Studies on Cell Adhesion and Recognition
III. The Occurrence of α-Mannosidase at the Fibroblast Cell Surface, and Its Possible Role in Cell Recognition

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ABSTRACT The occurrence of α-mannosidase activity at the surface of hamster embryo (NIL) fibroblasts is indicated by the following findings: (*a*) When NIL cells were incubated on the glass surfaces on which ovalbumin glycopeptides were covalently linked, a rapid release of free mannose from ovalbumin glycopeptides was observed as evidenced by analysis on gas chromatography/mass spectrometry. (*b*) Cell suspensions as well as intact cell monolayers hydrolyzed rapidly *p*-nitrophenyl-α-D-mannoside, and the time-course of the hydrolytic cleavage was linear from the moment of mixing of the substrate with the cells. The hydrolysis of the nitrophenyl glycosides of β-D-mannose, α-D-galactose, β-D-galactose, α-L-fucose, β-D-glucose, β-D-*N*-acetylgalactosamine and β-D-*N*-acetylglucosamine was negligible or more than ten times lower as compared with the hydrolysis of α-D-mannoside. (*c*) No released or secreted activity of mannosidase could be detected under the conditions used. (*d*) Studies using known proportions of broken cells in the incubation mixture indicated that >90% of the mannosidase activity measured was attributable to intact cells and not to broken cells or cell fragments. (*e*) Hydrolysis of *p*-nitrophenyl-α-D-mannoside by cell monolayers was inhibited, in the order of decreasing inhibitory activity, by yeast mannan, ovalbumin, α-1,4-L-mannonolactone, α-methylmannoside, and mannose-6-phosphate. High inhibitory activity of the mannan polysaccharide and of ovalbumin favored the presence of the mannosidase activity at the cell surface, as these substrates may not penetrate rapidly into the cells.

The following findings indicated that the cell surface mannosidase is mediating the cell adhesion based on the recognition of high-mannose-type glycopeptide: (*a*) Ovalbumin-coated plastic surfaces strongly promoted attachment and spreading of NIL fibroblasts, whereas the same ovalbumin coat did not promote attachment and spreading of some other cell types (BALB/c 3T3 fibroblasts and freshly prepared rat liver cells). (*b*) Digestion of ovalbumin with α-mannosidase greatly reduced the adhesion-mediating activity. (*c*) Cell adhesion to ovalbumin-coated surfaces was strongly inhibited by mannose tetrasaccharides, moderately by α-1,4-L-mannonolactone, and weakly by α-methylmannoside and mannose-6-phosphate. This order of the inhibitory activity for cell attachment is the same as that for the inhibition of mannosidic hydrolysis. The interpretation that the cell surface mannosidase is able to mediate cell adhesion is in agreement with previous studies suggesting that polyvalent glycosidase surfaces can promote cell adhesion to a degree similar to that caused by fibronectin and several lectins by interacting with their cell surface substrate sites (the accompanying papers of this series).

In a model study, glycosidases coated on plastic surfaces can function as a polyvalent enzyme surface that promotes cell adhesion because of a specific interaction between the enzyme surface and the cell surface substrates (see the preceding papers

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of this series [6, 23]). The intensity and the specificity of this type of cell adhesion reaction is comparable to that caused by fibronectin and lectin surfaces (23). Therefore, cell surface glycosidase activities should be taken into consideration as well as the cell surface lectin activities as the basis for recognition and adhesion phenomena between cells and those between cells and substrata. In fact, the polyvalent glycosidase surfaces may well be equivalent to the lectin surfaces, from the viewpoint of cellular interactions.

In the present communication, evidence is presented that an α-mannosidase activity occurs at the surfaces of hamster embryo (NIL) fibroblasts and possibly of baby hamster kidney (BHK) cells, but may be low or absent at the surfaces of BALB/c 3T3 cells. Furthermore, we suggest that the cell surface mannosidase contributes to the adhesion of NIL cells on ovalbumin-coated surfaces.

MATERIALS AND METHODS

Materials

Ovalbumin (grade V), fetuin (type III), crystalline bovine serum albumin, concanavalin A, and *Clostridium perfringens* neuraminidase (type IX, affinity purified) were purchased from Sigma Chemical Co. (St. Louis, Mo.). The sample of ovalbumin used was >95% pure on polyacrylamide gel electrophoresis. Fibronectin was purified from conditioned medium of NIL cells by gelatin affinity chromatography (8), and was >95% pure when estimated on polyacrylamide gel electrophoresis after protein staining. Mannosidase-digested ovalbumin was prepared essentially according to the method of Shepherd and Montgomery (30). Ovalbumin (5 mg) was dissolved in a buffer containing 10 mM NaCl, 50 mM Na acetate, 0.1 mM Zn acetate, pH 4.5 (in the total volume of 1 ml). To this were added 3 U of jack bean meal α-mannosidase (Sigma Chemical Co.; 20 U/mg protein), and the mixture was incubated at 37°C for 30 h, after which 3 U of the enzyme were again added, and the incubation was continued for another 30 h. The digested ovalbumin was applied on a column of Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden), which was eluted with Salt/Pi,[1] pH 7.4. Determination of enzyme activity (using *p*-nitrophenyl-α-D-mannoside in the hydrolysis buffer given above) and of protein (A_{280}) revealed that the mannosidase (~200,000 mol wt) was excluded from the column, clearly before elution of ovalbumin. Monosaccharide analysis of the digested ovalbumin revealed that 26% of mannose residues had been cleaved in the treatment. Polyacrylamide gel electrophoresis did not suggest degradation of the protein.

Ovalbumin and concanavalin A were iodinated, using the chloramine-T method (10). The iodinated proteins were dialyzed extensively at 4°C against Salt/Pi/water (1/1, vol/vol) and finally against Salt/Pi. The labeled proteins comigrated on polyacrylamide gel electrophoresis with the nonlabeled proteins, without signs of degradation.

Ovalbumin glycopeptides were prepared by pronase digestion of ovalbumin (1), and they were purified by gel filtration on a column of Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, Calif.). Mannose tetrasaccharides, Manα1 → 2Manα1 → 2Manα1 → 2Man and Manα1 → 3Manα1 → 2Manα1 → 2Man (all monosaccharide residues are D-pyranoses), were gifts from Dr. Shigeo Suzuki (Tohoku College of Pharmaceutical Sciences, Sendai, Japan). Yeast mannan (from baker's yeast), α-methyl-D-mannoside (grade III) and D-mannose-6-phosphate (grade I) were purchased from Sigma Chemical Co. and α-1,4-L-mannonolactone from Pfanstiehl Lab., Inc. (Waukegan, Ill.). "The most inhibitory solution" of mannosidase (aldonate-lactones) was prepared from α-1,4-L-mannonolactone after Levvy et al. (19). β-methyl-D-mannoside (isopropylate) was kindly donated by Professor Irwin J. Goldstein (University of Michigan).

p-Nitrophenyl-α-D-mannoside was purchased from Sigma Chemical Co. and Calbiochem-Behring Corp. (American Hoechst Corp., LaJolla, Calif.). Both preparations gave the same rate of hydrolysis. *p*-Nitrophenyl glycosides of β-D-mannose, α-D-galactose, β-D-galactose, α-L-fucose, β-D-glucose, and β-D-*N*-acetylglucosamine were purchased from Sigma Chemical Co., and *p*-nitrophenyl-β-D-*N*-acetylgalactosamine was purchased from Pierce Chemical Co. (Rockford, Illinois).

Preparation of Ovalbumin Glycopeptide Surfaces by Covalent Linking to Glass

Aminopropyl glass surfaces (37; J. C. Venter, personal communication) were prepared from 9-cm-diameter glass plates. The plates were boiled in 5% nitric acid for 1 h, and dried at 80°C. 10% γ-Aminopropyltriethoxysilane (Pierce Chemical Co.) in water was added (pH was brought to 3.5 with nitric acid), and the plates were incubated at 80°C for 3 h, washed with water, and dried overnight at 80°C.

Ovalbumin glycopeptide, purified on a Bio-Gel P-10 column, was coupled to aminopropyl glass surfaces either directly or after *N*-acetylation. The glycopeptide was *N*-acetylated in acetic anhydride and sodium bicarbonate at room temperature for 20 min. This was performed to block the free amino groups and to prevent the reaction of the glycopeptide carboxyl group to its own amino groups rather than to the aminopropyl glass.

The underivatized glycopeptides (1 mg hexose) or *N*-acetylated glycopeptides were dissolved in 10 ml water and added to a 9-cm-diameter aminopropyl glass plate that had been washed extensively with water. Coupling to the plate was achieved by adding 200 mg 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide HCl (Sigma Chemical Co.). Incubation was continued at room temperature for 40 h at pH 6. The degree of coupling to the glass was determined by sugar analysis after hydrolysis of the glass complex in 1 N HCl at 80°C for 5 h.

Cells and Cell Culture

NIL fibroblasts, BHK cells, and mouse 3T3 fibroblasts were cultured in Dulbecco's Modified Eagle's Medium supplemented with 5% fetal calf serum, 100 U penicillin G/ml and 0.1 mg streptomycin/ml in an atmosphere of 5% CO_2. Wistar rat liver cells were prepared according to the collagenase perfusion technique (28) and cultured, using Leibovitz's medium (18; L-15 medium, Grand Island Biological Co., Grand Island, N. Y.)

Enzyme Assays

Cell suspensions for glycosidase assays were prepared by dispersing confluent cell monolayers with 10 μg/ml crystalline trypsin (Worthington Biochemical Corp., Freehold, N. J.) in Ca- and Mg-free Salt/Pi for 20 min at 37°C (23). Trypsinization was stopped by adding an equal volume of Ca/Mg-free Salt/Pi containing 40 μg/ml soybean trypsin inhibitor (Sigma Chemical Co.). The cells were centrifuged and washed two more times in the solution containing soybean trypsin inhibitor (23). The cell suspensions were thereafter brought to pH 6.0 by washing two times with a phosphate-buffered salt solution having the following composition: 50 mM NaCl, 55 mM Na_2HPO_4, 10 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, titrated to pH 6.0 with 1 M HCl. Taking into account the higher osmotic pressure caused by the disodium phosphate as compared with sodium chloride (42), the osmotic pressure of this solution was equivalent to 130–140 mM NaCl. This solution showed a better buffering action than an ordinary Salt/Pi and was suitable for hydrolysis experiments.[2] Trial experiments indicated that after two washes of cell suspensions of pH of the supernate remained 6.0. When a trypsinized cell suspension was washed from this buffer and incubated at pH 6.0 for 40 min, 92% of cells excluded trypan blue, and no detachment of cells from confluent monolayers occurred in an incubation for 2 h at 37°C.

In hydrolysis experiments of nitrophenyl sugars by cell suspensions, the final concentration of cells was 2.7×10^6/ml and that of the substrate was 8 mM, in the total volume of 0.6 ml in the phosphate buffer, pH 6.0. After incubation at 37°C for the time period indicated, the cells were centrifuged, the supernate was transferred to 2.0 ml of 0.2 M Na_2CO_3, and the absorbance at 400 nm was measured. The absorbance given by the sugar solution incubated in the buffer only was subtracted from the measured value. Cells incubated in the buffer without added sugar did not cause any increase in the absorbance.

Hydrolysis of nitrophenyl sugars was also studied by use of cell monolayers. Cells were cultured on 5-cm-diameter plates for 4–7 d, until they were confluent. The cell monolayers were washed three times with 3 ml of the phosphate buffer, pH 6.0 (see above), and the substrate solution at 5 or 8 mM concentration, as indicated, was applied in 1.5 ml of the phosphate buffer. Incubation was continued for 20 minutes at 37°C, after which 1 ml of the medium was transferred to 2.0 ml of 0.2 M Na_2CO_3, and the absorbance increase at 400 nm was read. Because yeast mannan and ovalbumin absorb at 400 nm, appropriate blanks

[1] *Abbreviations used in this paper:* Salt/Pi, 137 mM NaCl/2.7 mM KCl/0.7 mM $CaCl_2$/0.5 mM $MgCl_2$/8.1 mM Na_2HPO_4/1.5 mM KH_2PO_4.

[2] Tris-HCl-buffered saline gave a low rate of hydrolysis, probably because inhibition of mannosidase by Tris-HCl, as was described previously (19). HEPES-buffered saline gave approximately the same rate of hydrolysis, but was inconvenient to use because of lower buffering effect at acidic pH values.

were used in the experiments of hydrolysis inhibition by these glycoproteins. Both cell suspensions and cell monolayers incubated in buffer alone did not show any measurable absorbance at 400 nm.

In hydrolysis experiments using ovalbumin glycopeptides covalently linked to glass (see above), the cell suspensions were prepared by trypsinization as for other assays, and buffered with the phosphate salt solution, pH 6.0. The cell suspensions, 5×10^6 cells/ml, were applied in 5 ml of the phosphate-buffered salt solution, pH 6.0, on 9-cm-diameter plates, to which glycopeptides had been linked or which had been treated with the reagents only. The cell suspensions were incubated on the plates for 20 min at 37°C, during which time the cells began to attach to both the glycopeptide surfaces and the blank plates (which contain amino groups having a cell attachment activity). The supernates were removed and passed through 4 ml of mixed bed resins (AG 501; Bio-Rad Laboratories). The columns were washed three times with 4 ml of methanol/water 9/1 (vol/vol). The filtrate and the eluates were combined, taken to dryness, and analyzed by gas chromatography and mass spectrometry after trimethylsilylation.

Adhesion Assays

Adhesion surfaces were prepared by adsorbing different proteins on microtiter well surfaces, devised for high-efficiency protein binding (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, Va.). Different proteins were adsorbed at concentrations of 3–50 μg/ml in 50 μl of Salt/Pi buffer, pH 7.4, for 2 h at room temperature. The wells were washed three times with 100 μl of Salt/Pi buffer and studied for adhesion-promoting activity. Adsorption characteristics of different iodine-labeled proteins (including fetuin and fibronectin) in these conditions have been studied previously (23). Ovalbumin was found to form a stable adsorbed layer on plastic; i.e., the activity of iodinated ovalbumin found adsorbed after a 2-h incubation followed by three washings (375 cpm/well) was essentially unchanged (359 cpm/well) even after being incubated in Salt/Pi for 0.5 h at room temperature, followed by washing three times with Salt/Pi.

Freshly confluent cell monolayers were trypsinized and washed from trypsin and soybean inhibitor solution as described under Enzyme Assays. The effects of this procedure on cell surface glycoproteins have been studied previously (23). The cells were suspended in Salt/Pi buffer, ph 7.4, and washed two more times. Adhesion reactions were started by adding 7.0×10^4 cells in 50 μl of Salt/Pi to 50 μl of the same buffer in microtiter wells. Two to four wells were prepared for each data point shown. As the aqueous solution of mannonolactone tends to be acidic because of the liberation of mannonic acid, 30 mM HEPES (Ultrol-grade; Calbiochem-Behring Corp.) was used to buffer the adhesion medium to pH 7.4 in some assays. The results were, however, the same, when either Salt/Pi or the HEPES-buffered Salt/Pi was used. The cell suspensions in buffered salt solutions were incubated for 1 h at 37°C (if not otherwise indicated), the medium was removed, and the nonattached cells were washed off by rinsing the wells three times with 100 μl of Salt/Pi, using a multiwell pipette (Titertek; Finnpipette, Helsinki, Finland). In inhibition studies with sugars, 200-μl proportions of cell suspension (at two times the cell density finally added to microtiter wells) were added to 200 μl of the buffered salt solution containing the sugar inhibitor at the concentration indicated. Samples (50 μl) of cell suspensions were transferred after 15 min at room temperature to microtiter wells containing the same concentration of the inhibitor (in 50-μl volume). The results were more constant when the assays were carried out in the way described above, compared with determinations in which the cells were directly added to microtiter wells containing the sugar solution, without allowing the inhibitors to bind to cells before starting the assay. All determinations were carried out in duplicate or triplicate. After the adhesion reaction, each well was estimated by microscopy for the extent of cell attachment and cell spreading. For quantification of the numbers of cells attached, the cells were labeled before the assays either with 1 μCi/ml [^{3}H]thymidine or 2 μCi/ml [^{3}H]proline (New England Nuclear, Boston, Mass.) in complete culture medium for 20 h. Radioactivity from the attached cells was solubilized for counting by rinsing the microtiter wells two times with 100 μl of 1% SDS in 0.5 N NaOH. Adhesion assays with liver cells were carried out in the way described above for fibroblasts, except that Leibovitz's medium was used instead of Salt/Pi, and the reaction time was 2 h. Under these conditions, an intense reaction was observed on fibronectin, lectin, and glycosidase surfaces (23).

In kinetics studies, [^{3}H]proline-labeled NIL cells from the same suspension were added simultaneously with the multiwell pipette to three different types of surfaces on the same multiwell plate, surfaces coated with concanavalin A, with ovalbumin, and with the buffer only (plastic surfaces). After the time intervals indicated, the medium was removed and the wells were rinsed two times with 100 μl of Salt/Pi, using the multiwell pipette. The wells were estimated by microscopy for cell attachment, and the numbers of cells attached were quantified from radioactivity as described above. Each time-point on each type of surface was analyzed in duplicate.

Analytical Methods

Hexose was determined by use of the orcinol reaction (21). Free hexoses were analyzed after trimethylsilylation (Tri-Sil; Pierce Chemical Co.) of the dried samples by gas chromatography (34) on a 3% SE-30 column (Supelco, Inc., Bellefonte, Pa.), using a temperature program from 160°–230°C at the rate of 4°C/min. Monosaccharide analysis of glycoproteins was performed after methanolysis and trimethylsilylation on the SE-30 column, using inositol as the internal standard (4). Mass spectra from the gas chromatography peaks were recorded, using a Finnigan 3300 gas chromatographer/mass spectrometer combined to a Finnigan 6110 data system (Finnigan Corp., Sunnyvale, Calif.).

Gel electrophoresis was carried out by use of 8% polyacrylamide slab gels containing 0.1% SDS according to the basic stacking gel procedure of Laemmli (17). Before analysis, the samples were heated in a boiling water bath for 5 min in the sample buffer containing 2% SDS and 5% 2-mercaptoethanol. Proteins were detected from radioactivity of the iodinated samples and from protein staining with Coomassie Blue R-250 (9). Protein standards for relative molecular weight estimation were hamster skeletal muscle myosin (200,000), bovine serum albumin (68,000), hamster skeletal muscle actin (45,000), and *Dolichos biflorus* lectin subunit (27,000).

RESULTS

Mannosidase of NIL Fibroblasts

HYDROLYSIS OF P-NITROPHENYL GLYCOSIDES BY CELL SUSPENSIONS AND BY CELL MONOLAYERS: The time-course of hydrolytic cleavage of *p*-nitrophenyl-α-D-mannoside by a trypsinized cell suspension is shown in Fig. 1. The specificity of the hydrolytic cleavage with respect to the sugar moiety was suggested by slow liberation of *p*-nitrophenol from *p*-nitrophenyl-β-D-galactoside (Fig. 1). Similar results were obtained when intact cell monolayers were used; *p*-nitrophenyl-α-D-mannoside was hydrolyzed much more rapidly than the *p*-nitrophenyl glycosides of β-D-mannose, α-D-galactose, β-D-galactose, α-L-fucose, β-D-glucose, β-D-*N*-acetylgalactosamine or β-D-*N*-acetylglucosamine. The rate of hydrolysis with these sugar substrates was similar when the analysis was performed at either pH 5.0 or 6.0. As shown in Table I, only the activity of α-mannosidase was significant for NIL cells under these conditions.

The rapid onset of the cleavage and the linearity of the hydrolysis with respect to time from the moment of mixing the substrate with the cells (Fig. 1) suggest the presence of an "outside" activity. This can be attributed to: (*a*) an activity released to the medium during the preparation of the cells, (*b*) an activity displayed by broken cells or cell fragments, or (*c*) an activity present at the surface of intact cells. Only the last

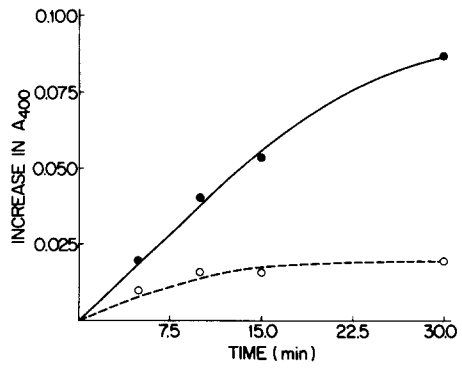

FIGURE 1 Time-course of the hydrolysis of nitrophenyl sugars (8 mM) by trypsinized NIL cell suspensions (2.7×10^6 cells/ml) in phosphate-buffered salt solution, pH 6.0, at 37°C (for more details, see Materials and Methods). ●, *p*-Nitrophenyl-α-D-mannoside; ○, *p*-nitrophenyl-β-D-galactoside.

TABLE I

Hydrolysis of Different p-Nitrophenyl Sugars by NIL Cells

Sugar	Increase in A_{400}	% Sugar released as compared with mannose hydrolysis
	nm	
α-D-Mannose	0.120	100
β-D-Mannose	0.011	9
α-D-Galactose	0.000	0
β-D-Galactose	0.009	8
α-L-Fucose	0.009	8
β-D-Glucose	0.000	0
β-D-*N*-Acetylgalactosamine	0.002	2
β-D-*N*-Acetylglucosamine	0.000	0

Different nitrophenyl sugar solutions (1.5 ml at 8 mM concentration) were incubated on 5-cm-diameter confluent NIL cell monolayers at pH 6.0 for 20 min at 37°C (for more details, see Materials and Methods). The reactions were stopped by adding a 1.0-ml sample of the nitrophenyl sugar solution to 2.0 ml of 0.2 M Na_2CO_3, and the increase in A_{400} was measured. The values given are averages from two determinations.

possibility (*c*) has been supported by various data, as will be described in paragraphs 4–7 of this section.

THE PH OPTIMUM OF THE MANNOSIDASE ACTIVITY: Dependency of the mannosidase activity of NIL cell monolayers on pH is shown in Fig. 2. The optimum pH was ~6; above that, the hydrolytic activity declined rapidly and was low or absent at pH 7.0 or 7.4. The activity curve as a function of pH was similar to that of the α-mannosidase purified recently by Tulsiani et al. (35) from rat liver Golgi membranes.

DOES THE MANNOSIDASE ACTIVITY DERIVE FROM SECRETION OR FROM BROKEN CELLS? As shown in Fig. 2, the hydrolysis of 5 mM *p*-nitrophenyl-α-D-mannoside by cell monolayers grown on 5-cm-diameter dishes causes an absorbance increase of ~0.10–0.15 at 400 nm in the method used. When the cell monolayers were incubated for 1 h at 37°C in the buffer alone, and the incubated buffer solution was tested for α-mannosidase activity in the same way as used for cell monolayers, no absorbance changes could be measured. Therefore, the enzyme must be associated with the cells; the results given above excluded the possibility that the α-mannosidase activity was secreted or released to the incubation medium.

Another possibility—that the mannosidase activity of NIL cells could be attributed to the presence of broken cells or cell fragments—was studied by use of the method recommended by Struck and Lennarz (33). Cells were broken by a Dounce homogenizer (Kontes Co., Vineland, N. J.) under microscope control, until no intact cells could be observed. This cell suspension ("100% broken cells") was mixed in various proportions with nontreated cells, keeping the total cell number constant, and the α-mannosidase activity was measured. A summary of these experiments is shown in Fig. 3. The important point of this result is the activity obtained by extrapolating to 0% broken cells (32). If an extrapolated activity becomes zero, it is not necessary to assume an outside activity in intact cells. As is evident from Fig. 3, no possibility exists to extrapolate the mannosidase activity to zero at 0% broken cells. Unexpectedly, the activity increased only slightly. A few possibilities can be considered as explanations for the only slight increase in the activity, when the cells are broken. For example, cells release competing substrates that would lower the measured activity; this type of effect was recently shown by Hoflack et al. (15) in the measurement of sialyltransferase activity of whole cells. Another possibility would be that the method used does not measure intracellular mannosidases, which, for example, could require lower pH values or the presence of detergent for the activity. It is also possible that cytosolic or Golgi enzymes are unstable when released into medium or solubilized by homogenization in the absence of detergent. Further study is necessary.

INHIBITION OF CELLULAR MANNOSIDASE ACTIVITY BY GLYCOPROTEINS AND BY MONOSACCHARIDES: Inhibition of hydrolysis of *p*-nitrophenyl-α-D-mannoside by ovalbumin and by yeast mannan is shown in Fig. 4*A*. The finding that the cellular mannosidase activity was inhibited by high molecular weight mannoside polymers, such as ovalbumin and yeast mannan, suggests that the hydrolytic activity is present at the cell surface. The inhibitory activity in the yeast mannan preparation used was shown to be nondialyzable, and is therefore attributable to the polysaccharide structure of the mannan. Also, incubation of a high concentration of iodinated ovalbumin (5 mg/ml) in the hydrolysis buffer, pH 6.0, for 0.5 h with

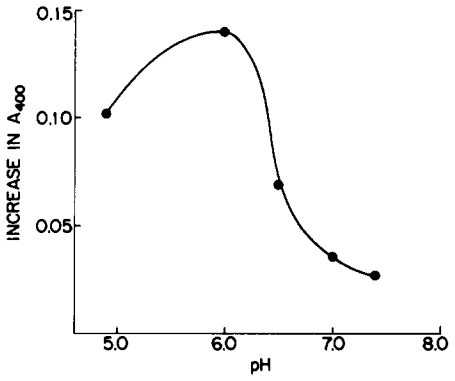

FIGURE 2 Dependency of the hydrolysis of *p*-nitrophenyl-α-D-mannoside on pH. A 5-mM solution of the sugar in 1.5 ml of phosphate-buffered salt solution was exposed to 5-cm-diameter intact NIL cell monolayers buffered to the same pH before the assay. After 20 min at 37°C, 1.0 ml of the solution was added to 2.0 ml of 0.2 M Na_2CO_3, and the absorbance increase relative to the sugar solution incubated in the buffer only was measured.

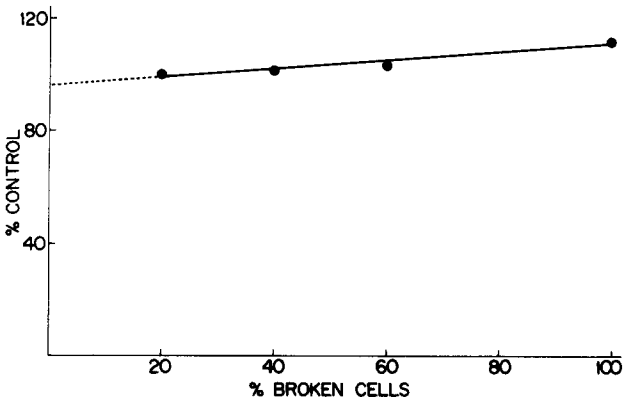

FIGURE 3 Effect of mechanically broken cells on the hydrolysis of *p*-nitrophenyl-α-D-mannoside as compared to the hydrolysis by trypsinized cell suspensions, into which no broken cells were added. Cells were broken with a Dounce homogenizer ("100% broken cells"), and were mixed in various proportions to the nontreated cell suspension ("cell number" is constant in all experiments). The experiments were carried out using an 8-mM substrate solution at 37°C for 20 min at pH 6.0 (see Fig. 1).

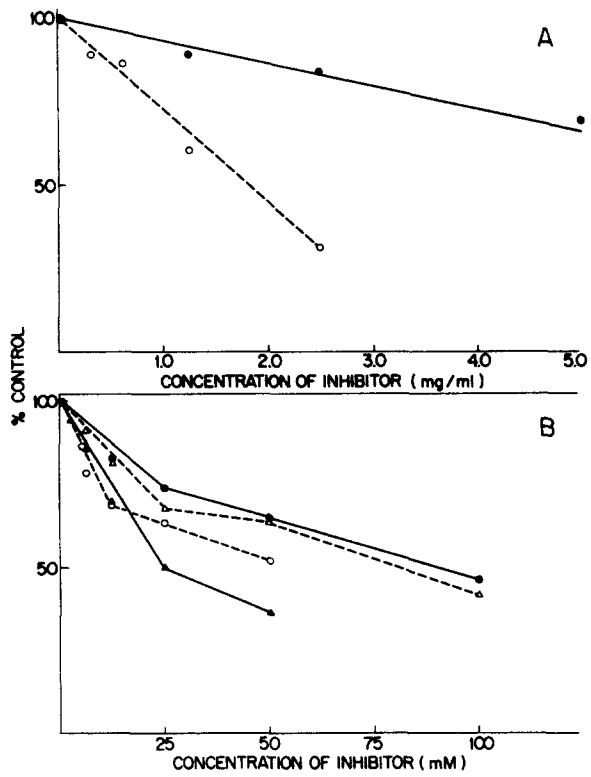

FIGURE 4 Inhibition of hydrolysis of 5 mM *p*-nitrophenyl-α-D-mannoside by glycoproteins and by monosaccharides. Intact NIL cell monolayers (diameter, 5 cm) were used in the experiments at pH 6.0 at 37°C for 20 min (see Fig. 2). (*A*) ●, Ovalbumin; ○, yeast mannan. Substrate solutions, into which various concentrations of glycoproteins had been mixed, were added upon cell monolayers, and the absorbances at 400 nm after 20 min were measured. The absorbances given by different nitrophenyl sugar-glycoprotein solutions that were incubated in the buffer only were subtracted from the measured values. (*B*) ●, α-Methyl-D-mannoside; △, D-mannose-6-phosphate; ▲, mixture of aldonate-lactones prepared by heating of the aldonate solution in acid pH; ▲, ○, α-1,4-L-mannonolactone.

a high amount of cells did not suggest penetration of ovalbumin into the cells. Thus, in an incubation with 100 μl of cells (packed volume) and 200 μl of buffer volume, 93% of ovalbumin radioactivity was recovered in the supernate after centrifugation, and only 7% was found in the cell pellet. Therefore, the sum of the radioactivity adsorbed on the cells and that absorbed into the cells was much lower than would be expected only from free diffusion into the cell volume (33%), suggesting that the cell membrane is able to exclude iodinated ovalbumin in the conditions used for mannosidase measurements. It should be noted that the inhibitory activity of ovalbumin must be higher than that of the mannan on a sugar basis, because the sugar content in ovalbumin is much lower (3–5% by weight; see reference 22).

Much higher concentrations of monosaccharides were needed for inhibition of the mannosidase by simple sugars as compared with ovalbumin or yeast mannan. For example, the concentration of mannose or α-methylmannoside needed to cause an inhibition to 60–70% of the control value (Fig. 4) was 30–40 times higher than that of ovalbumin, although exact comparisons cannot be made from the measurements with whole cells. In addition to natural high-mannose structures, *p*-nitrophenyl sugar is a much better substrate than α-methylmannoside. This kind of difference has been shown for the purified jack bean meal mannosidase (20). As expected, the lactone forms of mannonic acid were better inhibitors than α-methylmannoside (Fig. 4 *B*). However, the concentrations necessary for effective inhibition of cell surface mannosidase were higher than those described for other mannosidase activities from different sources (19). Also, the inhibitory solution prepared from the mannonate lactone mixture (a mixture of 1,4- and 1,5-lactones) was not as a potent an inhibitor as those described for some other mannosidase activities (Fig. 4 *B*; reference 19).

HYDROLYSIS OF MANNOSE FROM OVALBUMIN GLYCOPEPTIDES COVALENTLY LINKED TO A GLASS SURFACE: To further study the possibility that NIL cells contain a surface-exposed mannosidase activity, ovalbumin glycopeptides covalently linked to a glass surface were tested as the substrate. When a NIL cell suspension (5.0×10^6 cells/ml) was incubated on such a surface (5-ml volume on a 9-cm-diameter plate) for 20 min at 37°C, a liberation of mannose could be demonstrated in the supernate by direct trimethylsilylation after the supernate was passed through a mixed-bed ion exchange resin. A gas chromatogram of this experiment is shown in Figure 5 B_1. Because no mannose could be found from cell incubation on a plate without glycopeptide (C_1), it is concluded that mannose was liberated from the glycopeptide plate and not from the cells (Fig. 5). Mass spectra from the peaks having the retention times of mannose (peaks *I* and *II* in B_1) revealed the peaks that are characteristically intense in the mass spectra of trimethylsilylated hexoses (i.e., m/e 73, 147, 191, 204, and 217). Detection of a hexose ion (m/e 191) from a similar gas chromatographic analysis gave the same result; mannose could be detected from an incubation on the glycopeptide plate (Fig. 5 B_2) but not from an incubation on a plate treated with reagents only (Fig. 5 C_2). The amount of mannose liberated in 20 min from a plate containing ~14 μg hexose was ~0.6 μg, ~4% of total hexose coupled to the plate. Taking into account that ~40% of mannose in ovalbumin glycopeptides can be cleaved by α-mannosidase (30), the amount liberated in 20 min is high enough to suggest a significant mannosidase activity. Taken together with the results given above, it seems reasonable to conclude that an intense surface-exposed mannosidase activity is present in NIL cells.

Cell Adhesion to Ovalbumin-coated Surfaces

ATTACHMENT AND SPREADING OF FIBROBLASTS ON SURFACES COATED WITH OVALBUMIN: We have previously shown that different proteins can be adsorbed on plastic surfaces to yield protein layers bound in a way that is stable enough to be used for recognition-adhesion studies (23). Of the various glycoproteins studied, fetuin, asialofetuin, and glycophorin showed clearly lower adhesion-mediating activities than glycosidases, lectins, and fibronectin (23). However, an intense adhesion-promoting activity was observed for ovalbumin, as shown in comparison with fibronectin and fetuin in Fig. 6. In contrast to the surfaces of glycosidase, lectin, and fibronectin, all of which promoted adhesion of all kinds of cells tested (hepatocytes, 3T3, NIL, and BHK fibroblasts), the adhesion-promoting activity of the ovalbumin surface was cell-type specific. Whereas ovalbumin strongly promoted attachment and spreading of NIL cells (FIg. 6), and BHK cells also reacted fairly well, it did not promote attachment of 3T3 cells more than did bovine serum albumin or fetuin surfaces. No reaction could be observed for liver cells even after a 2-h incubation at 37°C. Therefore, the ovalbumin-promoted reaction is not attributable to a "general" cell surface affinity (as is the case of

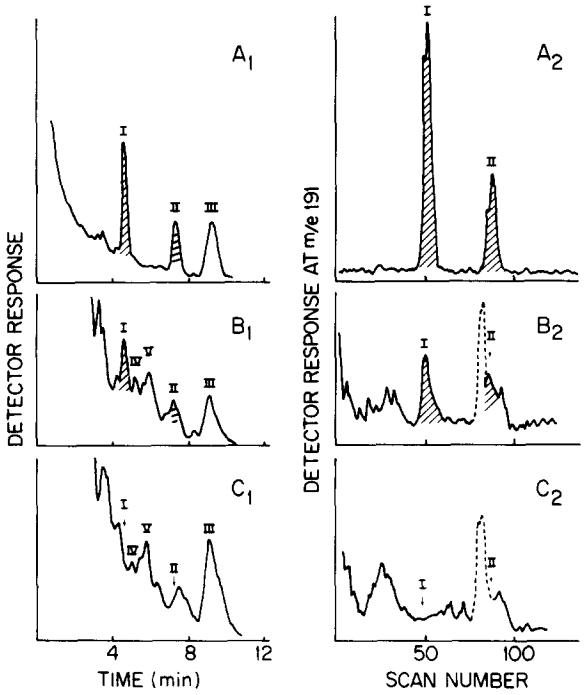

FIGURE 5 Gas chromatography and mass chromatography of sugars released on incubation of a NIL cell suspension on a glass plate to which ovalbumin glycopeptides had been covalently linked (for details, see Materials and Methods). In the experiment shown, 14-μg glycopeptide hexose was coupled to the plate. The charts A_1, B_2, and C_1 show the profiles detected by flame ionization, and the charts A_2, B_2, and C_2 show the profiles of ions with mass (m/e) of 191, scanned at 3-s intervals. Gas chromatography condition for all profiles is identical: 3% SE-30, 160°C. Peaks identified: *I* and *II*, peaks from trimethylsilylated mannose; *III*, trimethylsilylated galactitol (used as a standard); *IV* and *V*, unidentified peaks. A_1, standard sugar mixture (0.5 μg mannose, 0.5 μg galactitol); B_1, half of the supernate from a cell incubation of the glycopeptide surface and 0.5 μg galactitol; C_1, half of the supernate from a cell incubation on a surface derivatized without glycopeptide and 0.5 μg galactitol. The analysis *C* was run at a two times more sensitive setting than in *B*. The location of mannose elution is indicated by arrows (*I* and *II*). A_2 shows the mass ion profile scanned at m/e 191; sample is standard trimethylsilylated mannose. Peaks *I* and *II* represent α- and β-anomers, respectively. B_2, the mass ion profile at m/e 191 scan of the supernate from a cell incubation on the ovalbumin glycopeptide-glass surface, trimethylsilylated. The shaded peaks *I* and *II* are identified as α- and β-mannose, respectively. Other peaks and a dotted peak are unidentified material released from cells; C_2, the mass ion profile at m/e 191 scan of the supernate from a cell incubation on the *N*-acetylaminopropyl glass without ovalbumin glycopeptide. Mannose peaks are absent but other unidentified peaks are present as in B_2.

glycosidases and lectins coated on plastic surfaces), but might represent a more specific recognition-adhesion reaction resulting from a surface activity peculiar to NIL and BHK cells, both of which are hamster fibroblasts.

The adhesion on ovalbumin-coated surfaces was sensitive tc pH changes in the same way as the adhesion on fibronectin surfaces. The optimum pH was 7.0–7.4, and below 7.0 the activity declined rather rapidly (at pH 6.0, only slightly above that on nonreactive surfaces such as bovine serum albumin or fetuin surfaces). The activity was also low at pH 8.0. These changes probably reflect effects on the general adhesion mechanism, which is very sensitive to metabolic inhibition and toxic effects (6, 12). Also, preincubation of cells in the presence of 5 mM NaN_3, followed by a 1-h adhesion experiment in the presence of the same concentration of NaN_3 reduced the number of cells attached to 48% controls (as the mean of three determinations; the control cells were incubated in Salt/Pi, pH 7.4). Therefore, the adhesion reaction observed on ovalbumin surfaces is attributable to living cells.

Preincubation of cells with a high concentration of ovalbumin (5 mg/ml, 30 min at room temperature), followed by washing of the cells three times from the adhesion buffer, failed to produce any inhibition in ovalbumin-mediated cell adhesion. The number of cells attached (as the mean of three determinations of radioactivity) was 115% as compared with controls (incubated in the buffer only), when the cells were pretreated with ovalbumin in the way described above. Therefore, ovalbumin in solution does not form a stable interaction with the adhesion-mediating site. In this respect, ovalbumin-mediated adhesion is similar to the fibronectin-mediated reaction (6, 12), in striking contrast to lectin-mediated adhesion (6).

KINETICS OF OVALBUMIN-MEDIATED CELL ADHESION: Comparison of the time-course of ovalbumin-mediated cell adhesion to that of concanavalin A and plastic surfaces is shown in Fig. 7. As described previously for lectin-mediated adhesion reactions (6), there is little increase in the numbers of cells attached after 20–30 min on concanavalin A surfaces, whereas there is a continuous increase in cell attachment on ovalbumin surfaces up to at least 1 h. Thus, the kinetics of ovalbumin-mediated adhesion clearly resemble the kinetics described for fibronectin and glycosidase surfaces (6), which differ from those of lectin-mediated cell binding.

In the experiment shown in Fig. 7, 43 and 55% of added cells were bound to ovalbumin surfaces and concanavalin A surfaces, respectively, during 1 h (on the basis of recovery of cell radioactivities). The proportion of cells bound on ovalbumin surfaces is somewhat higher, when a higher concentration of ovalbumin (10 μg/ml in the experiment of Fig. 7) is used to coat the surfaces (see Fig. 11).

INHIBITION OF OVALBUMIN-MEDIATED CELL ADHESION BY SUGARS: Mannose tetrasaccharides clearly inhibited ovalbumin-mediated cell adhesion (Fig. 8), strongly suggesting an involvement of a mannosidic interaction in the reaction. Yeast mannan was also inhibitory at ~1 mg/ml.

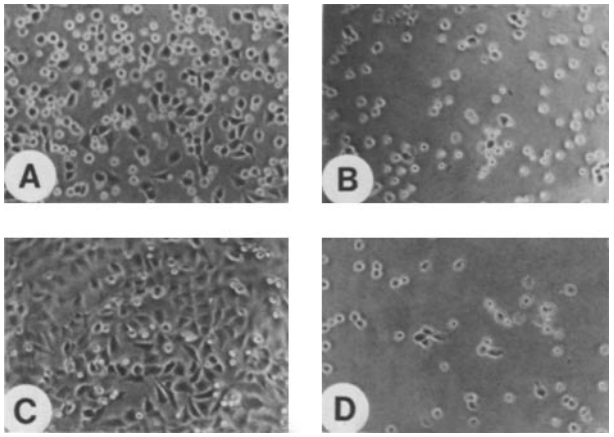

FIGURE 6 Attachment and spreading of NIL fibroblasts on different adhesion surfaces (for details of the analysis, see Materials and Methods). *A*, ovalbumin (50 μg/ml); *B*, ovalbumin (50 μg/ml) in the presence of 10 mM α-1,4-L-mannonolactone; *C*, fibronectin (10 μg/ml); *D*, fetuin (50 μg/ml).

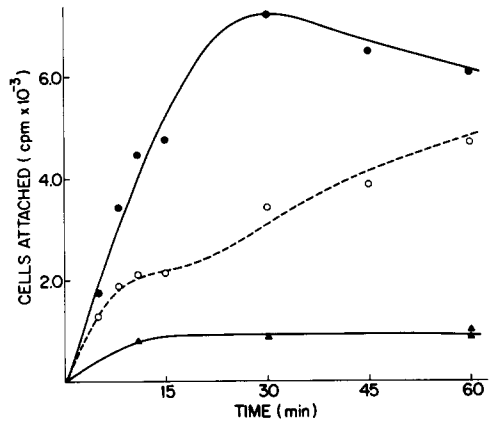

FIGURE 7 Kinetics of cell adhesion on different adhesion surfaces. [^{3}H]proline-labeled NIL cells from the same suspension were added simultaneously on different surfaces (representing the same time interval), using a multiwell pipette and different adhesion surfaces on the same multiwell plate. At the time intervals indicated, the nonattached cells were simultaneously washed off, using the multiwell pipette. The attached cells were solubilized and counted for radioactivity (for more details, see Materials and Methods). Concanavalin A (●; 10 μg/ml); ovalbumin (○; 10 μg/ml); plastic (▲; treated with Salt/Pi only).

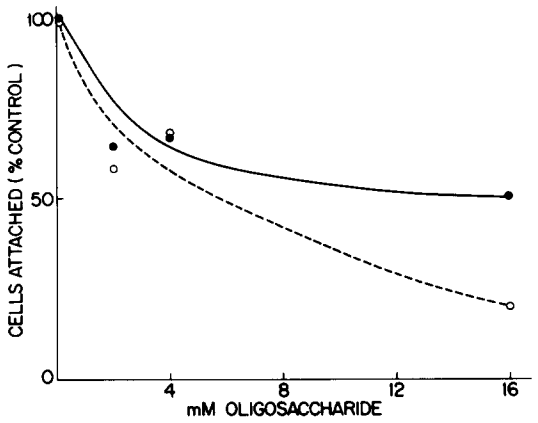

FIGURE 8 Inhibition of adhesion of [^{3}H]thymidine-labeled NIL cells on ovalbumin-coated (50 μg/ml) surfaces by mannose tetrasaccharides (for details of the analyses, see Materials and Methods). Manα1→2Manα1→2Manα1→2Man (α-1,2-mannotetraose) (●); Manα1→3Manα1→2Manα1→2Man (α-1,3-mannotetraose).

Therefore, the NIL cells recognize the sugar moiety of ovalbumin, most of which consists of high-mannose-type oligosaccharide chains (22).

A crystalline mannonolactone dissolved within a few minutes before the assay (the lactone form is stable at pH 7.4 only for a few hours; reference 19) gave a fairly strong inhibition of ovalbumin-mediated cell adhesion (Figs. 6 and 9). In contrast, the lactone incubated overnight in the HEPES-buffered Salt/Pi was found to be only weakly inhibitory. Thus, on the basis of radioactivity recovered from attached cells, the number of cells attached in the presence of 10 and 25 mM lactone were 88 and 85%, respectively, as compared with the controls (as the averages from two determinations; cf. Fig. 9). Similarly, this kind of lactone solution gave only a weak inhibition (85% of the control at 50 mM concentration) in the monolayer assay for mannosidic hydrolysis (see Fig. 4*B*). The lactone ring structure is therefore probably needed for the effect of α-1,4-L-mannonolactone in cell adhesion as in glycosidase inhibition (19). Furthermore, the inhibition by the lactone is a specific effect for the adhesion surface, because sialidase-mediated adhesion was resistant to this sugar (Fig. 9). As compared with α-methylmannoside, the mannonolactone has a clearly stronger inhibitory effect (Fig. 9). The β anomer of methylmannoside was not inhibitory (113% of the controls as the mean of two determinations at 100 mM concentration).

Comparison of the effects of mannose and mannose-6-phosphate (Fig. 10) suggested that mannose is a somewhat stronger inhibitor. However, the inhibitory effects of mannose, mannose-6-phosphate, and α-methylmannoside are all in the same concentration range (50–100 mM sugars). Galactose had no inhibitory effect, whereas *N*-acetylglucosamine was probably somewhat inhibitory at 100 mM concentration (Fig. 10). However, *N*-acetylgalactosamine showed a somewhat better inhibition at 100 mM concentration (data not shown), although the cause of this phenomenon is difficult to find. Some inhibitory effect of different sugars at high concentrations seems to occur for unspecific reasons on various adhesion surfaces (our unpublished observations). Alternatively, the amino sugar effect at high concentrations may relate to inhibitory effect of various amines in cell adhesion (29).

The effects of mannose-related monosaccharides on concanavalin A activity were studied using the binding of iodinated concanavalin A from solution onto cells as an indicator, because it is difficult to inhibit concanavalin A–mediated cell adhesion by specific sugars (23). As compared with ovalbumin-mediated cell adhesion, the effects of the monosaccharides were clearly reversed. Thus, mannose and α-methylmannoside

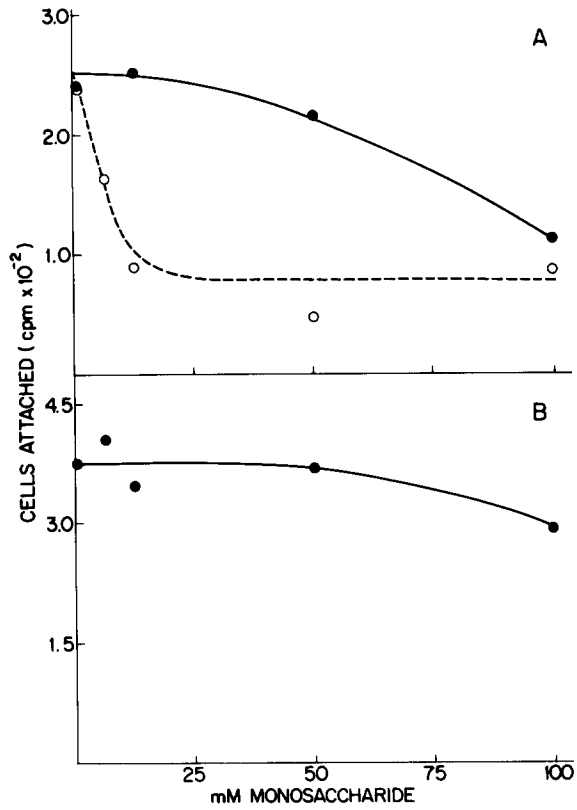

FIGURE 9 Inhibition of adhesion of [^{3}H]thymidine-labeled NIL cells on ovalbumin-coated surfaces by monosaccharides. (*A*) Ovalbumin surfaces (50 μg/ml); ●, α-methyl-D-mannoside; ○, α-1,4-L-mannonolactone. (*B*) Surfaces coated with *Clostridium perfringens* neuraminidase (10 μg/ml); ●, α-1,4-L-mannonolactone.

effectively inhibited binding of concanavalin A to the cells, whereas α-1,4-L-mannonolactone was not inhibitory (Table II).

EFFECT OF MANNOSIDASE DIGESTION ON THE ADHESION-MEDIATING ACTIVITY OF OVALBUMIN: The effect of extensive mannosidase digestion on the adhesion-mediating activity of ovalbumin is shown in Fig. 11. It is evident that the adhesion-mediating activity of ovalbumin was largely lost upon α-mannosidase digestion. Because both the intact ovalbumin and that extensively digested with α-mannosidase showed the same range of adsorption on the plastic surfaces,

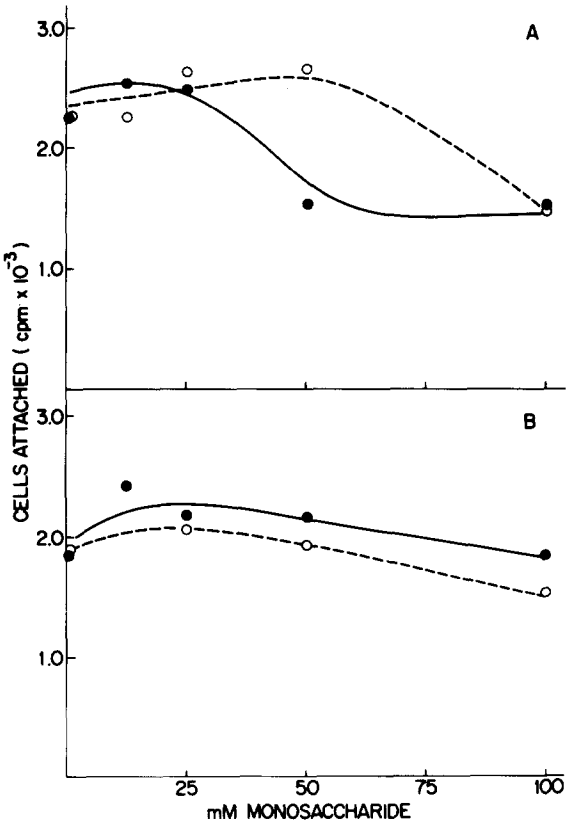

FIGURE 10 Effect of monosaccharides on the adhesion of [^{3}H]proline-labeled NIL cells (surfaces coated with 50 μg/ml ovalbumin). (*A*) ●, D-mannose; ○, D-mannose-6-phosphate. (*B*) ●, D-galactose; ○, *N*-acetyl-D-glucosamine.

TABLE II

Binding of Concanavalin A to Cells in the Presence of α-Methyl-D-mannoside, D-Mannose, and α-1,4-Mannonolactone

	Concanavalin A bound to cells	
Added sugar	Activity	% Control
	cpm × 10⁻³	
None (control)	10.6	100
α-Methyl-D-mannoside	4.3	41
D-Mannose	5.2	49
α-1,4-L-Mannonolactone	10.0	94

The sugars to be tested (0.1 M final concentration) were added to an [^{125}I]concanavalin A solution in Salt/Pi-30 mM HEPES, pH 7.4 (50 μg/ml final concentration). Thereafter, NIL cells in Salt/Pi-HEPES were added to give a final cell concentration of 1.0 × 10^6 cells/ml, the tubes were incubated for 60 min at room temperature, and the cells were washed three times by centrifugation at 800 g. The cell pellets were solubilized with 2% SDS, and transferred to scintillation vials. In control experiments without sugar haptens, ~10% of added radioactivity was bound to cells.

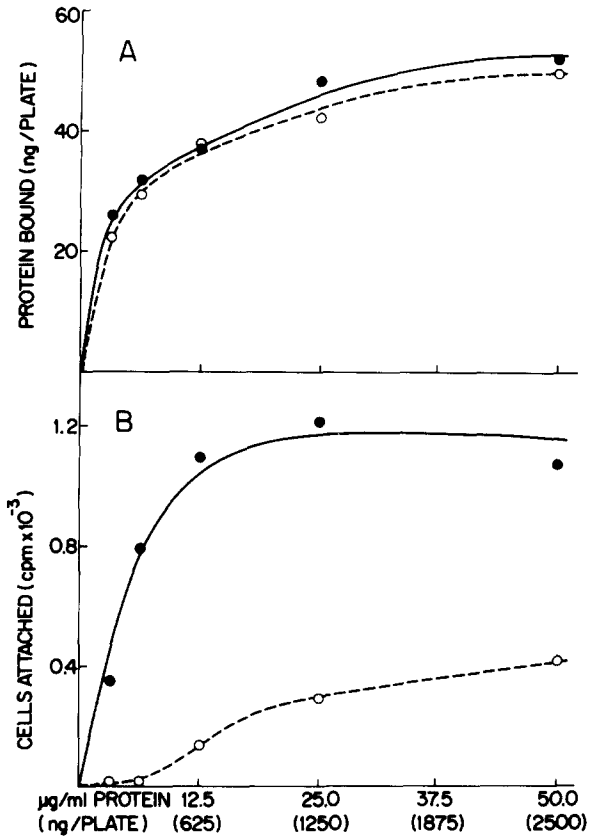

FIGURE 11 Binding of ovalbumin (●) and of mannosidase-digested ovalbumin (○) (see Materials and Methods) to plastic surfaces (*A*), and cell adhesion to surfaces coated with ovalbumin and with mannosidase-digested ovalbumin (*B*). (*A*) ^{125}I-labeled proteins were incubated on microtiter wells at different protein concentrations in 50 μl of Salt/Pi for 2 h at room temperature. The wells were washed three times with 100 μl of Salt/Pi, and the protein bound to surfaces was solubilized by rinsing the wells three times with 100 μl of 1% SDS in 0.5 N NaOH. The amounts of protein bound were calculated from the recovery of radioactivity. (*B*) Binding of [^{3}H]proline-labeled NIL cells on surfaces coated with ovalbumin and with mannosidase-digested ovalbumin. The surfaces were coated with the proteins as described for *A*, and tested for cell attachment activity.

the loss of cell-adhesion activity of ovalbumin by α-mannosidase treatment is attributable to reduced cell interaction with the enzyme-treated protein (see Fig. 11). Monosaccharide analysis of the mannosidase-digested ovalbumin suggested that ~30% of the mannose residues had been hydrolyzed. This result is in agreement with the studies of Shepherd and Montgomery (30, 31) suggesting that ~20% of mannose residues of ovalbumin are cleavable under conditions similar to those used in the present study.

DISCUSSION

To accept the concept of polyvalent glycosidase surfaces (23) as lectin-type mediators of cell recognition and adhesion (2, 3, 7), one must provide evidence for the occurrence of glycosylhydrolases at the cell surface. Previously, three types of evidence have suggested the possible occurrence of glycosidase activities at the cell surface. First, glycosidase activities can be found in plasma membrane—enriched fractions in membrane fractionation studies (38), and hydrolysis of some glycosides by whole cells has been suggested to be partially attributable to a cell surface enzyme (27). Second, glycosidases added to the

growth medium are taken up into the cells by a receptor-mediated endocytosis (14, 32). Third, antiglycosidase antibodies were recently reported to stain the cell surface (39).

The present studies have provided evidence that NIL fibroblasts are capable of hydrolyzing mannosyl residues of high-mannose-type glycoprotein (ovalbumin) when cells are incubated and contacted with the glycopeptide coat covalently affixed onto the glass surfaces (Fig. 5). The same cells are capable of rapidly hydrolyzing the mannosyl residue of *p*-nitrophenyl-α-mannoside when incubated with this substrate (Figs. 1 and 2; Table I). This hydrolysis reaction was effectively inhibited by ovalbumin and yeast mannan (Fig. 4), which cannot penetrate into cells. These results indicate that the cellular mannosidase activity detected by the procedures described above may not be attributable to penetration of the substrate into cells, but rather to the presence of α-mannosidase at the cell surface. However, two other possibilities were taken into consideration: that (*a*) the hydrolysis is attributable to an extracellular activity, but is caused by an enzyme rapidly released to the medium, and (*b*) the activity is attributable to broken cells or cell fragments and not to intact cells. The results clearly excluded these two possibilities as well. No enzyme activity was detectable in the medium preincubated with cell monolayers even after 1 h, although the hydrolysis could be detected in the presence of cells from the first few minutes (Fig. 1) of incubation. Thus, the possibility of released or secreted mannosidase can be excluded. Intact cell monolayers showed as much activity as trypsinized cells, and the addition of broken cells to the intact cell suspension did not greatly enhance the hydrolytic activity of the whole cell suspension (Fig. 3). These results excluded the second possibility that the activity could be attributed to the broken cells.

Ovalbumin clearly displayed a cell attachment and spreading activity, when NIL or BHK cells were plated on plastic surfaces coated with ovalbumin (Fig. 6). In contrast, BALB/c 3T3 cells or freshly prepared liver cells did not react with ovalbumin surfaces under the conditions used. Therefore, NIL cell reaction on ovalbumin surfaces may be influenced by a cell surface activity peculiar to this cell type. Because ovalbumin is generally regarded as a biologically inert protein (22), to propose that a cell surface activity mediates affinity to ovalbumin surfaces seems a reasonable interpretation.

Because ovalbumin-mediated cell adhesion was strongly inhibited by mannose oligosaccharides and the mannosidase-treated ovalbumin showed a reduced adhesion-mediating activity (Figs. 8 and 11), it seems reasonable to conclude that this adhesion reaction involves a mannosidic recognition. This result, however, does not exclude participation of other types of interactions in the adhesion reaction observed, such as ionic interactions that may be important in cell adhesion (7). However, desialylation of cells with neuraminidase did not increase or decrease adhesion on ovalbumin surfaces.

Whether the cell surface mannosidase contributes to ovalbumin-mediated cell adhesion is of major interest, because the similarity and the difference between a lectin-mediated and an enzyme-mediated cell adhesion have been studied recently (6, 23). Although the adhesion assay conditions (isotonic salt solution, pH 7.4) were low-hydrolytic or nonhydrolytic conditions for the cell surface α-mannosidase, this does not exclude the possibility that the mannosidase could mediate the adhesion reaction. Although the catalytic activity for hydrolysis of glycosides is in acidic pH range with a few exceptions, the sugar-binding activity, the basis of adhesion reaction, may well be expressed under physiological pH. In fact, sialidase and β-galactosidase strongly mediated cell adhesion under low-hydrolyzing or non hydrolyzing, physiological pH conditions (23). Mannosidase activity of BALB/c 3T3 cells was much lower than of NIL cells in coincidence with the low cell-adhesion capability on ovalbumin surfaces.

The idea that the cell surface mannosidase contributes to NIL cell adhesion on ovalbumin surface has been further strengthened by the following six observations:

(*a*) Both the cellular mannosidase activity and cell adhesion were inhibited by the natural mannosidic structures, mannonolactone, and simple mannosides in the same order of inhibitory activities (Figs. 4 and 8–10).

(*b*) The cell adhesion reaction was inhibited by a potent mannosidase inhibitor, α-1,4-L-mannonolactone, that did not inhibit the activity of concanavalin A, a "simple" sugar-binding protein (Fig. 9; Table II). The lactone effect observed was in agreement with a glycosidase-type inhibition, which requires the lactone ring structure (19), whereas the aldonate ion that rapidly forms from the lactone in aqueous solution was not inhibitory.

(*c*) Pretreatment of ovalbumin with α-mannosidase, followed by purification of the glycosidase-digested protein by gel filtration, clearly reduced the adhesion-mediating activity of ovalbumin (Fig. 11). Therefore, the adhesion-enhancing effect of the surface mannosidase cannot be attributed to cleavage of the mannosidic chains of ovalbumin during the adhesion reaction. This result also discounts the possibility that a cell surface mannosyltransferase would be involved in the adhesion reaction studied. The decrease in adhesion-mediating activity of ovalbumin upon mannosidase digestion can hardly be explained only on the basis of decrease of the total quantity of mannose on the adhesion surfaces (Fig. 11), but the exoglycosidase-available mannose residues seem to have a higher "specific activity" from the viewpoint of cell interactions than the mannose residues that are not available to the exoglycosidase.

It is interesting that the adhesion-mediating activity of ovalbumin, but not of mannosidase-digested ovalbumin, rises very steeply (by ~200% in terms of cell numbers attached) at the concentration range of ~3–10 μg/ml of protein used to coat the surfaces, although the actual quantity of ovalbumin on the adhesion surfaces increases at this concentration range by only ~40% (Fig. 11). Thus, only a slight or a moderate change in the quantity of oligomannosidic chains on the adhesion surface can have a striking effect on cell adhesion in the model used. This result is in agreement with the studies of Weigel et al. (41) using monosaccharides linked to gel particles as the model. These authors suggested that even slight quantitative changes at the threshold concentration range of surface carbohydrates can cause strong changes in cell behavior.

(*d*) Kinetic analysis of cell adhesion on ovalbumin-coated surfaces revealed a sigmoidal-type curve (Fig. 7), which was previously found as typical of glycosidase- and fibronectin-mediated reactions (6). The lectin-mediated cell adhesion was more rapid in the conditions used.

(*e*) Incubation of cells in suspension with a high concentration of ovalbumin, followed by washings, did not result in any inhibition of ovalbumin-mediated cell adhesion. Therefore, ovalbumin in solution does not form a stable interaction with the cell surface affinity site. A similar experiment was recently reported to give an inhibition for a lectin-like interaction suggested to be attributable to a glycosidase receptor (11). Thus, ovalbumin-mediated cell adhesion is probably strongly

dependent on the polyvalency of the immobilized substrate. We have previously suggested that the glycosidase-mediated adhesion is strongly dependent on the valency of the two interacting surfaces (6). This phenomenon must be important in vivo, because cell-to-cell and cell-to-substrate interaction could be regulated by the change of valency of the binding sites. The extracellular substrates of a lower valency than the membrane-bound substrates would be readily dissociable from the adhesion-mediating sites.

(*f*) It has recently become evident that certain fibroblastic cells have a cell surface glycosidase receptor that recognizes mannose-6-phosphate (32). However, mannose-6-phosphate was a somewhat weaker inhibitor of cell adhesion than α-methylmannoside and mannose (Figs. 9 and 10). On the other hand, mannose-6-phosphate inhibited mannose hydrolysis to approximately the same degree as mannose or α-methylmannoside (Fig. 4). Therefore, the inhibition of adhesion by mannose-6-phosphate is better explained by inhibition of the mannosidase activity than of a glycosidase receptor, if such a receptor would exist in NIL cells. Furthermore, ovalbumin was reported to be noninhibitory for glycosidase uptake (16).

The results of adhesion studies on mannoglycoprotein and the presence of a high mannosidase activity in NIL cells strongly suggest a specific cellular recognition through the surface-exposed mannosidase. It is possible that the cell surface mannosidase may contribute to cell-to-cell recognition between homologous cells, or it may be of importance for aberrant recognition displayed by malignant cells, which are known to have abnormal cell surface sugar structures (13, 40). Also, occurrence of high glycosidase activities in malignant cells has been suggested by Bosmann (5). Clearly, further studies on the influence of high surface glycosidase activity on cell behavior would be indicated at present.

The data presented in this study are, to our knowledge, the first evidence that a glycosidase activity, identified to be present at the cell surface, can mediate cell recognition. The specific cell adhesion-mediating capability of cell surface enzymes acting on cell surface substrates may not be restricted to glycosidases; proteases and glycosyltransferases might have similar properties. Such a function for cell surface glycosyltransferases has been suggested by Roseman (26). In fact, a somewhat similar situation with respect to enzymatic activity can be observed when one compares glycosidases and glycosyltransferases. Extracellular fluid lacks protons for the hydrolyzing activity of glycosidases and sugar nucleotides for the transferase reactions. Because the mechanism of the transferase reaction can be random (24), the glycosyltransferase may bind to the oligosaccharide chains in the absence of sugar nucleotides. Therefore, glycosyltransferases might show recognition activities similar to those of glycosidases in the absence of sugar nucleotides. We have no information as to whether NIL cells secrete sugar nucleotides. Also, even if the hydrolase or transferase reactions were effectively taking place, the cells would provide new substrate sites by active movement phenomena (25), which might allow the cells to adhere. It should be noted that the time of affinity contact (36) needed for an irreversible adhesion (6) is only ~0.5–1.0 h.

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