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Mannose 6-, fructose 1-, and fructose 6-phosphates inhibit human natural cell-mediated cytotoxicity

(natural killer cells/lysosomes/gelonin-monophosphopentamannose conjugate)

JAMES T. FORBES*, ROGER K. BRETTHAUER†, AND THOMAS N. OELTMANN*

*Department of Medicine, Division of Oncology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232; and †Department of Chemistry, University of Notre Dame, Notre Dame, Indiana 46556

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ABSTRACT *In vitro* human natural cell-mediated cytotoxicity (NCMC) to K-562, Molt-4, and F-265 cells is inhibited in a dose-dependent manner by mannose 6-phosphate, fructose 1-phosphate, and fructose 6-phosphate. This inhibition is not observed with mannose, glucose, fucose, glucose 6-phosphate, mannose 1-phosphate, galactose 1-phosphate, or galactose 6-phosphate. Preincubation of the effector cells, obtained from fresh whole blood, with mannose-6-phosphate, fructose-1-phosphate, or fructose-6-phosphate did not inhibit cytotoxicity, which indicated that these hexose phosphates are not nonspecifically toxic towards the effector lymphocytes. Mannose-6-phosphate and the stereochemically similar fructose-1-phosphate are more potent inhibitors than fructose-6-phosphate in terms of concentration required and time of onset of effect. Inhibition of cytotoxicity by mannose-6-phosphate varied with target cell type: F-265 is protected at much lower concentrations of mannose-6-phosphate (<1 mM) than is either Molt-4 or K-562. The inhibition of NCMC is also observed with the inhibitors of lysosomal function, NH_4Cl, and chloroquine. The presence of a functional mannose-6-phosphate receptor on target cells was demonstrated: (*i*) Gelonin, a seed protein that inactivates the eukaryotic ribosome but is *nontoxic* to intact cells, was covalently linked to monophosphopentamannose, and this conjugate was toxic to both K-562 and F-265 target cells, the latter being by far the more sensitive; and (*ii*) chloroquine, NH_4Cl, and mannose-6-phosphate all inhibited the toxicity of gelonin-monophosphopentamannose. These results suggest either that a cytolytic lymphokine contains a hexose phosphate residue and may be taken up by target cells through the lysosomal/mannose 6-phosphate pathway or that such a residue is involved in target cell–effector cell recognition.

The basis of specificity in natural cell-mediated cytotoxicity (NCMC) has remained undefined for a variety of reasons (1–3) such as variations in labeling efficiency and lysis of different target cells. In spite of these limitations, evidence has been produced for selective cytotoxicity (4). Most of these studies have suggested that NCMC is directed towards multiple antigens, some of which are shared by more than one target cell line (4, 5). Specificity has been further demonstrated by inhibition studies in which unlabeled target cells are included in the admixture of effector cells and isotopically labeled target cells (5–7). Crude extracts from target cells also have been demonstrated to specifically block the binding of effector cells with target cells (8, 9) and to prevent the lysis of target cells by effector cells (unpublished data). No information is available concerning the structure of those target antigens or the nature of their recognition by effector cells.

Crude cell extracts containing glycoproteins are effective in blocking both effector cell–target cell binding and NCMC, suggesting carbohydrate moieties as possible target structures. Stutman *et al.* (10, 11) have shown that murine NCMC can be inhibited by several sugars, including D-mannose, D-galactose, D-glucose, and L-fucose and have suggested that the recognition structure may be lectin-like in nature. The observation that a receptor on the surface of human fibroblasts specific for D-Man-6-*P* is responsible for the pinocytosis of β-glucuronidase (12) and α-L-iduronidase (13) makes such a sugar a logical suspect (14). It has been suggested that Man-6-*P* is a common recognition marker for intracellular transport of many lysosomal hydrolases (15). Such a marker also could serve as a mechanism of recognition between two cells such as effector and target in NCMC or could be involved in the lytic phase of this reaction.

In the present study we demonstrate that NCMC between effector cells from human peripheral blood and the target cell lines K-562, Molt-4, and F-265 can be inhibited by coincubation with Man-6-*P*, Fru-1-*P*, and Fru-6-*P*. This inhibition exhibits limited specificity because similar assays designed to test mannose, fucose, glucose, galactose, glucose 6-phosphate, mannose 1-phosphate, galactose 1-phosphate, and galactose 6-phosphate were without positive results.

MATERIALS AND METHODS

Target Cells. The target cell lines used in this study were K-562, a line derived from the pleural effusion of a patient with chronic myelogenous leukemia (16); Molt-4, a human T-cell line (17); and F-265, a normal human lymphoid cell line (18). These cell lines were maintained in stationary suspension culture in RPMI-1640 medium supplemented with 10 mM glutamine and 10% fetal calf serum.

Sugars. All sugars tested were obtained as sodium salts or neutral sugars from Sigma. Stock solutions made in RPMI-1640 medium were adjusted to pH 7.2 prior to use.

Assays for Cytotoxicity and Inhibition. Cytotoxicity assays were performed as described (19). Mononuclear peripheral blood leukocytes (PBL) were obtained from heparin-treated whole blood by one-step centrifugation on a Ficoll/Hypaque gradient. Cells were washed twice with a balanced salt solution and enumerated; viability was determined by trypan blue exclusion. PBL were then suspended to the desired concentration in RPMI-1640 medium supplemented with 10% (vol/vol) fetal calf serum and antibiotics (complete media). Target cells ($5–7 \times 10^6$ cells per ml) were incubated with 150 μCi (1 Ci = 3.7×10^{10} becquerels) $Na_2{}^{51}CrO_4$ for 45 min at 37°C. The labeled target cells were then washed twice with complete media and adjusted to a concentration such that the indicated number of target cells were contained in 0.050 ml. The reaction mixtures,

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Abbreviations: NCMC, natural cell-mediated cytotoxicity; PBL, mononuclear peripheral blood leukocytes; Man_5-6-*P*, monophosphopentamannose.

in 12 × 75 mm polystyrene tubes, were incubated in 5% CO_2/95% air at 37°C for 4 hr. All reactions were performed in quadruplicate. After the appropriate incubation time, the tubes were centrifuged at 1000 × *g* for 10 min, and the supernatants were harvested for determination of unbound isotope. Total radioactivity of an initial aliquot of the appropriate number of target cells was determined. Autologous controls (unlabeled target cells added to labeled target cells) served as spontaneous release values in all experiments, and any experiment in which this exceeded 10% of the incorporated label was rejected as being technically unsound. The percentage of NCMC was calculated by the following equation: % NCMC = (experimental release − spontaneous release) ÷ (total label − spontaneous release) × 100. Inhibition was tested in a similar manner except that the indicated concentrations of sugars, NH_4Cl, or chloroquine were present in the reaction mixture for the times indicated. The percentage of inhibition was calculated by the following formula:% inhibition = (%NCMC in absence of sugar − % NCMC in the presence of sugar) ÷ (% NCMC in absence of sugar) × 100.

Isolation and Purification of Gelonin. Seeds of *Gelonium multiflorum* were a gift from Sjur Olsnes. Gelonin was isolated from the seeds as described by Stirpe *et al.* (20). Briefly, the seeds were ground in a homogenizer with 8 vol of 0.14 M NaCl in 5 mM sodium phosphate (pH 7.4). The homogenate was stirred overnight at 4°C then centrifuged at 35,000 × *g* for 20 min. The cloudy yellow supernatant was then dialyzed against 5 mM sodium phosphate (pH 6.5), followed by removal of any precipitate by centrifugation. The crude extract was then applied to a CM-52 column (20 × 1.8 cm) equilibrated with 5 mM sodium phosphate (pH 6.5). The column was washed with buffer until the absorbance at 280 nm was zero and then eluted with a 400-ml linear gradient of 0–0.3 M sodium chloride in the same buffer. The fractions that contained gelonin (by $NaDodSO_4$ gel electrophoresis and inhibition of protein synthesis in a cell-free system) were pooled and concentrated. The yield was 6 mg from 5 g of seeds.

Synthesis of Gelonin-Monophosphopentamannose (Gelonin-Man_5-6-*P*). Man_5-6-*P* was prepared essentially as described (21) and has the following structure:

P-6-Man (α1→3) Man (α1→3) Man (α1→3) Man (α1→2) Man.

Hansenula holstii (NRRL Y-2448) phosphomannan (6 g) was hydrolyzed for 4 hr at 100°C in a 200-ml solution (pH 2.4) containing 2 g of KCl. After the hydrolysate was cooled and neutralized, a small amount of insoluble residue was removed by centrifugation. Barium acetate (2 g) was dissolved in the supernatant liquid, which was then made basic with sodium hydroxide. Addition of 20 ml of ethanol precipitated any unhydrolyzed phosphomannan and core material, and the precipitate was then removed by centrifugation. An additional 2 vol (400 ml) of ethanol were added to precipitate the barium salt of the pentasaccharide phosphate. The precipitate was dissolved in water, and again ethanol fractionated. The final product was dissolved in water by mild acidification, passed over a Dowex-50 (H+) resin, and neutralized with ammonium hydroxide. The yield was about 1.5 g after lyophilization.

The covalent linkage of the carbohydrate to gelonin was accomplished by reductive amination of a Schiff base between C-1 of the reducing terminal sugar residue and a free amino group on the gelonin protein by the methods of Schwartz and Gray (22). Man_5-6-*P* (0.2 M) was mixed with gelonin (15 mg/ml) and $NaCNBH_3$ (160 mM) in 50 mM *N,N*-bis(2-hydroxyethyl) glycine (pH 9), incubated for 24 hr at 37°C, and then dialyzed at 4°C against 10 mM Tris buffer (pH 7.4). The resulting coupled product was separated from unincorporated Man_5-6-*P* by gel filtration on a Sephadex G-100 column. There were approximately two phosphate groups incorporated per mol of gelonin.

Inhibition of Protein Synthesis in Target Cells by Gelonin-Man_5-6-*P*. Cells were incubated in the presence of gelonin-Man_5-6-*P* at the concentrations indicated for 16 hr at 37°C in a humidified O_2/CO_2 atmosphere. After the incubation, [^{3}H]leucine was added to each culture, and the incubation was continued for an additional hour. Trichloroacetic acid was then added to a final concentration of 10% (wt/vol). The resulting precipitate was collected by centrifugation, solubilized in 0.5 M NaOH, and precipitated once again with 10% trichloroacetic acid. This procedure was repeated twice more, the final precipitate was dissolved in 1 ml of NCS (Amersham), and the radioactive content was determined.

RESULTS

Inhibition of NCMC by Man-6-*P*, Fru-1-*P*, and Fru-6-*P*. Mannose, fucose, glucose, galactose, glucose 6-phosphate, and Man-6-*P* were tested for their ability to inhibit human NCMC to ^{51}Cr-labeled K-562 target cells. The results (Fig. 1) show that, of these sugars, only Man-6-*P* significantly inhibited cytotoxicity against K-562 cells in a dose-dependent manner. The inhibition of human NCMC to K-562 cells by hexose phosphates was much more striking than that mediated by neutral sugars. Table 1 presents data showing that Man-6-*P*, Fru-6-*P*, and Fru-1-*P* were potent inhibitors of NCMC, whereas glucose-6-phosphate, galactose-1-phosphate, galactose-6-phosphate, and mannose-1-phosphate mediated little or no inhibition. Inorganic phosphate was not inhibitory to NCMC against K-562 cells (data not shown). Kaplan *et al.* (12) suggested that the relative positions of P-1, the anomeric oxygen, and the O-4 of Fru-1-*P* are equivalent to the position of P-6, O-4, and O-2, respectively, of Man-6-*P*. The inhibition of NCMC by Fru-6-*P* may represent a conversion of this sugar to Man-6-*P* as the result of enzymes present in fetal calf serum or released from damaged or dead cells. When

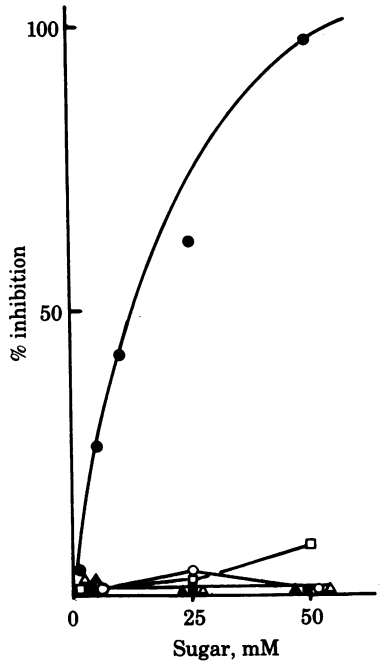

FIG. 1. Inhibition of NCMC (1 × 10^6 human PBL to 1 × 10^4 ^{51}Cr-labeled K-562 cells) by glucose (□), glucose 6-phosphate (○), mannose (■), fucose (△), galactose (▲), and Man-6-*P* (●). Cytotoxicity in the absence of sugar was 65%. (Values are the means of four replicate cultures.)

Table 1. Effect of hexose phosphates on NCMC to K-562 cells

Added compound, 50 mM	Percentage NCMC*	Percentage inhibition
None	70	—
Man-1-*P*	57	19
Man-6-*P*	22	69
Glc-6-*P*	62	11
Fru-1-P	18	74
Fru-6-*P*	20	71
Gal-1-*P*	60	14
Gal-6-*P*	57	19

* PBL (1×10^6) plus 1×10^4 ^{51}Cr-labeled K-562 cells were incubated together for 4 hr at 37°C in 1.0 ml of medium with the indicated sugar.

effector cells and target cells were coincubated for 1 hr prior to the addition of Man-6-*P*, Fru-1-*P*, or Fru-6-*P* at a final concentration of 25 mM, cytotoxicity was inhibited by Man-6-*P* and Fru-1-*P* maximally at the earliest time assayed. However, inhibition by Fru-6-*P*, after a latent period of more than 30 min, increased during the course of the assay (Fig. 2). This increase in inhibition with time by Fru-6-*P* is consistent with its enzymatic conversion to Man-6-*P*. Appropriate enzymes in the culture media also should convert glucose-6-phosphate and mannose-1-phosphate to Man-6-*P*. The fact that these hexose phosphates are not inhibitors of NCMC (Table 1) and the requirement for almost quantitative conversion of Fru-6-*P* to Man-6-*P* make this mechanism of inhibition by Fru-6-*P* less likely. The inhibition of NCMC by Fru-6-*P* may represent either incomplete specificity or a mechanism unrelated to Man-6-*P*. It also should be noted (Fig. 3) that Man-6-*P* and Fru-1-*P* inhibit NCMC at much lower concentrations than Fru-6-*P*.

Inhibition of NCMC Against Various Target Cells. The ability of Man-6-*P* to inhibit NCMC to other target cell types was also investigated. When Molt-4, K-562, and F-265 cells were radiolabeled and used as target cells, coincubation in the presence of Man-6-*P* caused inhibition of NCMC to all three target cell lines (Fig. 4). The inhibition of NCMC to K-562 and Molt-4 cells by Man-6-*P* was approximately equal. However, inhibition of NCMC against the cell line F-265 occurred at much lower concentrations of Man-6-*P* than in the instances of K-562 and Molt-4. The concentrations of Man-6-*P* that caused 50% inhibition were calculated to be 18 mM, 23 mM, and 0.75 mM for K-562, Molt-4, and F-265 cells, respectively (data not shown). This is especially noteworthy in that previous data (19) have suggested that Molt-4 and K-562 share cross-reacting target structures recognized by the effector cells of NCMC, whereas the cell line F-265 does not share these structures to the same extent. Levels of NCMC to K-562 and Molt-4 by the same effector cells have been shown to be remarkably similar (19).

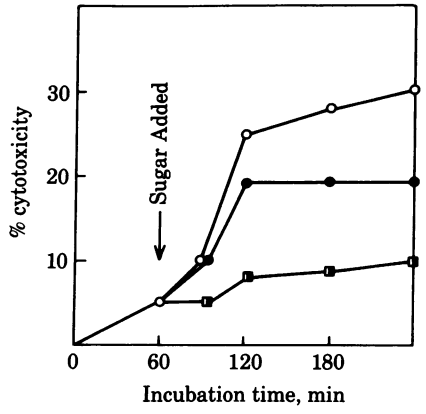

FIG. 2. NCMC by 1×10^6 human PBL to 1×10^4 ^{51}Cr-labeled K-562 cells alone (○) or with 25 mM Man-6-*P* (□), Fru-1-*P* (■), or Fru-6-*P* (●) added 1 hr after initiation of cultures. Samples were taken at 60, 90, 120, 180, and 240 min after the start of incubation. (Values are the means of four replicate cultures.)

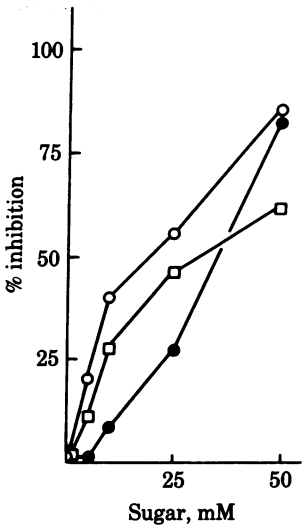

FIG. 3. Inhibition of NCMC by 1×10^6 human PBL to 1×10^4 ^{51}Cr-labeled K-562 cells by various concentrations of Man-6-*P* (○), Fru-1-*P* (□), and Fru-6-*P* (●). Cytotoxicity in the absence of sugars was 47%. (Values are the means of four replicate cultures.)

Addition of the sugar to effector cells prior to the addition of labeled target cells enhanced slightly the inhibitory activity (data not shown). Pretreatment of effector cells for 1 or 2 hr at 4°C or 37°C with Man-6-*P*, Fru-1-*P*, or Fru-6-*P*, followed by washing prior to the addition of radiolabeled target cells, had no effect on the level of cytolysis in subsequent assays of NCMC (data not shown). These results show that these sugars are not directly toxic to effector cells and that the inhibition of NCMC is not the result of nonspecific toxicity.

Man-6-*P* Receptor and the Role of the Lysosome in Cytotoxicity. In order to establish the presence of a Man-6-*P* receptor on the target cell and lysosomal involvement, the toxic, covalent conjugate composed of gelonin, a toxic seed protein, and Man_5-6-*P* was prepared. Gelonin is present in the seeds of *G. multiflorum* in high concentration and is easily isolated, exception-

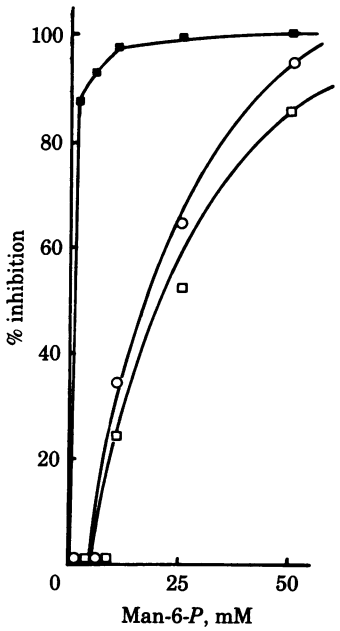

FIG. 4. Man-6-*P*-mediated inhibition of NCMC by 1×10^6 human PBL against 1×10^4 ^{51}Cr-labeled F-265 (■), K-562 (○), or Molt-4 (□) cells. Cytotoxicity in the absence of Man-6-*P* was 65% to F-265, 75% to K-562, and 44% to Molt-4 cells. (Values are the means of four replicate cultures.)

ally stable to chemical and physical treatments, and nontoxic to intact cells (20). Gelonin is a single chain glycoprotein with biological properties similar to those of the A-chains of abrin, ricin, and modeccin. Like these, gelonin inactivates the 60S ribosomal subunit in a cell-free translation system from rabbit reticulocytes. It has been estimated that one molecule of the toxin inactivates about 200 ribosomes per minute, indicating that it acts catalytically (20). In a cell-free system, gelonin is more toxic than the abrin A-chain. Gelonin is nontoxic towards whole cells presumably because it lacks the B chain, or haptomer, necessary for toxin binding. When chemically linked to a haptomer, such as concanavalin A, gelonin acquires toxic activity towards whole cells (20). Gelonin was coupled to Man_5-6-*P* by reduction of the Schiff base between the C-1 reducing terminal sugar residue and a free amino group of the gelonin protein. This conjugate should be toxic towards any cell type that has a functional Man-6-*P* receptor. The Man-6-*P* receptor would then direct the internalization of this normally nontoxic protein. Target cells K-562 and F-265 were incubated with gelonin-Man_5-6-*P*, labeled with [^{3}H]leucine, treated with 10% trichloroacetic acid, and analyzed for the radioactive content. The conjugate was toxic to F-265 cells at micromolar concentrations, whereas toxicity towards K-562 cells occurred at a much higher concentration of gelonin-Man_5-6-*P* (Fig. 5). Further, when Man-6-*P* was present in the incubation medium the gelonin-Man_5-6-*P* was nontoxic to both F-265 and K-562 cells. It is apparent, therefore, that the F-265 target cell line contains a functional Man-6-*P* receptor that is capable of delivering the toxic protein gelonin to the cytoplasm. The results with K-562 cell line are not quite as clear in view of the unusual shape of the dose–response curve.

In order to evaluate the role of the lysosome in the toxic phase of NCMC, the assays were conducted in the presence of either

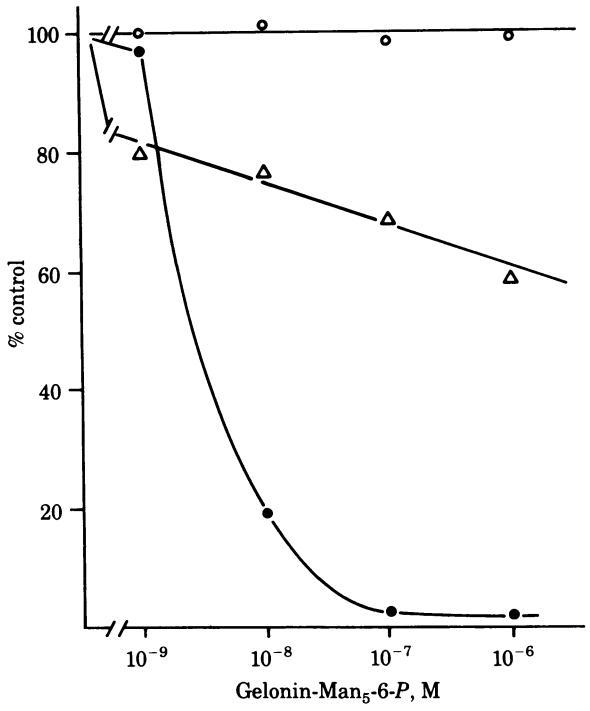

FIG. 5. Inhibition of protein synthesis in F-265 and K-562 cells by gelonin-Man_5-6-*P*. Cells (1×10^4 per ml) were incubated in complete medium with the indicated amounts of gelonin-Man_5-6-*P* for 17 hr. [^{3}H]Leucine was present during the final hour, and the radioactive content was determined. ●, F-265; △, K-562; ○, F-265 and K-562 in 10 mM Man-6-*P*.

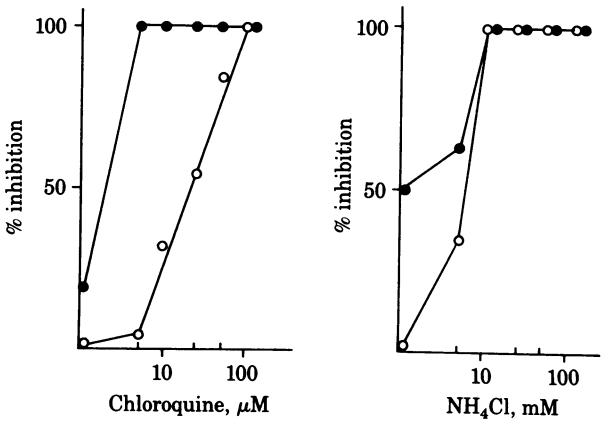

FIG. 6. Inhibition of NCMC by chloroquine (*A*) and NH_4Cl (*B*). Cells (10^6 PBL plus 10^4 ^{51}Cr-labeled target cells) were incubated with the indicated amounts of NH_4Cl or chloroquine. ○, K-562 cells; ●, F-265 cells. Cytotoxicity in the absence of NH_4Cl and chloroquine was 52% to K-562 cells and 22% to F-265 cells. (Values are the means of four replicate cultures.)

NH_4Cl or chloroquine. These two substances are both inhibitors of lysosomal function, and both target cells were spared the cytotoxic effects of effector cells when coincubated in the presence of either of these lysosomal inhibitors (Fig. 6). Thus, a role for the lysosome in NCMC is indicated.

DISCUSSION

The elucidation of specificity in NCMC is important in understanding the biology of this naturally occurring phenomenon. Prior attempts to identify the specificity of this reaction have utilized either various susceptible target cell types (4) or the competitive inhibition assay (5, 6). The data from these studies, while suggesting some specificity, have provided no clear indication of the nature of the target(s) involved. Several possibilities have been ruled out by these techniques, including murine leukemia virus determinants (23) and histocompatibility antigens (1–3, 24). Earlier reports by Stutman *et al.* (10, 11) have suggested that D-mannose may block natural cytotoxicity against Yac-1 or Meth A target cells in a murine effector cell system. These authors also reported inhibition in this system mediated by D-galactose, *N*-acetyl-D-glucosamine, and D-glucose. D-mannose was only slightly more effective than the others at 50–100 mM. Similar studies by these authors demonstrated that the same sugars did not inhibit cytotoxicity by allosensitized T cells.

In this study we have demonstrated that NCMC may be effectively inhibited by Man-6-*P*, Fru-1-*P*, and Fru-6-*P* but not by neutral sugars, in marked contrast to inhibition of murine NCMC reported by Stutman *et al.* (10, 11). We feel that the lower inhibitory concentration (Fig. 3) and shorter time of onset (Fig. 2) noted for Man-6-*P* and Fru-1-*P* suggest that the receptor is specific for these stereochemically related molecules and that inhibition by Fru-6-*P* may be due to its enzymatic conversion to Man-6-*P*. The increased sensitivity of F-265 cell line to the toxicity of gelonin-Man_5-6-*P* as compared to K-562 cells, in addition to their increased susceptibility to Man-6-*P* inhibition of NCMC provides strong but not unequivocal evidence that the effect of Man-6-*P* is on the target cell rather than the effector cell. The effector cells in this reaction may have on their membranes glycoproteins containing complex oligosaccharides capable of recognition by lectin-like receptors on the surface of the target cells, by analogy with the suggestion by Stutman. We assume that these receptors also recognize Man-6-*P* or Fru-1-

P, which are related stereochemically (12). This receptor also appears to recognize a product derived from Fru-6-*P*. This model would predict that coincubation in the presence of Man-6-*P* would block this receptor, decreasing the binding of effector cell to target cell, with a subsequent decrease in the level of cytotoxicity. An alternative is that the hexose phosphate receptor would not be the same as the natural cytotoxicity receptor but would be in close proximity.

The data presented here also may be interpreted as suggesting that a receptor for Man-6-*P* is involved in the cytolytic phase of this reaction. Thus, Man-6-*P* may prevent a molecule with lytic activity from binding to the appropriate receptor on the target cell. This is especially interesting because receptors for Man-6-*P* have been shown to be the mechanism by which certain lysosomal hydrolases can be reintroduced into some cells (12–14). In that case, just as here, Man-6-*P* and Fru-1-*P* are effective in blocking the process. The lytic effector molecule may contain a Man-6-*P* residue for which the target cell has a complementary binding site. Such an effector molecule could be targeted to the lysosome by recognition of Man-6-*P*. This molecule might then pass from the lysosome to the cytosol where its cytolytic action would be manifested. A similar mechanism was suggested by Roder *et al.* (25), who demonstrated that chloroquine, an inhibitor of lysosomal function (26), can inhibit NCMC in mice. We have found that 10 mM NH_4Cl and 10 μM chloroquine are both effective inhibitors of human NCMC. This strengthens the argument for involvement of a lysosomal pathway in the lytic event of NCMC. That this uptake pathway can be used to introduce toxic molecules into these target cells is demonstrated by the Man_5-6-*P*-directed internalization of the toxic seed protein, gelonin, into K-562 or F-265 cells. Recent evidence provided by Wright and Bonavida (27) has shown that murine spleen cells and human PBL can be stimulated to produce soluble mediators that preferentially lyse NCMC-sensitive target cells and not NCMC-insensitive targets. The lysis of Yac-1 by soluble mediators from stimulated mouse spleen cells was inhibited in the presence of 50 mM α-methyl-D-mannoside, D-galactose, and *N*-acetyl-D-galactosamine but not by glucose, arabinose, or L-sorbose. These data provide further support for the involvement of a carbohydrate in the lytic phase of NCMC.

In summary, we report here the inhibition of NCMC by Man-6-*P*, Fru-1-*P*, and Fru-6-*P*. The two most likely explanations for this observation are: (*i*) these hexose phosphates compete for binding to the recognition receptor on the target cell surface and prevent the subsequent binding of the effector cell to the target cell or (*ii*) the hexose phosphates prevent binding of a cytolytic effector molecule to the target cell and its subsequent uptake by the target cell.

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