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Synthesis and carbohydrate-binding activity of poly(ethyleneglycol)–*Ricinus communis* agglutinin I conjugates*†

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ABSTRACT

The synthesis of poly(ethyleneglycol) (PEG)–lectin conjugates was investigated to provide new reagents for evaluation as biological response modifiers. PEG was activated with 1,1′-carbonyldiimidazole (CDI), followed by conjugation with *Ricinus communis* I (RCAI) lectin. The resulting conjugates were heterodisperse with respect to molecular weight. Carbohydrate-binding activity was retained. The conjugates were separated by affinity chromatography into fractions differing in apparent carbohydrate-binding affinity. Conjugation of RCAI with PEG 4 (mol.wt. 3350) or PEG 6 (mol.wt. 8000) appeared to provide less hindrance of the lectin binding site compared to conjugates prepared with PEG 20 (mol.wt. 20 000). Results of free amine assays indicated that higher ratios of PEG to RCAI in conjugates correlated with loss of low-affinity binding and retention of high-affinity binding. The data showed the feasibility of preparing PEG-lectin conjugates for *in vivo* use.

INTRODUCTION

Dozens of plant and animal lectins have been described since the turn of the century[1]. Although the functions of these lectins are generally unknown, their carbohydrate-binding activity has made them useful tools to study the biology[2] and biochemistry[3] of glycoconjugates *in vitro*. Since the pioneering work of Nowell[4] on the mitogenic activity of phytohemagglutinin present in *Phaseolus vulgaris*, lectins have been major reagents used in the analysis of cellular components of the mammalian immune system[5,6]. While lectin-induced mitogenesis is usually characteristic of T lymphocytes[7], other cells of the immune system also bear surface receptors for various lectins[8]. The interaction of lectins with cells of the immune system induces or inhibits a variety of responses *in vitro*, which are indicative of cellular functions *in vivo*[9–11]. For example, lectins induce the synthesis and secretion of a variety of cytokines, a large family of proteins that are involved in the regulation of immune responses[12,13].

Many lectins have the potential for being powerful therapeutic and diagnostic reagents *in vivo*, owing to their carbohydrate-binding activity and specificity. There have been identified numerous plant, animal, and microbial lectins[1], which could be

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screened for these purposes. Some effort has already been made along these lines. A β-D-galactoside-binding lectin from the electric eel was reported to have therapeutic benefit in treating and preventing experimental, autoimmune myasthenia gravis in rabbits[14]. A β-D-galactoside-binding lectin from *Sarcophaga* (flesh fly) was found to induce the synthesis of human gamma interferon[15] and murine tumor necrosis-like factor[16] *in vitro*. An antitumor effect *in vivo* on murine tumors was also noted for the *Sarcophaga* lectin[17]. The β-D-galactoside-binding lectin from mistletoe was shown by Hajto *et al.*[18] to alter natural killer cell activity and large granular-lymphocyte quantity when injected into rabbits[18]. The same authors[18] also presented inferential data that the mistletoe lectin may have immunomodulatory activity *in vivo* in humans.

In order for a lectin to be useful *in vivo*, its toxicity and its immunogenicity must be reduced, its biologically active lifetime *in vivo* must be extended, and its ligand-binding activity must be retained and perhaps altered to improve specificity. These are basically the same problems that must be solved with recombinant and natural, human-derived, biological response modifiers destined for clinical use in humans[19–21]. Although lectins may be presumed to lack target-cell specificity and, hence, not be applicable *in vivo*, this problem has also been emphasized with respect to endogenous growth factors[22].

Our first approach to solving these problems with lectins was the synthesis and characterization of poly(ethyleneglycol)–*Ricinus communis* agglutinin I (PEG–RCAI) conjugates. PEG conjugation has been used to alter the properties of many proteins[23]. The reduction in toxicity, immunogenicity, and clearance rate of certain proteins linked to PEG has led to clinical trials for some PEG–enzyme conjugates[24]. The choice of RCAI for the studies reported herein was based primarily upon its stability and ease of isolation, and the presence of receptors on cells of the immune system.

EXPERIMENTAL

Materials. — RCAI was isolated as previously described[25]. Hemagglutination assays were carried out in microtiter plates with 2% rabbit erythrocytes[26]. PEG 4, PEG 6, PEG 20 (mol.wt. 3350, 8000, and 20 000, respectively), and Spectrapor dialysis tubing were obtained from Fisher Scientific (Rochester, NY). 1,1′-Carbonyldiimidazole (CDI) was obtained from Sigma Chemical Co. (St. Louis, MO), and Bio-Gel P-2 from Bio-Rad. Centricon 10 devices were obtained from Amicon, Danvers, MA.

Methods. — U.v. spectra were recorded with a Perkin–Elmer Lamda 4A spectrophotometer; the spectra with their machine-printed ordinate and adscissa values are directly reproduced here. The RCAI content of samples used for spectral analysis was estimated by use of the RCAI extinction coefficient (A_{280}^{1cm} of a 1.0 mg/mL solution, 1.22). Free amino groups were determined[27] by reaction with 2,4,6-trinitrobenzenesulfonate with lysine as a standard. Protein was determined with a Coomassie Blue dye-binding method (Bio-Rad Laboratories, Richmond, CA).

Sodium dodecylsulfate–poly(acrylamide)gel electrophoresis (SDS–PAGE) was carried out with the Pharmacia Phast system using 8–25% acrylamide Phast gels and silver staining according to the manufacturer's directions (Pharmacia LKB Biotech-

nology Inc., Piscataway, NJ). Samples were boiled in 10mM Tris I mM EDTA–2.5% SDS–5% mercaptoethanol, pH 8.0, prior to analysis.

PEG was activated with CDI, dialyzed, and freeze-dried according to the method of Beauchamp *et al.*[28]. In some experiments, activated PEG was passed through a column of Sephadex G-25 (Pharmacia), equilibrated with water, as an alternative to dialysis.

PEG 20–RCAI conjugation. — Activated PEG 20 (0.5 g) was dissolved in 10mM $Na_2B_4O_7$, pH 8.5, to give a volume of 2.5 mL. RCAI (15 mg) and lactose (90 mg) were added, and the solution was stirred for 72 h at 4°.

PEG 6–RCAI conjugation. — Activated PEG 6 (0.25 g), RCAI (6.2 mg), and lactose (37 mg) were dissolved in 10mM $Na_2B_4O_7$, pH 8.5 (1.0 mL), and the solution was stirred for 72 h at 4°. Conjugation was also carried out as described for PEG 20.

PEG 4–RCAI conjugation. — Activated PEG 4 (0.27 g) was dissolved in 10mM $Na_2B_4O_7$–0.1M lactose, pH 8.5 (2.2 mL), and RCAI (12.5 mg) was added. The solution was stirred for 72 h at 4°.

Lactose was removed from the conjugates by dialysis in standard tubing against 0.10M NaCl–0.05M PO_4–0.05% NaN_3, pH 7.3 (phosphate-buffered saline solution, PBS), or by passage through a Bio-Gel P-2 column, equilibrated with 0.10M NaCl–0.05M Tris–0.05% NaN_3, pH 7.3 (TBS).

RESULTS

The carbohydrate-binding activity of conjugates was assessed by affinity chromatography on acid-treated Sepharose 6B[25]. Such acid treatment increases the binding affinity and capacity for lectin–Sepharose interaction (Fig. 1). Two pools were obtained for each type of PEG conjugate, one nonbinding pool washed off the column with PBS and a bound pool eluted with 0.1M lactose. The nonbinding pool contained detectable hemagglutinating activity, as did the lactose-eluted pool after dialysis to remove lactose. The lactose-eluted pools contained 40, 72, and 26% of the recovered protein for PEG 4, PEG 6, and PEG 20 conjugates, respectively (Table I).

The pools were concentrated with Centricon 10 devices and dialyzed against 0.15M sodium chloride. The hemagglutinating activity of the dialyzed pools was quantitated by two-fold serial titration. The results are shown in Table I. The specific hemagglutinating activity (ratio of titer to A_{280}) of the nonbound pools for PEG 6 and PEG 20 conjugates was less than that for the bound pools. All PEG conjugate pools had a specific hemagglutinating activity less than that for free RCAI.

In order to gain evidence for the presence of PEG or RCAI in the conjugate fractions, u.v. spectra were recorded for the conjugates, PEG, activated PEG, RCAI, and CDI (see Figs. 2–5). CDI had a major absorption peak at 209 nm (Fig. 2A,C). No absorption above 238 nm was detected. The u.v. spectrum for RCAI was typical of a glycoprotein (Fig. 2B,D); an absorption peak at 280 nm was present. An absorption peak at 202 nm with a shoulder at 222 nm, typical of absorption due to peptide bonds and glycosidically-bound carbohydrate, was present.

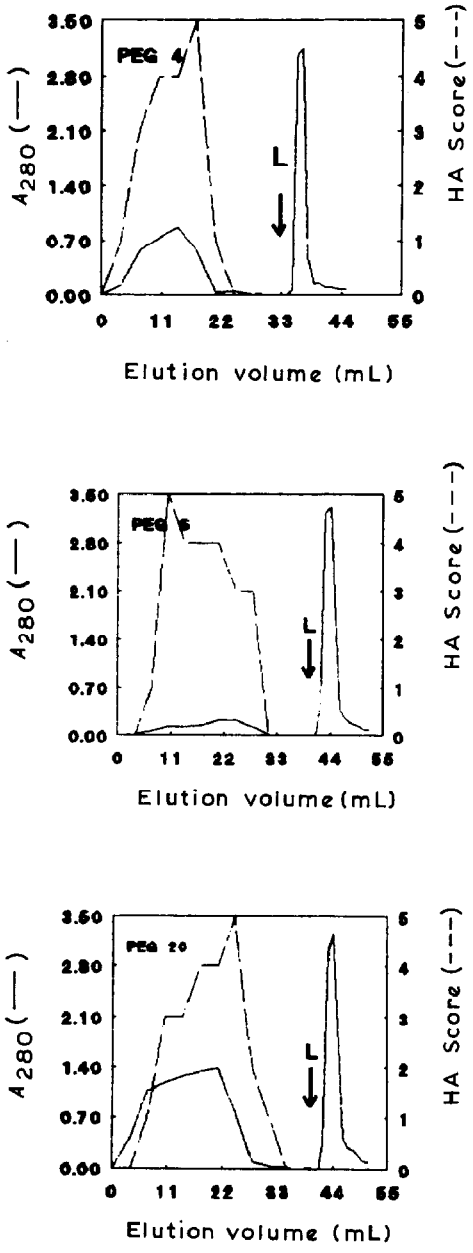

Fig. 1. Affinity chromatography of PEG–RCAI conjugates on acid-treated Sepharose 6B. After loading, the affinity columns (bed volume, 3.5 mL) were washed with PBS, followed by elution with 0.1M lactose. The chromatography was carried out at 4°. Fractions were assayed for protein (A_{280}) and hemagglutinating activity. The lactose-eluted fractions were negative for hemagglutinating activity until removal of lactose by dialysis. Load volume; 15, 25, and 20 mL for PEG 4, PEG 6, and PEG 20 conjugates, respectively.

TABLE I

Protein content and hemagglutinating activity of PEG–RCAI conjugate fractions from affinity chromatography

Sample[a]	*Proportions of nonbound and bound fractions*[b] (%)	*Hemagglutinating activity (HA)*		*Ratio of titer to* A_{280}
		A_{280}[c]	*Titer*	
PEG 20 nonbound	74	1.88	32	17
PEG 20 bound	26	1.69	128	76
PEG 6 nonbound	28	0.080	2	25
PEG 6 bound	72	1.546	128	83
PEG 4 nonbound	60	0.868	16	18
PEG 4 bound	40	0.657	16	24
RCAI		6.38	1024	162

[a] Nonbound fraction was washed off the affinity column with PBS. Bound fraction was eluted with 0.1M lactose. [b] Relative proportion (%) of nonbound and bound fractions determined by A_{280}. [c] A_{280} of samples titered for hemagglutinating activity.

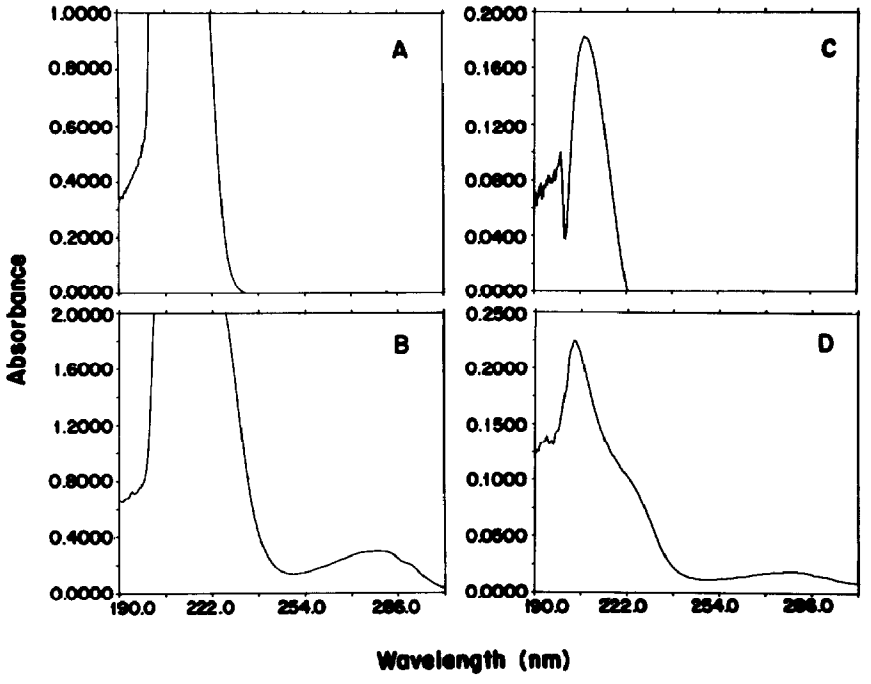

Fig. 2. U.v. spectra of CDI and RCAI; CDI was dissolved in 0.15M NaCl and RCAI was dialyzed against 0.15M NaCl prior to analysis: (A) CDI (0.10 mg/mL), (B) RCAI (0.25 mg/mL), (C) CDI (0.004 mg/mL), and (D) RCAI (0.020 mg/mL).

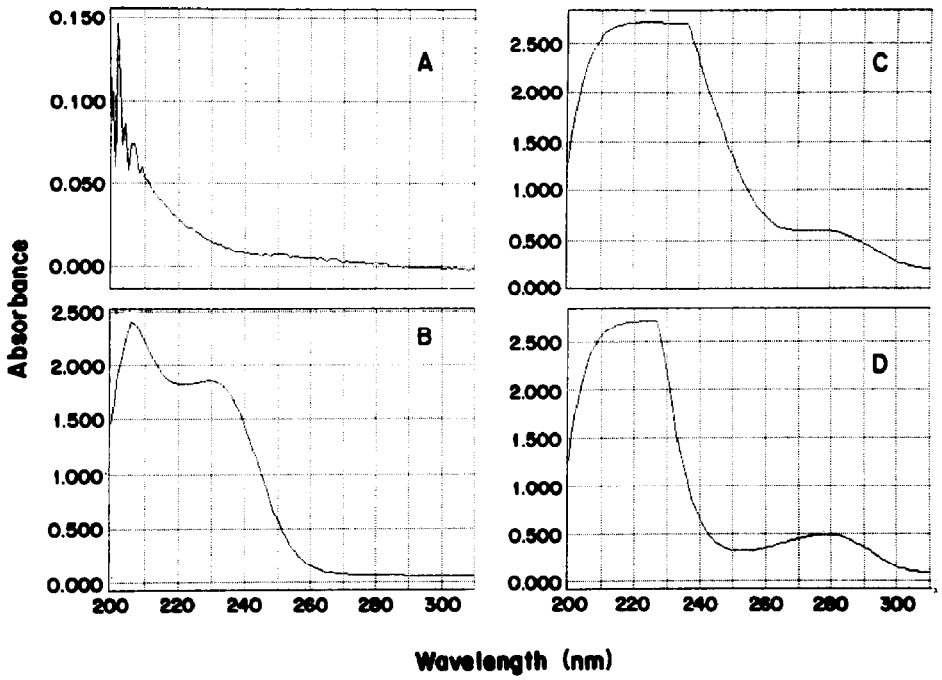

Fig. 3. U.v. spectra of PEG 4 and its derivatives; all samples were dialyzed against 0.15M NaCl prior to analysis: (A) PEG 4 (5 mg/mL), (B) activated PEG 4 (1.25 mg/mL), (C) nonbound PEG 4–RCAI conjugate (0.7 mg RCAI/mL), and (D) lactose-eluted PEG 4–RCAI conjugate (0.54 mg RCAI/mL).

The spectra for PEG 4 and its derivatives are shown in Fig. 3. At high concentration, PEG 4 showed a weak, poorly-defined absorption at 200–210 nm (Fig. 3A). Activated PEG 4 showed a strong absorption peak at 206 nm and a slightly weaker one at 230 nm (Fig. 3B). PEG 4–RCAI conjugate, which did not bind to acid-treated Sepharose but which was hemagglutinating-active, showed a major absorption peak in the short u.v. range characteristic of activated PEG 4, and a weaker absorption peak at 280 nm characteristic of RCAI (Fig. 3C). The expected absorption minimum at 250 nm was not apparent. PEG 4–RCAI conjugate, which bound to the affinity column and was eluted with lactose, had an absorption spectrum (Fig. 3D) similar to that of the nonbound conjugate. However, the bound conjugate appeared to have less absorption in the short u.v. spectrum, relative to that at 280 nm, when compared to the nonbound fraction. The absorption minimum at 250 nm was evident.

Native and activated PEG 6 had absorption spectra (Fig. 4 A,B) similar to those for PEG 4. PEG 6–RCAI conjugate, which did not bind to the affinity column, contained too little protein to generate an absorption peak at 280 nm (Fig. 4C). However, the u.v. absorption spectrum for the conjugate which bound to acid-treated Sepharose showed a prominent absorption peak at 280 nm (Fig. 4D) due to the presence of RCAI.

In contrast to PEG 4 and 6, native PEG 20 had a major absorption peak at 192 nm (Fig. 5A). This might have been due to a contaminant since the PEG 20 solution had a

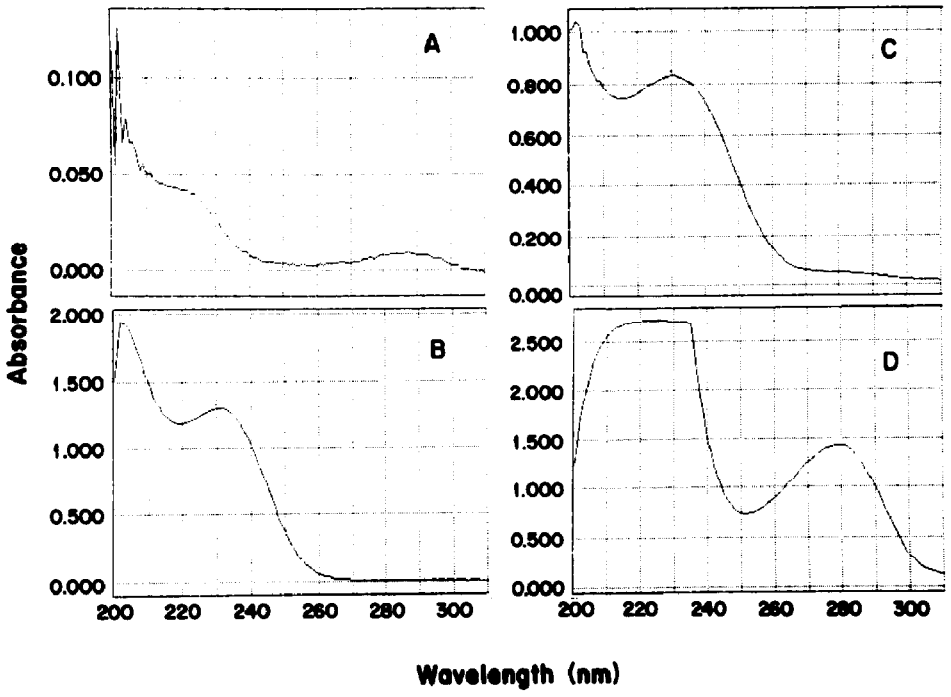

Fig. 4. U.v. spectra of PEG 6 and its derivatives; all samples were dialyzed against 0.15M NaCl prior to analysis: (A) PEG 6 (5 mg/mL), (B) activated PEG 6 (2.5 mg/mL), (C) nonbound PEG 6–RCAI conjugate (0.07 mg RCAI/mL), and (D) lactose-eluted PEG 6–RCAI conjugate (1.27 mg RCAI/mL).

very pale-yellowish coloration. Activated PEG 20 had a u.v. absorption spectrum (Fig. 5B) similar to those for activated PEG 4 and 6. The PEG 20 conjugate fraction, which did not bind to the affinity column, had a major absorption peak at 280 nm (Fig. 5C), which demonstrated the presence of RCAI in this fraction. The PEG conjugate fraction, that was eluted from the affinity column with lactose, had a u.v. absorption spectrum (Fig. 5D) similar to that of the nonbound fraction.

It should be noted that the u.v. spectra shown for the PEG–lectin conjugates may be influenced by the presence of unreacted imidazolylcarbamate groups in PEG which appear to absorb at 230 nm.

The specific-hemagglutinating activity of the PEG conjugates is given in Table II. Protein values were obtained by the Bio-Rad dye-binding assay. The PEG 4 conjugate bound and nonbound fractions had equivalent hemagglutinating activity and this was about three-fold less than that for RCAI. The PEG 6 nonbound fraction had negligable hemagglutinating activity and low protein content. The bound PEG 6 conjugate had hemagglutinating activity slightly less than that for RCAI. The nonbound PEG 20 conjugate had hemagglutinating activity similar to that of the PEG 4 pools, whereas the bound PEG 20 conjugate had hemagglutinating activity similar to that of the bound PEG 6 conjugate.

The various pools were assayed for free amino groups. The results are given in

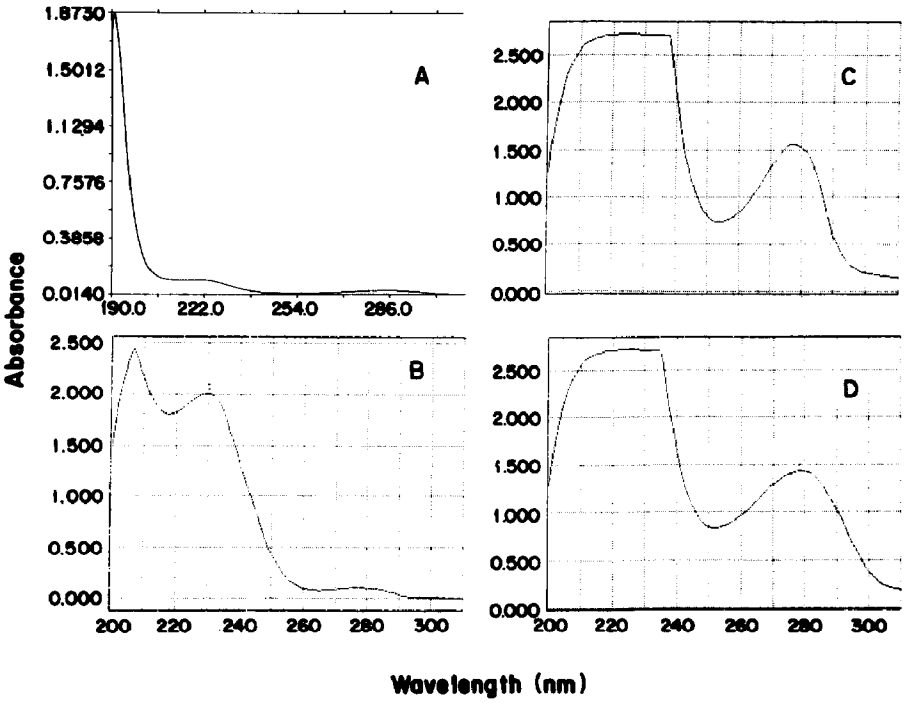

Fig. 5. U.v. spectra of PEG 20 and its derivatives; all samples were dialyzed against 0.15M NaCl prior to analysis: (A) PEG 20 (5 mg/mL), (B) activated PEG 20 (2.5 mg/mL), (C) nonbound PEG 20–RCAI conjugate (1.54 mg RCAI/mL), and (D) lactose-eluted PEG 20–RCAI conjugate (1.39 RCAI/mL).

TABLE II

Hemagglutinating activity and free amino group content of PEG–RCAI conjugate fractions from affinity chromatography

Sample	*Protein concentration*[a] *(μg of protein/mL)*	*Amino group content*[b] *[(μmol of amino group/μg of protein) × 10^{-4}]*
PEG 20 nonbound	3.3	[c]
PEG 20 bound	1.5	4.20
PEG 20 FT 1[d]	>32	[c]
PEG 6 nonbound	5.0	[c]
PEG 6 bound	1.5	7.20
PEG 4 nonbound	3.7	1.02
PEG 4 bound	3.5	8.65
RCAI	1.2	4.19

[a] Determined by dye-binding assay, at hemagglutination end-point. [b] Determined by the 2,4,6-trinitrobenzenesulfonate reaction. [c] None detected. [d] A column breakthrough fraction which was hemagglutinating-negative.

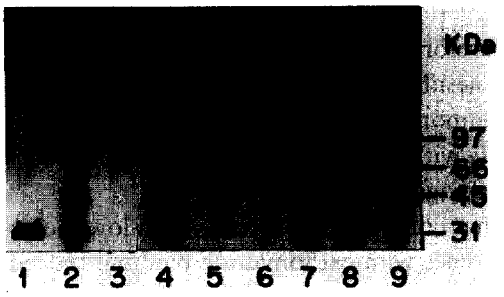

Fig. 6. SDS–PAGE of PEG–RCAI conjugates. The samples were reduced in sodium dodecyl sulfate as described in the Experimental section and subjected to electrophoresis on 8–25% acrylamide Phast gels, followed by silver staining: Lane 1, RCAI; lane 2, lactose-eluted PEG 6 conjugate; lane 3, nonbound PEG 6 conjugate; lane 4, lactose-eluted PEG 20 conjugate; lane 5, nonbound PEG 20 conjugate; lane 6, nonbound PEG 20 conjugate lacking hemagglutinating activity; lane 7, lactose-eluted PEG 6 conjugate; lane 8, lactose-eluted PEG 4 conjugate; and lane 9, nonbound PEG 4 conjugate. Molecular-weight markers are indicated.

Table II. It is evident that the PEG conjugates that bound to the affinity column had a higher content of free amino groups than did the corresponding nonbound fractions. In some cases, the conjugates had a free amino group content equal to or greater than that of RCAI. This discrepancy may be due to underestimated values for protein content of the conjugates. Conjugation of RCAI through its amino groups to activated PEG may depress the binding of Coomassie dye by RCAI, thus leading to underestimation of protein. Activated PEG did not react with 2,4,6-trinitrobenzenesulfonate and binding of Coomassie dye by PEG was not evident.

To further characterize PEG–RCAI conjugates, various fractions were analyzed by SDS–PAGE (Fig. 6). RCAI is composed of two different subunits of similar molecular mass which were not resolved in this system. All of the conjugate fractions were polydisperse. In some cases, a fraction of the conjugate polymers in a sample did not migrate through the stacking gel. Some conjugate fractions appeared to contain free RCAI. This was especially prominent for the lactose-eluted PEG 6 and 20 conjugates.

DISCUSSION

Lectins have been unable to achieve their potential for being powerful biological-response modifiers *in vivo* because of their toxicity, immunogenicity, and rapid clearance[29–31]. In view of this limitation, we have begun studies to develop lectin derivatives that may have the potential for therapeutic application. We describe herein the synthesis and some characteristics of PEG–RCAI conjugates.

We have carried out the successful synthesis of PEG–RCAI conjugates that retain carbohydrate-binding activity. The various PEG conjugates were separated into two different hemagglutinating-active fractions, differing in their binding affinity for acid-treated Sepharose. Each fraction was heterodisperse with respect to molecular weight, indicating that the ratio of PEG to molecule of RCAI was highly variable. It is possible that oligomers of RCAI were also obtained *via* crosslinking through activated PEG.

The results of affinity chromatography suggested that some of the conjugates had hindered carbohydrate-binding sites and therefore did not bind to acid-treated Sepharose. The results of the 2,4,6-trinitrobenzenesulfonate assay for free amino groups indicated that the nonbound fractions were more highly conjugated with PEG than were the bound fractions. However, reactivity with high-affinity receptors was retained by the nonbound fractions as revealed by hemagglutination assay.

Steric considerations would suggest that conjugation of lectins with low-molecular-weight PEG might result in less interference with carbohydrate binding. Indeed, comparison of the affinity chromatography results showed that a larger proportion of the PEG 4 and PEG 6 conjugates were capable of binding to acid-treated Sepharose, as compared to the PEG 20 conjugate.

The results of SDS–PAGE indicated that free RCAI was present in some of the conjugates. However, the electrophoretic system used here did not separate the two different RCAI subunits. Therefore, an alternative conclusion is that one of the RCAI subunits is more susceptible to reaction with activated PEG than the other. Under some conditions, this might result in one subunit not being conjugated with PEG. Future studies will resolve this possibility.

In summary, the results presented herein showed that carbohydrate-binding PEG–lectin conjugates can be synthesized and a portion of these conjugates have altered binding affinity. Alternate affinity adsorbents and gradient elution techniques may yield more homogeneous conjugate fractions for analysis. The behavior of PEG-lectin conjugates *in vitro* and *in vivo* remain to be determined and compared with the rather broad specificity of action of current biological-response modifiers[20,21].

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