinities of antibodies elicited by mPEG-HSA for unconjugated molecules of

10-kDa mPEG or PEG diol differ by a factor of 30 in serum from the illustrated rabbit (Figure 4A), and the affinities of antibodies elicited by mPEG₂-interferon-alpha for multi-PEGylated mPEG-HSA and HO-PEG-HSA differ by more than three orders of magnitude (Figure 5).

The ensemble of data presented here and in our previous publications suggests that clinically important decreases in the immunoreactivity of a next generation of PEGylated drugs (PharmaPEG[®] conjugates), including proteins, liposomes, nucleic acids, viruses, and red blood cells, may be attainable by the use of monofunctionally activated hydroxyPEGs instead of methoxyPEGs in their synthesis.^{2,16} Such decreases in immunoreactivity are likely to result in better tolerated PEGylated drugs, more durable clinical benefits, and fewer dropouts caused by accelerated clearance with loss of efficacy. ♦

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BIOGRAPHIES



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