Natural cytotoxic cells against solid tumors in mice: Blocking of cytotoxicity by D-mannose

(natural killer cells/natural cytotoxicity)

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ABSTRACT Natural cytotoxic (NC) and natural killer (NK) cells have been defined by their ability to lyse certain solid or lymphoid tumor targets in vitro, without prior sensitization. Our present studies describe an attempt to characterize the structures involved in the effector–target recognition leading to tumor cell lysis. Addition of the monosaccharide D-mannose to the NC cell assay significantly blocked cytotoxicity of the fibrosarcoma Meth A target by the effector cells at 50 mM and lower concentrations. D-Galactose showed blocking activity in one of five experiments, only at 50 mM. L-Fucose, D-glucose, and N-acetyl-D-glucosamine did not affect NC cell cytotoxicity at similar concentrations. All of the sugars tested inhibited NK cell lysis of the lymphoma YAC-1 target. None of the sugars affected killing of the appropriate target by allosensitized cytotoxic T lymphocytes. The blocking of NC-mediated cytotoxicity was not due to a direct toxic action of the sugars on the effector cells. These findings suggest that, in the NC system, recognition involves lectin-like structures with a specificity for D-mannose (or D-galactose, or both), whereas, in the NK system, such lectin-like structures are less restricted. Such structures appear not to be involved in the specific cytotoxicity mediated by T cells.

Although there has been extensive characterization of many features of natural cell-mediated cytotoxicity (CMC) in mice, especially of the "natural killer" (NK) type (1–3), little is known about the recognition structures or target sites involved in the effector–target cell interaction leading to lysis. In addition, when different tumor targets are studied, some heterogeneity of effector cells is found, and subtypes such as the "natural cytotoxic" (NC) cells, which react with adherent target cells derived from nonlymphoid tumors, have been described (4). In both the NK and NC systems, targets may be susceptible or resistant to lysis by such effector cells (1–4). Competitive ("cold-target") inhibition studies with NK cells (1–3) and assays that determine the binding of effector cells to sensitive targets (5) have suggested a certain degree of specificity of recognition. In addition, crude cell extracts, probably glycoprotein in nature, from NK-susceptible targets can inhibit or block the binding of effector NK cells to the appropriate susceptible target (6).

In the present study we show that some simple sugars can block NC- and NK-mediated CMC in vitro, with a preferential effect for D-mannose in the NC system. On the other hand, none of the sugars tested, even at higher concentrations, had any effect on cytotoxicity mediated by allosensitized T cells. These results suggest that simple sugars, probably as part of complex membrane glycoproteins or glycolipids, act as recognition sites for NC and NK cells.

MATERIALS AND METHODS

Animals. BALB/c and C57BL/6J mice were bred in our colony; see ref. 4 for further details. All experiments were done with mice of both sexes, between 8 and 12 weeks of age.

Target Cells. For details on Meth A, a cell line derived from a BALB/c chemically induced fibrosarcoma, see ref. 4. YAC-1 cells were obtained from G. Cudkowicz (State Univ. of New York, Buffalo, NY). EL4 was obtained from P. Ralph (Sloan-Kettering Institute, Rye, NY).

Sugars. All the sugars tested (see Tables 1 and 2) were obtained from Sigma. Stock solutions were made in medium (see details below), and sterilized by filtration prior to use.

NC Assay. The NC proline assay has been described in detail (4). Briefly, 10^4 [3H]proline-labeled Meth A target cells were allowed to adhere to the wells of a 96-well microplate (Falcon), for 24 hr, in 0.15 ml of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1% L-glutamine, 1% penicillin/streptomycin, and 1% nonessential amino acids. The sugars, diluted in this medium, were added in 50-μl amounts, followed by the effector spleen cells at 100:1, 50:1, and 10:1 effector-to-target (E:T) ratios. The plates were incubated for 24 hr at 37°C in 5% CO2 in air and washed extensively, and the wells were punched into scintillation vials. Hyamine hydroxide and scintillation fluid were added and the radioactivities were determined. For further details on target cell labeling, effector cell preparation, and counting procedures, see ref. 4. Five or six replicate test wells and controls were used to calculate mean cytotoxicity. Percent cytotoxicity was calculated by using the following equation (4):

\[
\text{% cytotoxicity} = 100 - \left( \frac{\text{cpm effector + targets}}{\text{cpm targets alone}} \right) \times 100.
\]

NK and Cytotoxic T Lymphocytes (CTL) Assay. The 51Cr release assay has been described (4, 7). Briefly, 2 x 10^4 51Cr-labeled target cells (YAC-1 for NK and EL4 for CTL) were added to wells of a 96-well microplate in 0.05 ml of the medium described above. The sugars were added in 0.05 ml, followed by the effector cells in 0.1 ml. The plates were then centrifuged 3 min at 250 g and incubated (37°C in 5% CO2) for 4 hr. After the incubation, the plates were centrifuged (10 min at 250 g) and 0.1 ml of medium was removed from each well and assayed for radioactivity in a Packard scintillation counter. Maximal releasable 51Cr was measured after target cell lysis with 1% Nonidet P-40. Percent cytotoxicity was calculated by the following formula:

Abbreviations: CMC, cell-mediated cytotoxicity; NK, natural killer cells; NC, natural cytotoxic cells; CTL, cytotoxic T lymphocytes; E:T, effector:target (ratios).

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CTL Generation. CTL were generated as described (7). Briefly, 7 × 10⁶ BALB/c spleen cells were cocultured (37°C in 5% CO₂) with 3 × 10⁶ irradiated [1500 roentgens (0.4 coulomb/kg)] C57BL/6 spleen cells in 2 ml of RPMI 1640 medium supplemented as above plus 57 µM 2-mercaptoethanol in wells of a 24-well Linbro plate (Flow Laboratories, McLean, VA). After 5-day culture the cells were harvested by pipette, washed, and assayed for cytotoxicity in the above ⁵¹Cr release assay using cells of EL4, an H-2b C57BL/6 lymphoma, as the targets.

RESULTS

CMC by NC Cells is Blocked by D-Mannose and D-Galactose. Table 1 shows a representative experiment on the effects of some simple sugars at 50, 20, and 10 mM on CMC by NC cells against adherent tumor targets (4). D-Mannose had a significant blocking effect at all concentrations tested, and the same significant blocking of CMC was observed at 50:1 and 10:1 E:T ratios (data not shown). D-Galactose produced significant blocking of CMC only at 50 mM, whereas the other sugars tested had no effect. Similar results were observed in five experiments: in four experiments D-mannose showed significant blocking of NC-mediated cytotoxicity at the three concentrations, whereas D-galactose showed blocking at only 50 mM and in only one of the five experiments. The other sugars produced no effect. N-Acetyl-D-galactosamine at those same concentrations was also without blocking effect in two experiments (data not shown). At higher concentrations (200 and 100 mM) all sugars blocked NC-mediated CMC; however, maximal blocking was observed only with D-mannose. For example, at 100 mM, D-mannose produced 98% inhibition of CMC, whereas L-fucose, D-galactose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, and D-glucose produced CMC inhibition ranging from 40 to 59% of the control values.

At the concentrations used in Table 1, the toxicity of the sugars alone for the target cells was less than 2%, after 24-hr incubation. At 100 mM, toxicity ranged from 2% to 10%, with D-mannose always in the upper 5–10% range.

Inhibition of NC-Mediated CMC by D-Mannose Is Not Due to Toxicity to the Effector Cells. When the effector spleen NC cells were preincubated for 24 hr in the presence of 100 mM D-mannose (and the other sugars used in Table 1), extensively washed, and subsequently used in the CMC assay against the Meth A targets, percent CMC at 100:1 E:T ratios was 43% for the controls preincubated without sugars and 45% for the spleen cells preincubated with D-mannose or the other sugars. On the other hand, the addition of 50 mM D-mannose to the effector and target mixture produced 59 and 53% reduction of CMC in the two cases. Thus, the effector NC cells preincubated with D-mannose were still functional and were still able to be blocked by the sugar in the CMC assay.

It is obvious that due to the length of the NC cytotoxicity assay, these pretreatment experiments cannot be considered as arguing against a recognition unit for D-mannose on the effector cells, and only indicate that D-mannose and the other sugars are not inhibiting NC-mediated CMC by a trivial toxicity to the effector cells. Similarly, the blocking was not due to alterations in ionic strength in the culture medium, because 20 and 50 mM L-glutamine had no effect on NC-mediated CMC (data not shown).

CMC by NK Cells Is Blocked by Simple Sugars Without Selectivity for D-Mannose. Table 2 shows the effect of some sugars on the CMC by NK cells, tested against lymphoma target cells in suspension in a short-term assay (1–3). All of the sugars produced blocking of NK-mediated CMC at 50 and 20 mM, and all sugars, with the exception of D-glucose, also blocked at 10 mM. Similar results were observed in five additional experiments. The same type of blocking of NK activity was observed at 50:1 and 10:1 E:T ratios (data not shown).

CMC by Allosensitized CTL Is Not Affected by Sugars. Table 2 also shows that the specific CMC by allosensitized CTL was not affected by 50 mM sugars, and that only at 100 mM was blocking by D-galactose and D-glucose observed. The same lack of blocking by the sugars was observed at 10:1 and 1:1 E:T ratios (data not shown). On the other hand, it is well established that CTL-mediated CMC can be readily blocked by the specific

<table>
<thead>
<tr>
<th>Sugar</th>
<th>% CMC at different sugar concentrations (and % reduction from controls)</th>
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<tbody>
<tr>
<td></td>
<td>0 mM</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>17 (47)</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>32</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>18 (44)</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>32</td>
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Spleen cells from normal BALB/c mice at 100:1 E:T ratios (also 50:1 and 10:1, not shown) were incubated in the presence of the indicated final concentrations of sugars and ⁵¹Cr-labeled YAC-1 targets for 4 hr, for the NK assay. The CTL were generated by in vitro immunization of BALB/c spleen cells with C57BL/6 irradiated cells and tested against ⁵¹Cr-labeled EL4 target cells in a 4-hr assay; data are shown for 5:1 E:T ratios (10:1 and 1:1 also tested).

* P < 0.05 compared to controls, by Student’s t test.
† P < 0.01.
antigen presented either as intact cells (8, 9) or as subcellular preparations (10). The effects of sugars on allosensitized CTL-mediated CMC were studied only marginally in determining the sugar requirements for support of CMC in the presence of dialyzed fetal calf serum (11). In such experiments, glucose, but not galactose or fructose, seemed to be required for appropriate expression of CMC (11).

As was indicated earlier, both NK and CTL activities are measured in short-term assays, whereas NC activity requires a longer assay (4, 12). Thus, if some nonspecific toxic effects of the sugars on targets, effector cells, or both are involved in the present phenomenon, such effects would be manifest in the long-term assay. However, the "selective" blocking by D-mannose (and D-galactose) was observed only in the long-term assay, suggesting that the blocking of NC-mediated CMC is specific for those sugars. This would support our view that NC-mediated CMC (as well as NK-mediated) may be directed against sugar-containing structures on the target cell membrane.

**DISCUSSION**

It is well accepted that CTL-mediated CMC can be specifically blocked either by covering the relevant antigenic sites on the targets with antibody or by competitive saturation of the receptor sites on the effector cells by the appropriate antigen; these studies were done mostly with allosensitized CMC (8–10). Other procedures that inhibit CMC in a nonspecific way include addition of a variety of metabolically inhibitors or procedures that prevent contact between effector and target cells (9). In the NK system, the "specificity" of the lytic event has been studied by competitive (or "cold-target") inhibition using a variety of susceptible targets (1–3) or by NK effector–target binding assays (5, 6, 13). However, there is no clear agreement concerning the nature of the structures being recognized on the targets by the NK cells (1–3, 5, 6). In both the NK (1–3) and the NC (4, 12) systems, the CMC reactivity appears not to be directed against murine leukemia virus determinants. Other target cells susceptible to NC-mediated CMC, such as Meth A, can act as cold-target inhibitors for NK-susceptible targets, such as YAC-1 (unpublished results), suggesting that comparable structures determine target cell susceptibility to NC and NK cells.

From the present studies showing that NC-mediated CMC can be effectively blocked by relatively low concentrations of D-mannose (and D-galactose), we propose that the NC effector cells have a "lectin-like" receptor that recognizes neutral sugars on the surface of the target cell, sugars that probably are part of complex glycoproteins or glycolipids of the membrane (14). Saturation of the binding sites by the sugars prevents the appropriate effector–target interaction and lysis is inhibited. Whether the effect is on binding to the target or on the lytic process itself requires further study. Direct contact between NC effectors and targets is required for lysis to take place (unpublished results). For the NK cells, which show blocking by sugars besides D-mannose and D-galactose, a similar lectin-like structure with less specificity for a determined sugar can be postulated. Variation in sugar specificity and avidity of lectins has been described (15). For CTL, such lectin–sugar interactions appear not to be involved in the lytic process, which is dependent on antigen-specific receptor structures (8–10).

Three other interrelated interpretations for the observed results can also be considered: (1) The reverse possibility that the lectin-like sugar receptors may be on the target cells cannot be excluded. If such is the case, the target would bind the effector cells via a lectin-like structure and such binding would lead to lysis. Two variations of this possibility are (ii) that the effecter cells are actually "recognizing" the lectin-like structure on the target (i.e., the effector cells will have "receptor" for the lectin-like structure and not for the sugars; however, binding of the appropriate sugar to the lectin on the target would interfere with the recognition by the effector) and (iii) the lectin-like receptor that binds some sugars is on the target cells but is not the relevant determinant and, once having reacted with the appropriate sugar(s), would block CMC via a steric hindrance; thus, the lectin-like receptor would not be related to the NC or NK-recognition structure but would be in close proximity to it. Further studies are required to determine the mechanism of the blocking of natural CMC by sugars. The facts that susceptibility or resistance to NC killing is, in part, a property of the target (4) and that the capacity to kill certain tumor targets is under genetic control for both NC (4) and NK systems (1–3) do not mitigate for or against the two (or four) possible interpretations. On the other hand, it has been shown that target recognition and binding are necessary but not sufficient for NK (13), NC (unpublished results), and CTL-mediated CMC (9), which may partially support the idea of a recognition unit on the effector cells. Similarly, tumor cells (as well as other cells) do display surface sugars that make them agglutinable by a variety of lectins (14, 15) and that may be related to the structures recognized by NC and NK cells.

Although the NK (1–3) and NC (4, 12) systems of CMC do not fit conventional immunologically specific responses (i.e., no immunological memory, no H2 antigen restriction for lysis, "constant" levels of effector cells, regulation by interferon, etc., see refs. 1–4), our present working hypothesis is that the sugars are acting by saturation of a lectin-like recognition unit on the effector cells.

The role of surface sugars in cell sociology (16) is generally accepted. Interactions between lectin-like structures and either simple sugars or complex glycoproteins or glycolipids from the cell membrane are well-established phenomena that appear important in cell adhesion and aggregation (17–20), platelet activation (19), cell differentiation (17, 21, 22), binding of virus and bacteria to host cells (23–27), virus-induced malignant transformation (16, 28, 29), phagocytosis (30), and fertilization (31). All but the last reference deal with systems involving mammalian cells. Interactions involving D-mannose or mannose-containing glycoproteins have been described for teratocarcinoma cell–cell adhesion (20), attachment of *Escherichia coli* to epithelial cells (26) or macrophages (27), murine sarcoma virus transformation (28), and phagocytosis (30). All these systems used concentrations of sugars comparable to or higher than those used in the present paper for demonstration of the effects.

It is tempting to postulate that a type of interaction similar to that noted above may be involved in the recently described phenomenon of "natural cytoxicity" against tumor cells (1–3), certain normal tissues (3) or exogenous viral or bacterial infections (32). Especially because all of these "natural" responses appear to be mediated by a family of closely related effector cells, which do not have T, B, or macrophage characteristics, and of which NK and NC cells represent the prototypes. In addition, it is tempting to speculate that similar mechanisms may be involved in the regulatory role of interferon on these natural cytoxicities (1–3), especially because the interaction of interferon with the carbohydrate portion of the membrane gangliosides can influence its activity (33).

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