Lectin Binding Studies on Murine Peritoneal Cells: Physicochemical Characterization of the Binding of Lectins from *Datura stramonium*, *Evonymus europaea*, and *Griffonia simplicifolia* to Murine Peritoneal Cells[1]

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Purified ^{125}I-labeled lectins from *Datura stramonium*, *Evonymus europaea*, and *Griffonia simplicifolia* (I-B$_4$ isolectin) were used to analyze changes in the expression of carbohydrates on the surface of resident (PC) and thioglycollate-stimulated murine (C57B/6J) peritoneal exudate cells (PEC). The lectins from *D. stramonium*, *E. europaea*, and *G. simplicifolia* I-B$_4$ bind specifically to PEC with relatively high affinity ($K_d = 5.65 \pm 1.08 \times 10^{-7}$ M, $1.08 \pm 0.12 \times 10^{-8}$ M, and $1.33 \pm 0.15 \times 10^{-7}$ M, respectively). Assuming a single lectin molecule binds to each cell surface saccharide, the number of receptor sites per cell ranged for different cell samples from 22.3 to 50.0×10^6, from 3.8 to 4.8×10^6, and from 2.0 to 16.8×10^6 for *D. stramonium*, *E. europaea*, and *G. simplicifolia* I-B$_4$ lectins, respectively. There were approximately 3- to 7-fold, 16- to 20-fold, and 2- to 20-fold increases in binding capacity for *D. stramonium*, *E. europaea* and *G. simplicifolia* I-B$_4$, respectively, compared to the binding to resident, peritoneal cells. Scatchard plots of the binding of all three lectins to PEC were linear, suggesting that the receptor sites for these lectins are homogeneous and noninteracting. The binding capacity of these lectins to PEC was unchanged after trypsin digestion of cells. The expression of carbohydrates on the surface of PEC was also monitored by an agglutination assay. PEC were agglutinated by all three lectins whereas PC either were not agglutinated or were agglutinated only at high lectin concentrations. On the basis of our knowledge of the carbohydrate binding specificity of the *D. stramonium* and *G. simplicifolia* I-B$_4$ lectins, we postulate that, parallel with thioglycolate stimulation, there is an increase in the number of *N*-acetyllactosamine residues and terminal α-D-galactosyl end groups. The blood group B, and H type 1 determinants—DGalα1,3[LFucα1,2]DGalβ1,3(or 4)DGlcNAc and LFucα1,2DGalβ1,3DGlcNAc, respectively, as well as DGalα1,3DGalβ1,3(or 4)DGlcNAc—may be considered to be possible receptors for the *E. europaea* lectin. These glycoconjugates, present on the surface of peritoneal exudate cells, provide new chemical markers for studying the differentiation of resident peritoneal cells. © 1986 Academic Press, Inc.

In previous communications (1, 4) we reported a difference between stimulated and resident mouse peritoneal cells in the expression of carbohydrate receptors. The α-D-galactosyl-specific isolectin (I-B$_4$) from *Griffonia simplicifolia* seeds (2) bound to stimulated murine PEC but not to resident PC,[2] as measured by a fluorescence-labeling

[2] Abbreviations used: BSA, bovine serum albumin; DNase I, deoxyribonuclease I from bovine pancrease; PBS, 0.01 M phosphate buffer (pH 7.1) with 0.15 M NaCl (unless stated otherwise this buffer contained 3 mM azide and was free of divalent cations); PC, resident peritoneal cells; PEC, peritoneal exudate cells;

technique (1). A second lectin of complex carbohydrate binding specificity from *Evonymus europaea* seeds (3) was bound by murine PEC but not by resident PC, as measured by an agglutination assay (4).

The primary cell population of PEC consists of macrophages. Since macrophages are involved in many immunological reactions (5), questions arise as to whether this explicit expression of differentiation-related carbohydrates on their surface is of biological significance and whether these carbohydrates play a definitive role in some of the biological functions of these cells. Other investigators have postulated that carbohydrates are essential for macrophage-lymphokine interaction as well (6–8). Thus the expression of lectin-binding glycoconjugates on the plasma membrane of macrophages may be directly related to specific macrophage functions.

In this paper we report on the physicochemical binding characteristics of both of the above lectins, as well as a third lectin from *Datura stramonium* (9), to resident PC and stimulated PEC. These binding parameters should prove helpful in the identification, purification, and structural elucidation of the cell surface carbohydrate receptors themselves, as well as in studies of their potential function in macrophages. We have already observed that the GS I-B$_4$ isolectin can promote macrophage-tumor cell interactions leading to enhanced lectin-induced, macrophage-mediated cytotoxicity of tumor cells *in vitro* (10).

MATERIALS AND METHODS

Lectins. D. stramonium, E. europaea, G. simplicifolia I-B$_4$, and *G. simplicifolia* IV lectins were purified as described earlier (9, 11–13).

Seeds of *D. stramonium* were obtained from Matthaei Botanical Gardens, University of Michigan; *E. europaea* seeds were collected from wild-growing shrubs in the neighborhood of Wroclaw, Poland; and *G. simplicifolia* seeds were obtained from Ghana.

Carbohydrates. Methyl α-D-galactopyranoside and lactose were products of Pfanstiehl Laboratories, Waukegan, Illinois. *N*-Acetyllactosamine was available in this laboratory from a previous study (9).

PMSF, α-toluenesulfonyl fluoride. Trypsin-TPCK, trypsin treated with L-(tosylamido 2-phenyl)-ethyl chloromethyl ketone.

Chemicals. Na^{125}I (carrier free) was obtained from New England Nuclear/DuPont, Boston, Massachusetts. Chloramine-T and sodium metabisulfite were from J. T. Baker Chemical Company, Philipsburg, New Jersey. Dioctyl phthalate and dibuthyl phthalate were from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin; bovine serum albumin was purchased from Miles Laboratories, Napierville, Illinois. Azocoll was from Calbiochem, La Jolla, California; Bio-Gel P-60 (50–100) mesh was from Bio-Rad Laboratories, Richmond, California.

Enzymes. Trypsin-TPCK was from Worthington Biochemical Corporation, Freehold, New Jersey. Enzyme activity was checked with Azocoll as substrate according to the procedure described in the Calbiochem–Behring Catalog (1984). Deoxyribonuclease (DNase) I from bovine pancrease was from Boehringer-Mannheim Biochemicals, Indianapolis, Indiana.

Isolation and preparation of macrophages. Macrophages were isolated by peritoneal lavage as described previously (14) from C57BL/6J female mice, 4–6 weeks of age (Jackson Laboratories, Bar Harbor, Maine). Resident macrophages were obtained from normal, untreated mice while inflammatory macrophages were obtained from animals 4 days after injection with 1 ml sterile thioglycollate (Difco, Detroit, Mich.). The cells were harvested and sedimented at 300g for 5 min at 4°C. Contaminating erythrocytes were lysed in 0.17 M Tris-0.14 M NH$_4$Cl for 10 min at 4°C. The cells were centrifuged (as above) and resuspended in PBS. The cells were washed twice in PBS, and reconstituted to 2×10^6/ml in PBS with 1 mM PMSF.

Lectin iodination. D. strammonium and *E. europaea* lectins were iodinated by the chloramine-T method as described by Hunter and Greenwood (15). Briefly, the lectin (10–24 µg in 20 µl of PBS) was diluted with 40 µl of 0.5 M potassium phosphate buffer, pH 7.4, in a 1-ml vial fitted with a multidose septum. Na^{125}I (0.9–1.9 mCi) was added followed by 30 µg of chloramine-T in 30 µl of 0.05 M phosphate buffer, pH 7.4, and the reaction mixture was shaken for 1 min. The iodination was quenched by the addition of sodium metabisulfite (10 µg in 10 µl of 0.05 M phosphate buffer, pH 7.4).

D. stramonium lectin labeled with ^{125}I was separated from the reactants on Bio-Gel P-60 (50–100 mesh), suspended in 0.01 M potassium phosphate with 0.15 M NaCl and 1/10,000 merthiolate, pH 7.4, and pretreated with 5% BSA. The gel was placed in a 5-ml disposable pipet with glass wool added to the bottom. Lectin was eluted with PBS buffer; 0.5-ml fractions were collected. *E. europaea* lectin was freed from reactants by extensive dialysis against several changes of PBS.

G. simplicifolia I-B$_4$ isolectin was iodinated by the iodogen procedure (16) except that NaI was omitted from the reaction. Briefly, a vial (1 ml) was coated with 36 µg of iodogen in CH$_2$Cl$_2$, and 240 µg of *G. simplicifolia* I-B$_4$ isolectin in PBS was added. The mixture was shaken for 5 min in an ice-water bath.

The iodination reaction was stopped by removing the reaction mixture from the vial. The lectin was separated from the reactants on a Bio-Gel P-60 column prepared as above. This iodination procedure resulted in lectin with a specific activity of 5.3×10^6 cpm/µg.

Iodinated lectins retained their carbohydrate-binding properties as indicated by the fact that binding was abolished by specific hapten inhibitors.

For binding studies, radiolabeled lectins were diluted with unlabeled lectins in order to obtain the required specific radioactivity. The specific radioactivity for *D. stramonium* was 1.97×10^5 cpm/µg; for *E. europaea*, 1.15×10^6 cpm/µg; and for *G. simplicifolia* I-B$_4$ isolectin, 2.7×10^5 cpm/µg. All binding experiments were carried out in triplicate without carbohydrate inhibitor and in duplicate with inhibitor, and the mean values were calculated. The difference between parallel results did not exceed 6%.

Binding assays. Binding experiments were carried out in 1.5-ml polypropylene microcentrifuge tubes (Curtin Matheson Scientific, Inc., Houston, Tex.) precoated with BSA by incubation for at least 1 h with PBS containing BSA (10 mg/ml). ^{125}I-Labeled lectin solutions were centrifuged before each assay for 10 min at 15,600g at 4°C, to remove traces of precipitate. The reaction mixture in a final volume of 250 µl of PBS containing 10 mg of BSA/ml was incubated with increasing amounts of ^{125}I-labeled lectin and 5–20 $\times$ 10^4 cells. The assay with *G. simplicifolia* I-B$_4$ isolectin also contained 0.1 mM CaCl$_2$. The tightly capped tubes were rotated at 4°C for 1 h and then centrifuged for 1 min at top speed in an Eppendorf microfuge. Solutions were carefully overlayered with 0.15 ml of a mixture of dioctyl phthalate and dibutyl phthalate in a ratio of 1:1 (v/v) and centrifuged again for 1 min as above. Subsequently, the upper layer was aspirated and the oil was carefully overlayered with 1 ml of PBS containing BSA 1 mg/ml. The tubes were centrifuged, the upper layer was removed by aspiration, and the washing procedure was repeated. The radioactivity was counted in an automatic gamma counter from Tracor Analytic, Elk Grove Village, Illinois. For the *E. europaea* lectin the only difference in the procedure was that before addition of 0.15 ml of oil, 1 ml of PBS containing BSA 1 mg/ml was added and mixed (to dilute the lactose solution), and the oil was overlayered. Binding of *D. stramonium*, *E. europaea*, and *G. simplicifolia* lectins was determined in the presence and absence of specific hapten inhibitors: 10 mM *N*-acetyllactosamine, 15 mM lactose, and 10 mM methyl α-D-galactoside, respectively. ^{125}I-Labeled lectins were preincubated with inhibitors for $\frac{1}{2}$ h at room temperature. These values were subtracted from the total lectin bound in the absence of inhibitors to obtain specific lectin binding.

Statistical analysis of binding data. Curves were fitted and binding parameters were estimated by a nonlinear regression method using iteratively reweighted least squares as described in BMDP Statistical Soft-

ware (17). The calculations were performed on the Amdahl 5860 computer at The Computing Center of The University of Michigan using a BMDP3R program. The fitted curves were plotted according to Scatchard (18).

Trypsin digestion of PEC. Unless otherwise stated, cells were kept in plastic tubes in an ice-water bath and all solutions were at 4°C. Trypsin solutions were freshly prepared and their activity was checked with Azocoll. The reaction mixture (final volume 0.5 ml) consisted of 0.01 M Tris–HCl buffer, pH 7.2, with 0.15 M NaCl and 3 mM sodium azide and contained up to 20×10^6 cells and 2.5 to 25.0 µg of trypsin-TPCK. Reaction mixtures were incubated at 37°C for 3 min and quenched by transfer to an ice-water bath, and, at the same time, 5 µl of 0.1 M PMSF was added to digested cells to quench the reaction. Six microliters (0.12 mg) of DNase I was added to both tubes and cells were incubated at room temperature for 12 min until clumps of cells disappeared. Control cells without enzyme were assayed in parallel and contained 1 mM PMSF. Control and digested cells were washed four times with 3 ml of PBS containing 1 mM PMSF and centrifuged each time at 700g for 5 min. Cells were counted on a Coulter counter, adjusted to the concentration required, and used for the binding studies.

No loss of cells occurred after digestion with 2.5 µg of trypsin compared with control cells. However, digestion with 25.0 µg of trypsin caused 47% of loss of cells.

Lectin agglutination of PEC. Single-cell suspensions of mouse cells (25 µl) at concentrations of approximately 6×10^6 cells/ml in PBS were mixed with 50 µl of varying concentrations of lectin solution, the volume was adjusted to 100 µl with PBS, and the mixtures were incubated for 2 h at 4°C in the 96-well plastic tissue culture plate from Vangard International, Inc., Neptune, New Jersey. After incubation the cell suspensions were examined by phase-contrast microscopy at 128× magnification. A serological scale of 0 to ++++ was used to estimate the degree of clumping. Lectin was omitted in the control sample. For inhibition of agglutination, 100 µl of the solution contained decreasing, twofold dilutions of inhibitor and lectins: *D. stramonium*, *E. europaea*, and *G. simplicifolia* I-B$_4$ at concentrations of 16, 16, and 50 µg/ml, respectively, were preincubated for 30 min at room temperature. The solutions were cooled at 4°C for 10 min and 25 µl of PEC or PC suspension was added and mixed and agglutination was assayed.

RESULTS

Binding of Lectins to PEC

Figures 1A to 3A show that the specific binding of lectins to PEC increased as increasing amounts of ^{125}I-labeled lectins were incubated with a constant number of
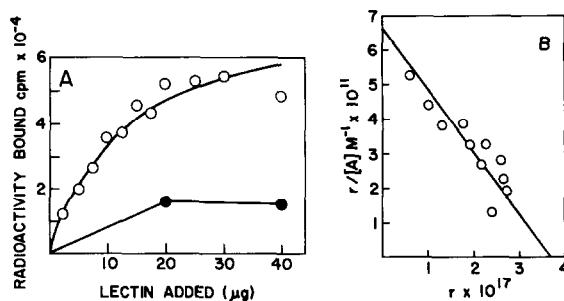
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FIG. 1. Panel A: Specific bindings of ^{125}I-*Datura stramonium* lectin to (○) PEC and (●) PC as a function of ^{125}I-*D. stramonium* lectin concentration. Each point is the mean of triplicate determinations from one experiment. Points are experimental and the curve is fitted as described under Materials and Methods. SD for each point did not exceed ±8% of the predicted value. Murine PEC (1.19×10^5) and murine PC (1.0×10^5) were taken for binding measurements. Cells were pooled from two or three animals. Panel B: Scatchard plot of the fitted curve from panel A; r, moles of lectin bound per cell; [A], molar concentration of free lectin. Calculations are based on M_r 86,000 for *D. stramonium* lectin (25–27).

cells. Nonspecific binding, which represents ^{125}I-labeled lectin not displaced by haptenic sugar, was determined in the presence of lectin-specific inhibitors (not shown). This binding was subtracted from the total binding in the absence of specific inhibitors; the resulting specific binding was saturable for all three lectins; i.e., the binding asymptotically approached a maximum value as described by the hyperbolic function which is characteristic of ligand-receptor interactions.

The specific binding was analyzed by Scatchard (18) plots (Figs. 1B–3B). The absence of curvature in the plots suggests that the carbohydrate binding sites of all three lectins are noninteracting and homogeneous. All three curves extend beyond the inflection point (half total binding) in the semilogarithmic plot (not shown) as described by Klotz (19); the data therefore provide reliable parameters for the binding capacity for these three lectins.

Free energy binding of lectins ($\Delta G°$) at 4° (pH 7.1) to PEC was calculated from the association constants and are shown in Table I. Among the three lectins studied, the highest $\Delta G°$ was found for the *E. europaea* lectin (−10.1 kcal) and the lowest (−7.9 kcal) for the *D. stramonium* lectin.

Binding of ^{125}I-Datura stramonium Lectin to PC and PEC

Specific binding of the ^{125}I-*D. stramonium* lectin to PC and PEC is shown in Fig.

1A. The nonspecific binding of lectin to cells was determined in the presence of 10 mM *N*-acetyllactosamine and represented ≤23% of total binding to PEC (not shown). The average dissociation constant (K_d) was determined to be $5.65 \pm 1.08 \times 10^{-7}$ M and the number of binding sites per cell, assuming a single lectin molecule binds to each receptor, was calculated to be $22.3 \pm 1.9 \times 10^6$ per cell (Table II). The number of receptor sites per cell ranged from 22.3×10^6 to 50.0×10^6 for different cell samples, a 2.2-fold variation. The PEC had more receptor sites for this lectin than any of the three lectins studied.

Binding of *D. stramonium* lectin to resident (PC) was only 15 to 33% of that determined for the stimulated cells (PEC) as seen when comparing the highest and lowest numbers of receptor sites on PEC for *D. stramonium* lectin (Fig. 1A, Table II). Thus, stimulation of PC with thioglycollate resulted in approximately a 3- to 7-fold in-

TABLE I

BINDING OF LECTINS TO MURINE PEC: ASSOCIATION CONSTANTS K_a AND $\Delta G°$, AT 4°C AND pH 7.1

Lectin	K_a (M^{-1})	$\Delta G°$ (kcal)
Datura stramonium	1.8×10^6	−7.9
Evonymus europaea	9.2×10^7	−10.1
Griffonia simplicifolia I-B$_4$	7.5×10^6	−8.7

TABLE II

RECEPTOR DENSITY PER STIMULATED CELL FOR *Datura stramonium, Evonymus europaea*, AND
Griffonia simplicifolia I-B₄ LECTINS AS OBSERVED ON DIFFERENT CELL SAMPLES

PEC sample number	Number of receptors per cell		
	D. stramonium	*E. europaea*	*G. simplicifolia* I-B₄
1[a]	$22.3 \pm 1.9 \times 10^6$	$4.8 \pm 0.2 \times 10^6$	$16.8 \pm 0.8 \times 10^6$
2[b]	$50.0 \pm 0.3 \times 10^6$	$3.9 \pm 0.1 \times 10^6$	$4.0 \pm 0.3 \times 10^6$
3[b]	$41.4 \pm 0.4 \times 10^6$	$3.8 \pm 0.1 \times 10^6$	$2.0 \pm 0.2 \times 10^6$
4[b]	nd[c]	$3.9 \pm 0.4 \times 10^6$	nd

[a] Data derived from a Scatchard plot of the complete curve.

[b] The number of receptor sites was calculated from a series of two to three points on the plateau (saturation region) of the incomplete curve and from the affinity of lectins (determined in completed, fitted curves). Thus it was possible to use, for new data, the same nonlinear regression method with reweighted least squares, as described under Materials and Methods.

[c] Not determined.

crease in the expression of the *D. stramonium* lectin's receptors on the cell surface.

Binding of ^{125}I-Evonymus europaea Lectin to PC and PEC

Specific binding of ^{125}I-*E. europaea* lectin to PC and PEC is shown in Fig. 2A. The nonspecific binding of lectin to cells was determined in the presence of 15 mM lactose and represented ≤32% of total binding to PEC (not shown). The average dissociation constant (K_d) was determined to be

$1.08 \pm 0.12 \times 10^{-8}$ M and the mean number of binding sites, assuming a single lectin molecule binds to each receptor, was calculated to be $4.8 \pm 0.2 \times 10^6$/cell. The number of *Evonymus* lectin receptors on PEC showed little variation among individual cell samples compared to the variation in numbers of receptor sites for the *D. stramonium* and *G. simplicifolia* lectins (Table II).

Resident PC specifically bound small, but clearly detectable, amounts of the ^{125}I-*E. europaea* lectin. This represented only 5 to
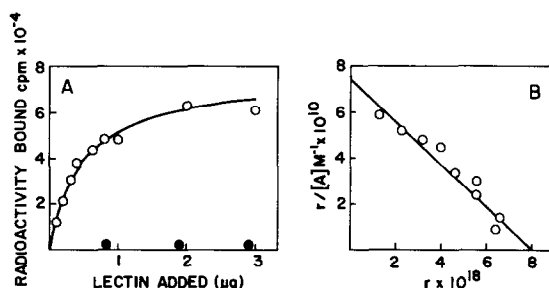

FIG. 2. Panel A: Specific binding of ^{125}I-*Evonymus europaea* to (O) PEC and (●) PC as a function of ^{125}I-*E. europaea* lectin concentration. Each point is the mean of triplicate determinations from one experiment. Points are experimental and the curve is fitted as described under Materials and Methods. SD for each point did not exceed ±6% of the predicted value. Murine PEC (5.0×10^4) and murine PC (5.0×10^4) were taken for binding measurements. Cells were pooled from two to three animals. Panel B: is the Scatchard plot of the fitted curve from panel A. Symbols are the same as in Fig. 1A. Calculations are based on M_r 166,000 for *E. europaea* lectin (3).

6% of the specific binding of lectin to the inflammatory PEC. Thus, stimulation of resident PC resulted in a 16- to 20-fold increase in the number of cell surface receptors for this lectin.

Binding of ^{125}I-Griffonia simplicifolia I-B$_4$ Isolectin to PC and PEC

Specific binding of ^{125}I-*G. simplicifolia* I-B$_4$ to PC and PEC is shown in Fig. 3A. The nonspecific binding was determined in the presence of 10 mM methyl α-D-galactoside and represented $\leqslant$7% of total binding (not shown). We determined that the *G. simplicifolia* I-B$_4$ binds specifically to intact PEC with relatively high affinity (K_d 1.33 $\pm$ 0.15 $\times$ 10^{-7} M) and the mean number of binding sites was calculated to be 16.8 $\pm$ 0.8 $\times$ 10^6 per cell (Table II). The variation in the number of receptor sites from individual cell samples (Table II) was approximately 8-fold.

Specific binding of *G. simplicifolia* lectin to the resident PC ranged from 5 to 42% of the specific binding of the lectin to inflammatory PEC. Thus, as seen with the other lectins, *in vivo* stimulation of PC with thioglycollate resulted in a significant increase of *G. simplicifolia* receptors (2.4- to 20-fold) on the cell surface.

Lectin Binding to Tryspin-Treated Inflammatory PEC

Lectin binding was carried out on PEC digested with 2.5 and 25.0 μg of trypsin (Figs. 4A–C). The results indicate that receptors for all three lectins appear to be trypsin resistant. Results of binding studies showed that the capacity of each lectin to bind to trypsin-treated PEC was independent of the enzyme concentration used for digestion. This 10-fold increase in enzyme concentration was used to overcome possible resistance of receptor to enzyme digestion. However, the latter amount of enzyme caused extensive damage of cells, as observed by significant cell clumping and up to 47% loss in cell number.

Lectin Agglutination of PC and PEC

As we have previously shown, *E. europaea* lectin caused agglutination of PEC but not of PC (4). In comparison, it is evident from Table III that *D. stramonium* and *G. simplicifolia* I-B$_4$ lectins also agglutinate PEC. PC were not agglutinated by *G simplicifolia* I-B$_4$ at concentrations up to 200 μg/ml. One sample of PC was agglutinated by a 16-fold higher *Datura* lectin concentration than required for agglutination of PEC, and one sample was not ag-
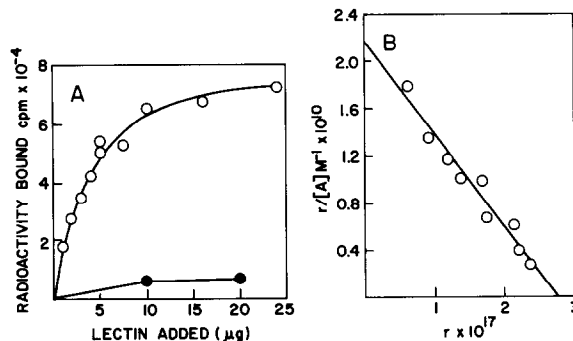

FIG. 3. Panel A: Specific binding of ^{125}I-*Griffonia simplicifolia* I-B$_4$ isolectin to ($\bigcirc$) PEC and ($\bullet$) PC as a function of ^{125}I-*G. simplicifolia* I-B$_4$ lectin concentration. Each point is the mean of triplicate determinations from one experiment. Points are experimental and the curve is fitted as described under Materials and Methods. SD for each point did not exceed $\pm$5% of the predicted value. Murine PEC (1.0 $\times$ 10^6) and murine PC (2.0 $\times$ 10^5) were taken for binding measurements. Cells were pooled from two or three animals. Panel B: Scatchard plot of the fitted curve from Panel A. Symbols are the same as in Fig. 1A. Calculations are based on M_r 114,000 for *G. simplicifolia* I-B$_4$ isolectin (28).
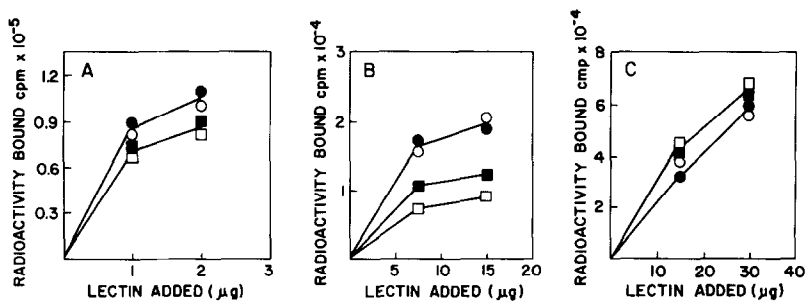
FIG. 4. Specific binding of ^{125}I-labeled lectins to (□, ○) untreated PEC from two different cell samples and to trypsin-treated PEC with 2.5 μg (■) and (●) 25.0 μg of enzyme. Panel A: *Evonymus europaea.* Panel B: *Griffonia simplicifolia* I-B₄. Panel C: *Datura stramonium.*

glutinated at the same lectin concentration. Agglutination of PEC was inhibited completely by *D. stramonium-* and *G. simplicifolia* I-B₄-specific inhibitors, 7 mM N-acetyllactosamine and ≤2.6 mM methyl α-D-galactoside, respectively.

These agglutination results on PEC and the lack or poor agglutination of PC by these three lectins are in agreement with the results of quantitative, binding determinations. *G. simplicifolia* IV lectin, which is blood group Le^b specific (13), did not agglutinate either PEC or PC at a concentration of 348 μg/ml.

DISCUSSION

In this study we have demonstrated a quantitative difference between resident and thioglycollate-stimulated murine peritoneal cells in the expression of carbohydrates on their cell surface as reflected by the binding of lectins from *D. stramonium, E. europaea,* and *G. simplicifolia.* These results are in agreement with previous observations (1, 4). The physicochemical parameters of binding, namely, the dissociation constant (K_d) and the average number of receptors per stimulated cell, have also been determined.

These studies were carried out on whole peritoneal cell populations which consist predominantly of macrophages. The reason for this is that so-called "purified" macrophages actually represent a heterogenous cell population as suggested from previous observations, at least with respect to the presence of carbohydrate receptors

on their surface (1, 20). Until suitable techniques for separations of these cell subpopulations can be worked out, the difference between a whole population of stimulated cells and a designated subpopulation cannot be evaluated.

The number of receptor sites determined in this study for each lectin is tentative

TABLE III

AGGLUTINATION OF RESIDENT AND THIOGLYCOLLATE-STIMULATED MURINE PERITONEAL CELLS BY LECTINS

Lectin	Peritoneal cells	Lectin concentration (μg/ml)[a]
Datura stramonium	Resident	>32
		32
	Stimulated	2
		4
		2
Evonymus europaea	Resident	>32
		>32
	Stimulated	2
		4
Griffonia simplicifolia I-B₄	Resident	>200
		>200
	Stimulated	50
		12
Griffonia simplicifolia IV	Resident	>348
	Stimulated	>348

[a] Each number represents the result from one experiment and shows the minimal lectin concentration in the reaction mixture required for + agglutination.

and can be corrected after purification of a given cell population. This represents an average number of receptors per cell. However, one has to keep in mind that lectin receptors are not uniformly distributed among particular cells; in other words, the density of receptors varies from cell to cell as was shown by de Water et al. for wheat germ agglutinin receptors on mouse peritoneal macrophages (20). The value determined for the number of receptor sites also represents the least number of receptors based on the assumption that one lectin molecule binds to one receptor; this disregards the valence of the lectins, e.g., two and four carbohydrate binding sites/molecule, respectively, for the D. stramonium and G. simplicifolia I-B$_4$ lectins. The valence of E. europaea lectin has not yet been determined.

The number of receptors per cell varied from one cell sample to another. The least variance in the number of receptor sites per cell was shown for the Evonymus lectin receptor; the greatest variance was shown for the Griffonia I-B$_4$ isolectin. This variation could result from a number of factors, e.g., metabolic state or growth phase of the cell population. However, in all cases, the number of lectin receptors increased upon in vivo stimulation of the cells.

Questions which arise regarding the origin of the several- to tenfold increase in the number of carbohydrate receptor sites include: (a) Is there an increase in a subpopulation of preexisting peritoneal cells without a change in the number of receptor sites per cell? (b) Is there an increase in the number of receptor sites per cell? (c) Is there an increase in both a and b? (d) Is there a de novo synthesis of a qualitatively different species of glycoconjugate?

E. europaea and G. simplicifolia lectins both can react with carbohydrate determinants containing terminal α-D-galactosyl residues (2, 3). However, the specificity of the E. europaea lectin is very complex: it can react with both fucosylated (3) and nonfucosylated determinants (unpublished data, manuscript in preparation). An important question regards whether both lectins bind to the same, or to different, carbohydrate determinants on the PEC

surface, and whether or not the Evonymus lectin receptor is fucosylated. Studies of Handa et al. (21) showed the presence of blood group B-active glycolipids in rat peritoneal macrophages. These glycolipids, if present in mouse macrophage membranes, might be responsible for the binding of the Griffonia and Evonymus lectins to the PEC.

Receptors for all three lectins were resistant to trypsin digestion. Incubation of cells with high concentrations of trypsin resulted in extensive damage and loss of cells. The fact that lectin binding following trypsin treatment was unchanged suggests that the receptors might be glycolipids and/or trypsin-resistant glycoproteins. This conclusion agrees with previous observations (4) which also showed that Evonymus lectin receptors were trypsin and pronase resistant. However, Maddox et al. (1) reported the sensitivity of the Griffonia lectin receptor to trypsin preparations. This is contrary to our present results. The trypsin preparations used by Maddox et al. (1) did not contain TPCK. This suggests that G. simplicifolia lectin receptor could be digested by contaminating proteolytic enzymes and not by trypsin itself. In support of this conclusion is our observation that the Griffonia lectin receptor was gradually destroyed during cell storage and this loss could be prevented by the addition of the proteolytic enzyme inhibitor PMSF. The binding capacity of cells stored at 4°C in PBS containing 1 mM PMSF for the G. simplicifolia B$_4$ isolectin was unchanged during 12 days. The receptor for D. stramonium lectin was very sensitive to storage, even in the presence of 1 mM PMSF. Overnight storage caused 35% loss of PEC binding capacity. In a previous report, the increased agglutinability of PEC by Evonymus lectin was also observed after trypsin and Pronase digestion (4). Inasmuch as we found that trypsin digestion did not change the lectin binding capacity of PEC one can assume that this increase in agglutinability is probably due to an increase in affinity.

One can also ask whether binding of lectin is a process restricted to the cell surface or whether it is associated with endocytosis

and/or pinocytosis? The following observations exclude the endo- and pinocytosis of lectins: (a) lectin binding experiments were conducted at 4°C which abolishes both endo- and pinocytosis (22); (b) PEC agglutinated by *Evonymus* lectin could be dissociated after 2 h into single-cell suspensions by addition of specific sugar inhibitor (4). That the binding of the three lectins employed in this study, to PEC, is a cell surface-mediated phenomenon is indicated by the fact that lectin binding is abolished by specific haptenic sugars. Furthermore, digestion of PEC with coffee bean α-galactosidase both abolished the binding of the *Griffonia* lectin and changed the affinity of the *Evonymus* lectin to PEC (unpublished data; manuscript in preparation).

Mercurio *et al.* (23) reported the appearance of a GM_1 ganglioside species on thioglycollate- and BCG-activated macrophages, a glycolipid which was absent on resident ones. However, until its structure is determined, one cannot determine whether or not it is responsible for binding to three of the lectins we have studied.

G. simplicifolia lectin is highly specific for α-D-galactosyl residues (1). From these results as well as from the previous observation that α-galactosidase-treated PEC lost their ability to bind the lectin (1) we conclude that activation of PC by thioglycollate is accompanied by an increse in the number of α-D-galactosyl residues on the cell surface. The binding of the *G. simplicifolia* I-B_4 isolectin to other cells (e.g., human type B erythrocytes) has been studied. The dissociation constant determined in the present studies is close to those reported by Hayes and Goldstein (24). However, the number of receptor sites per cell on PEC is one to two orders of magnitude greater than on erythrocytes.

The minimal determinant for high-affinity binding to *D. stramonium* lectin is *N*-acetyllactosamine (9); therefore one can assume the presence of *N*-acetyllactosamine units (single or repeating, linear or branched) in the PEC oligosaccharides.

E. europaea lectin has a very complex carbohydrate binding specificity in that it can bind both fucosylated blood group

B and H, tetra- and trisaccharides, respectively (3), as well as the nonfucosylated trisaccharide DGalα1,3DGalβ1,4D-GlcNAc (unpublished data, manuscript in preparation). The *Evonymus* lectin shows decreasing affinity for the following structures: DGalα1,3[LFucα1,2]DGalβ1,3(or 4)DGlcNAc > DGalα1,3[LFucα1,2]-DGal > LFucα1,2DGalβ1,3(or 4)DGlcNAc = DGalα1,3DGalβ1,4DGlcNAc. Because the blood group H type 2 determinant (LFucα1,2DGalβ1,4DGlcNAc) on PEC is unlikely (4), other structures should be considered as possible receptors for the *Evonymus* lectin.

The carbohydrate receptors described in this communication provide new chemical markers for the various stages of PC differentiation. In order to determine the possible biological significance, if any, of the carbohydrate determinants detected by these three lectins it is of importance to determine their structure(s). These studies are in progress.

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