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Immunology

Disruption of oligosaccharide processing in murine tumor cells inhibits their susceptibility to lysis by activated mouse macrophages

(nonspecific tumoricidal activity/deoxynojirimycin/interferon-γ/surface carbohydrates/P815 cells)

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ABSTRACT **The components of tumor cell surfaces that participate in the recognition and lysis of these cells by activated macrophages have not been identified. One plausible hypothesis is that these components are specific carbohydrate structures. As an initial test of this hypothesis, I have made use of the oligosaccharide processing inhibitors 1-deoxynojirimycin (dNM) and 1-deoxy*manno*jirimycin (dMM). dNM is an inhibitor of the glucosidases involved in the initial steps of oligosaccharide processing. dMM inhibits mannosidase I. P815 cells incubated in the presence of 1–2 mM dNM for 24 hr synthesized mature glycoproteins that contained glucosylated high-mannose asparagine-linked oligosaccharides instead of complex forms. The glucosylated oligosaccharides were present in trypsin digests of the cell surface. The dNM treatment resulted in a diminution in the amount of surface galactose residues as evidenced by neuraminidase/galactose oxidase/NaB^3H_4 labeling of surface glycopeptides. It did not, however, inhibit protein synthesis or alter the surface polypeptide profile of the tumor cells. P815 and R1− cells incubated in the presence of 1–3 mM dNM for 24 hr were considerably less sensitive to lysis by interferon-γ-activated macrophages than were cells incubated in control medium. At a dNM concentration of 3 mM, a 71% inhibition of P815 cell lysis was observed. Similarly, P815 and R1− cells incubated in the presence of 2 mM dMM were also less sensitive to macrophage-mediated lysis than were control cells. The inhibitors did not affect cell viability, growth, or gross morphology. These observations suggest that complex asparagine-linked oligosaccharides on tumor cell surfaces may participate in recognition and lysis by activated macrophages.**

In response to appropriate stimuli, macrophages can lyse syngeneic, allogeneic, and xenogeneic tumor cells but not normal cells, a phenomenon referred to as "nonspecific tumoricidal activity" (1). This phenomenon may represent an important component of host resistance to neoplastic growth (2). Moreover, amplification of macrophage-mediated tumoricidal activity could provide an effective means for the treatment of some types of malignant disease (3). In fact, recent studies on tumor necrosis factor, a product of activated macrophages, have emphasized the significance of the macrophage in tumor cell killing (4, 5).

Despite the importance of macrophage-mediated cytotoxicity, little is known about the *mechanism* by which activated macrophages recognize tumor cells. The interaction between macrophages and tumor cells is a nonphagocytic event involving intimate cell–cell contact or "binding" (1, 6). Binding is prerequisite for lysis (7, 8). Binding also mediates tumor cell recognition, and it may concentrate the macrophage products involved in tumor cell lysis (9).

The recognition structures on tumor cells involved in macrophage interactions are present on the cell surface and are shared by tumors of disparate origins (10). Most likely, these components increase in number as a result of oncogenic transformation, thereby constituting a common structural feature of tumor cell surfaces. However, the biochemical identities of the recognition structures have not been defined. Carbohydrates are a major class of surface components that could mediate tumor cell recognition by activated macrophages. It is well established that oncogenic transformation alters the structure and expression of cell surface carbohydrates (11). In fact, one of the most characteristic and ubiquitous changes in oncogenic transformation is an increase in the size of complex asparagine (Asn)-linked oligosaccharides (12). This quantitative change results from an increase in oligosaccharide "branching" or "antennary structure" (13). The functional correlates of these oligosaccharide alterations have not been elucidated.

We have begun to examine the possibility that specific Asn-linked oligosaccharides on tumor cell surfaces are involved in the recognition and lysis of tumor cells by activated macrophages. Asn-linked complex oligosaccharides are synthesized by a common pathway involving the transfer of a large precursor oligosaccharide from a dolichol donor to an appropriate asparagine residue in a nascent polypeptide (14). Subsequent to transfer, a series of enzymatic processing steps are initiated by the removal of the three glucose residues by glucosidases I and II. Additional processing steps involve the removal of specific mannose residues and the addition of *N*-acetylglucosamine, galactose, sialic acid, and fucose residues to form mature complex oligosaccharides (see Fig. 1 for additional details). The glucose analogue 1-deoxynojirimycin (dNM) is a specific inhibitor of glucosidases I and II (17). Cells incubated in the presence of dNM synthesize mature glycoproteins that contain immature glucosylated oligosaccharides. The mannose analogue 1-deoxy*manno*jirimycin (dMM) inhibits mannosidase I (18). Similar to the effects of dNM, cells incubated in the presence of dMM synthesize mature glycoproteins containing high-mannose Asn-linked oligosaccharides. The approach employed in the present set of experiments involved characterization of the biochemical alterations induced by dNM in murine tumor cells and the assessment of the effects of dNM and dMM on the susceptibility of these tumor cells to lysis by activated peritoneal macrophages.

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Abbreviations: dNM, 1-deoxynojirimycin; dMM, 1-deoxy*manno*jirimycin; endo H, endo-β-*N*-acetylglucosaminidase H.

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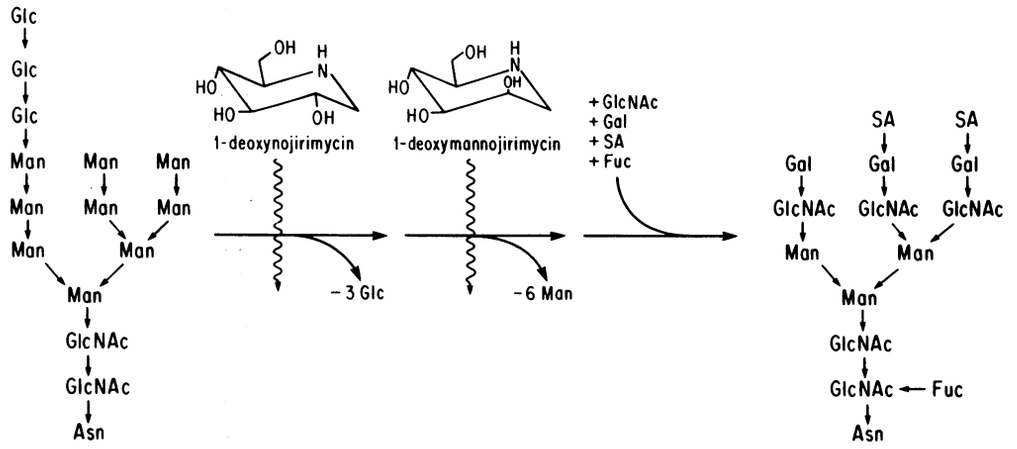

FIG. 1. Salient features of the biosynthesis of Asn-linked oligosaccharides. A large precursor oligosaccharide with the composition $Glc_3Man_9GlcNAc_2$ is synthesized on a dolichol-linked precursor and transferred *en bloc* to an appropriate asparagine residue in a nascent polypeptide. This large precursor oligosaccharide is enzymatically processed or "trimmed" by the initial removal of the three glucose residues by glucosidases I and II. Subsequently, four (α1-2)mannose residues are removed by mannosidase I. The removal of an (α1-3)- and an (α1-6)mannose residue is triggered by the addition of an *N*-acetylglucosamine residue. Finally, *N*-acetylglucosamine, galactose, fucose, and sialic acid (SA) residues are added to form mature complex oligosaccharides. Additional details and structures can be found in ref. 15 and 16. The glucose analogue 1-deoxynojirimycin (dNM) is a specific inhibitor of the glucosidases involved in the trimming of the precursor oligosaccharide (17). The major effect of dNM on oligosaccharide biosynthesis is to prevent processing of the precursor oligosaccharide such that mature glycoproteins containing asparagine glycosylation sites acquire oligosaccharides with the composition $Glc_{1-3}Man_{7-9}GlcNAc_2$ instead of complex forms. The mannose analogue 1-deoxy*manno*jirimycin (dMM) is a specific inhibitor of mannosidase I. dMM treatment results in the synthesis of mature glycoproteins containing high-mannose Asn-linked oligosaccharides (18).

MATERIALS AND METHODS

Mice. Female C57BL/6 mice obtained from The Jackson Laboratory at 7 weeks of age were used in all experiments.

Macrophages. Thioglycollate-elicited peritoneal macrophages were obtained as described previously (19) from mice that had been injected 4 days before sacrifice with 1.5 ml of a 4% (wt/wt) solution of thioglycollate broth (Difco). This procedure yielded 2–3 $\times$ 10^7 peritoneal cells, of which 80–90% were macrophages as determined by Wright–Giemsa staining.

Approximately 5×10^5 thioglycollate-elicited macrophages in 100 μl of minimal essential medium containing 10% heat-inactivated fetal bovine serum were plated in 6-mm wells of a 96-well microtiter plate. After 30 min at 37°C, an additional 100 μl of medium containing *Escherichia coli*-derived recombinant interferon-γ (Genentech, South San Francisco, CA) at 32 units/ml and lipopolysaccharide (*Salmonella typhosa* 0901; Difco) at 5 ng/ml was added to each well. The microtiter plates were incubated at 37°C for 5 hr, and then the wells containing macrophage monolayers were washed vigorously with warm medium and used in the cytolysis assays described below.

Tumor Cell Lines. The following murine tumor cell lines were used: P815, a mastocytoma derived from DBA/2 mice, and R1−, a murine T-cell lymphoma that does not express H-2 antigens on its surface (20).

The tumor cells were maintained *in vitro* in RPMI medium containing 10% heat-inactivated fetal bovine serum and 2 mM glutamine (control medium). For experiments, cells (10^6) in the exponential phase of growth were incubated at 37°C in 12 ml of control medium or medium containing various concentrations of dNM or dMM. The dNM and dMM were gifts of Bayer Chemical (Federal Republic of Germany). After 24 hr of incubation, the cells were washed several times with control medium and were used in the experiments described below.

Cytolysis Assay. The susceptibility of tumor cells, maintained in either control medium or medium containing inhibitors, to lysis by activated macrophages was assessed by an 18-hr $Na_2{}^{51}CrO_4$ (^{51}Cr; New England Nuclear) release assay (19). The assay was carried out in triplicate in 6-mm wells of a 96-well microtiter plate containing 5×10^5 activated macrophages and 5×10^4 ^{51}Cr-labeled tumor cells (see above). The spontaneous release of ^{51}Cr from the tumor cells during the course of the assay was less than 25% of the total label incorporated. The percent net cytolysis is defined as [released cpm in experimental wells − cpm spontaneous release)/(total cpm releasable − cpm spontaneous release)] $\times$ 100.

Metabolic Labeling. P815 cells were incubated in either control medium or medium containing dNM, as described above. After 1 hr, [2-^{3}H]mannose (0.5 mCi; American Radiolabeled Chemicals, St. Louis, MO; 1 Ci = 37 GBq) was added to each population of cells, and the cells were incubated for an additional 23 hr. The cells were washed in phosphate-buffered saline (0.01 M phosphate, pH 7.2/0.15 M NaCl/1 mM Ca^{2+}/1 mM Mg^{2+}) and solubilized in 1% $NaDodSO_4$. Glycoproteins were precipitated by the addition of cold acetone as described (21). The precipitated glycoproteins were digested exhaustively with Pronase (Calbiochem) for 72 hr (19). The Pronase glycopeptides were digested further with endo-β-*N*-acetylglucosaminidase H (endo H) for 24 hr (22), and the products were chromatographed on Bio-Gel P-4 (−400 mesh; Bio-Rad).

In addition, P815 cells incubated in the presence of dNM and [^{3}H]mannose for 24 hr, as described above, were washed and treated with 0.1% trypsin (GIBCO) in phosphate-buffered saline for 10 min at room temperature. The cells were pelleted by centrifugation and the supernatants containing tryptic glycopeptides were digested with Pronase and endo H (19). The digests were chromatographed on Bio-Gel P-4 (−400 mesh). Trypan blue staining was employed to assess the viability of the cells after the trypsin treatment.

Surface Labeling. P815 cells were incubated in control medium or in medium containing 2 mM dNM for 24 hr, washed with phosphate-buffered saline, and incubated in 1 ml of phosphate-buffered saline containing 40 μg units of *Vibrio cholerae* neuraminidase (Calbiochem) and 15 units of galactose oxidase (Worthington) for 1 hr at 37°C (19, 21). Subsequently, the cells were washed in phosphate-buffered saline and were incubated for an additional 30 min at room tem-

perature in 1 ml of phosphate-buffered saline containing 1 mCi of NaB^3H_4 (10 Ci/mmol; American Radiolabeled Chemicals). The cells were washed in phosphate-buffered saline and solubilized in 1% $NaDodSO_4$. Acetone precipitates were digested with Pronase as described (19). The Pronase digests were chromatographed on Bio-Gel P-6 (200–400 mesh).

P815 cells incubated in normal medium or in medium containing 2 mM dNM were surface iodinated (23) with ^{125}I (carrier free; New England Nuclear NEZ 033) and lactoperoxidase (Sigma). $NaDodSO_4$ extracts of the iodinated cells were analyzed by polyacrylamide gel electrophoresis and autoradiography.

RESULTS

dNM Disrupts Oligosaccharide Processing in P815 Cells. P815 cells were incubated in the presence of [2-^{3}H]mannose for 24 hr either in control medium or in medium containing dNM. Glycopeptides were obtained from these cells by exhaustive Pronase digestion. After endo H treatment, the glycopeptides were separated on the basis of size by gel filtration chromatography on Bio-Gel P-4 (−400 mesh). Endo H cleaves the glycosidic bond between the *N*-acetylglucosamine residues of the chitobiose core of precursor and high-mannose Asn-linked glycopeptides but not of complex glycopeptides (22). Thus, endo H treatment resolves complex from high-mannose structures in gel filtration analyses. As shown in Fig. 2, the largest high-mannose oligosaccharide observed in control cells migrates in a position corresponding to $Man_9GlcNAc$. In contrast, the largest high-mannose oligosaccharides observed in the presence of dNM migrate in positions corresponding to $Glc_1Man_9GlcNAc$ and $Glc_2Man_9GlcNAc$. Since mannose processing can proceed in the absence of glucose removal (e.g., see ref. 24), it is quite possible that the oligosaccharide species observed in Fig. 2 are $Glc_3Man_7GlcNAc$ and $Glc_3Man_8GlcNAc$. It is also apparent from Fig. 2 that, in the presence of increasing concentrations of dNM, the ratio of high mannose to complex peaks (migrating near the exclusion volume V_e) is increased markedly. These data indicate that dNM blocks the maturation of high-mannose to complex oligosaccharides in P815 cells.

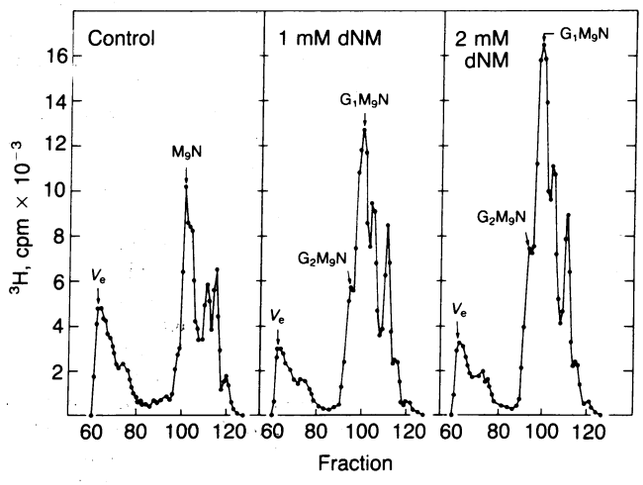

FIG. 2. Effect of dNM on the biosynthesis of Asn-linked oligosaccharides in P815 cells. P815 cells in the exponential phase of growth were incubated in flasks containing either control culture medium or medium containing dNM for 1 hr. Subsequently, 0.5 mCi of [^{3}H]mannose was added to the flasks and the cells were incubated for an additional 23 hr at 37°C. Cell lysates were digested exhaustively with Pronase and treated with endo H. The Bio-Gel P-4 (−400 mesh) glycopeptide elution profiles of the Pronase-digested and endo H-treated samples are shown for cells incubated in control medium (*Left*), medium containing 1 mM dNM (*Center*), medium containing 2 mM dNM (*Right*). The migration positions of the standards $Man_9GlcNAc$ (M_9N), $Glc_2Man_9GlcNAc$ (G_2M_9N), and $Glc_1Man_9GlcNAc$ (G_1M_9N) are indicated. The exclusion volume of the column is indicated by V_e.

dNM Modifies the Surface Carbohydrate Profile of P815 Cells. Data were obtained to determine if dNM disrupts the *surface* glycopeptide profile of P815 cells. P815 cells incubated in the presence of [^{3}H]mannose for 24 hr either in control medium or in medium containing 2 mM dNM were subjected to mild trypsin digestion under conditions in which no cell lysis was evident (as evidenced by trypan blue staining). The trypsin-treated samples were digested exhaustively with Pronase, incubated in the presence of endo H, and analyzed on Bio-Gel P-4. The gel filtration profiles obtained (data not shown) were similar to those shown in Fig. 2—i.e., the largest peaks observed in control cells corresponded to $Man_9GlcNAc$ and $Man_5GlcNAc$, and the largest peaks in dNM-treated cells corresponded to $Glc_1Man_9GlcNAc$ and $Glc_2Man_9GlcNAc$.

In addition to the above experiment, control and dNM-treated cells were surface labeled by the neuraminidase/galactose oxidase/NaB^3H_4 method (25), and Pronase digests of the labeled cells were analyzed by gel filtration chromatography on Bio-Gel P-6 (Fig. 3). This method labels galactose (and *N*-acetylgalactosamine) residues at the nonreducing termini of oligosaccharide chains, as well as galactose (and *N*-acetylgalactosamine) residues that are penultimate to sialic acid residues. It is apparent from the data in Fig. 3 that a marked reduction in the number of terminal and subterminal surface galactose (or *N*-acetylgalactosamine) residues is observed in cells incubated in the presence of dNM in comparison to cells incubated in control medium.

dNM Does Not Alter Protein Synthesis or the Surface Polypeptide Profile in P815 Cells. To evaluate the effect of dNM on protein synthesis, P815 cells were incubated in the presence of [^{35}S]methionine (New England Nuclear) for 24 hr either in control medium or in medium containing 2 mM dNM. The amount of radioactivity present in trichloroacetic acid precipitates obtained from both populations of cells was determined. The presence of dNM in the medium did not inhibit protein synthesis in P815 cells by more than 10% in several independent experiments (data not shown).

The effect of dNM on the surface polypeptide profile of P815 cells was assessed by polyacrylamide gel electropho-

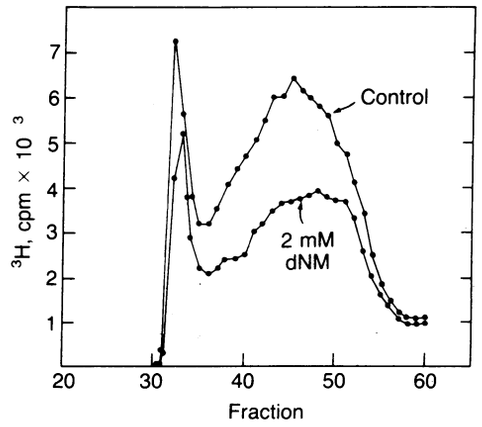

FIG. 3. Effect of dNM on the surface carbohydrate profile of P815 cells. P815 cells in the exponential phase of growth were incubated in either control medium or medium containing 2 mM dNM for 24 hr. The cells were washed and surface labeled by the neuraminidase/galactose oxidase/NaB^3H_4 method. Cell lysates were digested exhaustively with Pronase and the glycopeptides were chromatographed on Bio-Gel P-6 (200–400 mesh).

resis and autoradiography of cell lysates obtained from ^{125}I/lactoperoxidase-labeled cells incubated for 24 hr in control medium or in medium containing 2 mM dNM. As seen in Fig. 4, no major differences are evident in the surface polypeptide profile of control and dNM-treated cells.

dNM Inhibits Cytolysis of P815 and R1− Murine Tumor Cells by Activated Mouse Peritoneal Macrophages. Murine tumor cells were incubated in the presence of dNM for 24 hr and their susceptibility to lysis by activated murine macrophages was compared to that of control cells by using an 18-hr ^{51}Cr-release assay. Specifically, two different murine tumor cell lines, the mastocytoma P815 and the lymphoma R1− [which does not express surface H-2 (20)] were incubated in control cell culture medium or in the presence of medium containing various concentrations of dNM for 24 hr. The cells were washed, labeled with ^{51}Cr, and added to monolayers of interferon-γ activated peritoneal macrophages. At the concentrations used in these experiments, dNM did not affect cell viability, growth, or gross morphology. Additional evidence to indicate that dNM had no deleterious effects on the target cells is indicated by the fact that the spontaneous release of ^{51}Cr during the course of the 18-hr assay was not altered by the dNM treatment.

The cytolysis data are presented in Table 1. The data indicate that a marked inhibition of cytolysis is observed for both P815 and R1− cells treated with dNM. Moreover, as indicated by the results obtained with P815 cells, the degree of inhibition observed is a direct function of the dNM concentration.

The inhibitory effect of dNM on cytolysis was reversible. P815 cells exposed to medium containing dNM for 24 hr followed by incubation in control medium reacquired the susceptibility to lysis characteristic of control cells within 48 hr (data not shown).

Mannosidase I Inhibitor dMM Reduces Cytolysis of P815 and R1− Murine Tumor Cells by Activated Macrophages. P815 and R1− cells were incubated in the presence of 2 mM dMM for 24 hr, and their susceptibility to macrophage-mediated lysis was compared to that of control cells (Table 1). Significant inhibition of cytolysis was observed in the presence of dMM for both P815 and R1− cells. Specifically, a 30% inhibition of macrophage-mediated lysis was observed for both P815 and R1− cells incubated in medium containing 2 mM dMM in comparison to control populations of these cells incubated in the absence of dMM. However, this inhibition was not as marked as that observed with 2 mM dNM (Table 1).

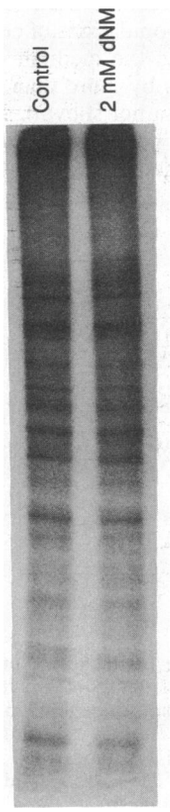

FIG. 4. Effect of dNM on the surface polypeptide profile of P815 cells. P815 cells in the exponential phase of growth were incubated in either control medium or medium containing 2 mM dNM for 24 hr. The cells were washed and surface labeled by the ^{125}I/lactoperoxidase method. Detergent ($NaDodSO_4$) extracts of the cells were analyzed by polyacrylamide gel electrophoresis (10% gel) and autoradiography.

Table 1. Effects of oligosaccharide processing inhibitors on macrophage-mediated lysis of murine tumor cells

Cells	Condition	Net cytolysis, %	Inhibition, %
P815	Control	41.1	—
	1 mM dNM	29.1	29.2
	2 mM dNM	21.4	47.9
	3 mM dNM	11.9	71.0
	2 mM dMM	28.5	30.7
R1−	Control	37.7	—
	2 mM dNM	12.3	67.4
	2 mM dMM	26.0	31.0

Murine tumor cells were incubated in control medium (RPMI with 10% fetal calf serum) or in this medium containing either dNM or DMM for 24 hr. The cells were washed, labeled with ^{51}Cr, and added to monolayers of interferon-γ-activated mouse macrophages. The results presented are representative of the results obtained in several different experiments. The number of experiments represented for each condition is as follows: P815 control, 4; 1 mM dNM, 1; 2 mM dNM, 4; 3 mM dNM, 3; 2 mM dMM, 2; R1− control, 2; 2 mM dNM, 1; 2 mM dMM, 2.

DISCUSSION

Understanding macrophage–tumor cell interactions will require a thorough description of the cell surface components involved. The components of tumor cell surfaces involved in recognition and binding by macrophages most likely increase in number as a result of oncogenic transformation and constitute a ubiquitous structural feature of tumor cell surfaces (1, 9). The results presented here provide initial evidence for the participation of specific carbohydrate sequences on tumor cell surfaces in macrophage–tumor cell interactions. Specifically, we have demonstrated that perturbation of Asn-linked oligosaccharide processing in murine tumor cells renders the tumor cells considerably less sensitive to macrophage-mediated lysis than tumor cells not treated with such inhibitors.

The possibility exists that carbohydrates may play a secondary role in macrophage–tumor cell interactions. For example, oligosaccharides may affect the conformation of a specific surface protein that is the primary recognition structure. Such a secondary role cannot be excluded in light of the existing data. However, *preliminary* reports from other laboratories suggest that the binding of tumor cells by macrophages may be mediated by galactose-containing carbohydrates on tumor cell surfaces (26, 27). Thus, one plausible, though not exclusive, interpretation of the existing data is that specific carbohydrates on tumor cell surfaces are essential for the effective recognition and lysis of tumor cells by activated macrophages.

In the present study, we have made use of inhibitors of two processing enzymes involved in the synthesis of complex Asn-linked oligosaccharides, dNM and dMM. Unlike tunicamycin, which blocks all Asn-linked glycosylation (28),

dNM and dMM allow glycosylation of proteins. In contrast to the effects of tunicamycin, therefore, proteins are not degraded more rapidly, nor are they prevented from reaching the cell surface, in the presence of these processing inhibitors (29). Moreover, although tunicamycin has been observed to inhibit glycolipid biosynthesis (30), dNM had no such inhibitory effect in P815 cells, at the concentrations employed in the present experiments (G. Schwarting and A.M., unpublished data). For these reasons, dNM and dMM are useful tools for examining the involvement of complex Asn-linked oligosaccharides in cellular functions.

One of the most reproducible changes of oncogenic transformation is an increase in the size of complex Asn-linked oligosaccharides (12, 13). This alteration results from a quantitative increase in oligosaccharide branching or antennary structure (13). Transformed cells are characterized by an increase in triantennary oligosaccharides containing the GlcNAc(β1-6)Man(α1-6) branch and in tetraantennary oligosaccharides [all of which contain this branch (31)]. These increases are compensated for by decreases in high-mannose oligosaccharides, biantennary oligosaccharides, and triantennary oligosaccharides containing the GlcNAc(β1-4)Man(α1-3) branch (31). Although such structural alterations constitute a characteristic feature of tumor cell surfaces, their functional correlates are not known. In view of the data presented in this paper, it is possible that highly branched Asn-linked oligosaccharides serve as recognition sites for activated macrophages. Several structural features could contribute to the preferential recognition of highly branched oligosaccharides. They include (*i*) the distribution, or density, of terminal sugars [e.g., galactose and sialic acid (32)], (*ii*) the spatial conformation of the oligosaccharide (33, 34), and (*iii*) specific carbohydrate sequences such as the GlcNAc(β1-6)Man(α1-6) branch (31, 35).

In summary, the data presented provide an experimental basis for the rigorous testing of the hypothesis that specific carbohydrate structures on tumor cell surfaces participate in recognition and lysis by activated macrophages.

Note. Castanospermine (1,6,7,8-tetrahydroxyoctahydroindolazine) is an inhibitor of glucosidase I (24). A 56% inhibition of macrophage-mediated lysis was observed for P815 cells incubated in the presence of castanospermine (Calbiochem) at 20 μg/ml for 24 hr in comparison to cells incubated in control medium.

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