Glycophorin A Protects K562 Cells from Natural Killer Cell Attack

ROLE OF OLIGOSACCHARIDES

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Glycophorin A is a protein with an abundant glycosylation (60% carbohydrate by weight), and studies have suggested that resistance of target cells to natural killing may be correlated with the level of glycophorin A expression. To assess the role of glycophorin A and of its carbohydrates in sensitivity to lysis by natural killer (NK) cells, the glycoprotein was inserted into the membrane of K562 target cells using electroporation. Peripheral blood lymphocytes were used as effector cells. When glycophorin A was inserted into the membrane, the level of resistance to NK cell attack increased with the number of glycophorin A molecules electroinserted. The resistance to lysis was not due to a defect in target cell-effector cell binding. Electromodulation of glycophorin A did not cause a decrease in the expression of either "positive signals" for NK cells (such as CD71, CD15, and CD32 antigens) or cellular adhesion molecules (CD18, CD29, CD54, and CD58). Furthermore, electroinsertion of glycophorin A did not trigger any "negative signals," such as class I HLA antigen expression. Finally, it was shown that the sialic acid and O-linked oligosaccharides of glycophorin A did not play any role in its effect against NK cells. Conversely, the unique N-linked oligosaccharide was shown to be essential for resistance to occur.

Natural killer (NK) cells are generally considered part of the first host defenses against neoplastic and infectious diseases. NK cells are CD3−large granular lymphocytes and can be functionally defined as cells that mediate non-histocompatibility-restricted killing of some target cells. These lymphocytes are spontaneously cytotoxic against some tumors and virally infected cells via nonspecific mechanisms. In addition, NK cells are able to kill certain normal cells in vitro.

Although there has been extensive characterization of many features of NK cells, the NK cell receptors and their ligands on the target cell surface have remained elusive for a long time. Thus, the sensitivity of target cells has been considered alternatively as being due to the expression of immature structures and viral antigens or to an increased expression on the cell surface of normal glycoproteins such as transferrin receptor (CD71) and CD32 antigen (3–5), of glycolipids such as asialo-GM₂ and G_M₂ (6, 7), of oligosaccharides such as 3-fucosyl-N-acetyllactosamine (CD15 antigen) (8), and of peptides such as a 42-kDa polypeptide (9). These molecules could act as "positive signals" on NK cells. Conversely, it has been shown that a high level of cell membrane sialylation decreases the sensitivity of target cells (6, 10). More recently, it has been demonstrated that the expression of MHC class I molecules protects tumor cells (predominantly those of lymphoid origin) against NK cell attack (11–13), acting as a "negative signal" for NK cell-mediated lysis. Moreover, inhibitory receptors for NK cell activation have been identified, and they bind MHC class I molecules (14, 15). Finally, in the past year, a natural killer cell receptor proved to be specific for ubiquitous oligosaccharides and triggers the NK cell cytolytic mechanism (16). In conclusion, the target molecules involved in recognition of target cells by NK cells may contain peptidic or carbohydrate residues that can activate or inhibit the NK cell-mediated killing (17).

In addition to specific NK ligand(s), cell adhesion molecules (such as CD54, CD58, CD56, lymphocyte function-associated antigen 1, and CD2) are also involved in cellular cytotoxic mechanisms (18, 19). However, these accessory molecules do not appear to represent NK ligands by themselves, but rather as strengthening target cell-effector cell conjugates after the initial cognate interaction between the target cell and the NK cell (19).

Recently, it has been established that resistance of K562 cells to NK cells can be correlated with an increase of glycophorin A on the cell surface (20, 21). However, it has never been suggested that this glycoprotein was capable of protecting tumor cells against lysis by NK cells. The insertion of this molecule into the membrane of cells sensitive to NK cell attack gives a direct measure of its involvement in NK cell-mediated lysis. In addition, the abundant glycosylation of this molecule allows us to use an experimental approach to determine the role of the glycoprotein sugars in the mechanism of resistance to NK cell-mediated lysis.

In this report, glycophorin A was inserted into the K562 cell membrane. This was achieved by electroinsertion, which has recently been used to insert proteins with a membrane spanning sequence into mouse red cell membranes (22) and into nuclear cells (23, 24). We demonstrate that such modified cells are resistant to NK cell-mediated lysis. The carbohydrate nature of the molecular entity that is involved in this modulation has also been determined.

MATERIALS AND METHODS

Cells—K562 cells were grown at 37°C in a humidified 5% CO₂ incubator. The culture medium was Eagle's minimum essential medium (MEM 0111, Eurobio, Paris) supplemented with 10% fetal calf serum (Boehringer, Mannheim, Germany), penicillin (100 IU/ml), streptomycin (100 mg/ml), and L-glutamine (0.58 mg/ml). The cell density was maintained between 2 × 10⁶ and 1.6 × 10⁷ cells/ml.

Peripheral blood mononuclear cells from normal human volunteers (CD71) and CD32 antigen (3–5), of glycolipids such as asialo-GM₂ and G_M₂ (6, 7), of oligosaccharides such as 3-fucosyl-N-acetyllactosamine (CD15 antigen) (8), and of peptides such as a 42-kDa polypeptide (9). These molecules could act as "positive signals" on NK cells. Conversely, it has been shown that a high level of cell membrane sialylation decreases the sensitivity of target cells (6, 10). More recently, it has been demonstrated that the expression of MHC class I molecules protects tumor cells (predominantly those of lymphoid origin) against NK cell attack (11–13), acting as a "negative signal" for NK cell-mediated lysis. Moreover, inhibitory receptors for NK cell activation have been identified, and they bind MHC class I molecules (14, 15). Finally, in the past year, a natural killer cell receptor proved to be specific for ubiquitous oligosaccharides and triggers the NK cell cytolytic mechanism (16). In conclusion, the target molecules involved in recognition of target cells by NK cells may contain peptidic or carbohydrate residues that can activate or inhibit the NK cell-mediated killing (17).

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Peripheral blood mononuclear cells from normal human volunteers
were isolated on Ficoll-Hypaque (MSL, Eurobio). Peripheral blood lymphocytes (PBL) were obtained after peripheral blood mononuclear cell depletion of adherent cells by 1 h of incubation at 37 °C in plastic Petri dishes.

Chemicals—The pulsing buffer contained 125 mM sucrose, 69 mM KCl, 1 mM MgCl₂, and 10 mM potassium phosphate buffer, pH 7.4. Its low ionic content reduced the joule heating associated with the electric field. The washing phosphate-buffered saline (PBS) contained 125 mM K₂HPO₄, 8.3 mM NaH₂PO₄/H₂O, and 125 mM NaCl, pH 7.4. All chemicals were from Sigma. Glycophorin A type MN (GPA) and asialo-glycoporin were obtained from Sigma.

Electroinsertion—Electroinsertion was carried out as described previously for Chinese hamster ovary cells (24) using a CNRS electropulsor (I ouan, St. Herblain, France) able to deliver square wave pulses, the parameters of which (voltage, pulse duration, and number and frequency of pulses) can all be adjusted separately. The pulses were monitored using a 15-MHz oscilloscope (Enertec, St. Etienne, France). Electrodes were parallel and flat with an anode-cathode distance of 1.5 mm. Since electroinsertion is a back-effect of electroporation (24), various values of field intensity and pulse number and duration were applied to K562 cells to obtain both maximum viability and membrane permeabilization. Optimal conditions were one square wave pulse of 7 ms duration at 0.6 kV/cm. Consequently, electroinsertion was performed using K562 cells (10⁶), which were incubated at 37 °C for 15 min with glycophorin A or asialoglycoporin in a total volume of 18 μl and then submitted to a permeabilization pulse. After pulse application, the cells were washed three times in PBS (400 × g for 4 min at room temperature) and analyzed by immunofluorescence. A control sample was subjected to all these steps, except that no pulse was applied. Cell viability was checked by using the trypan blue exclusion test after pulsing (always >95%) and by observing cell growth and cell viability 24 h (always >98%) after pulsing.

Immunofluorescence Assay—GPA, asialo-glycoporin, transferrin receptor (CD71), CD15 antigen, MHC class I antigen, and adhesion molecules were detected by an indirect staining method using, respectively, an anti-GPA mouse monoclonal antibody (mAb) that also stains the asialoglycoporin and that binds to amino acids 27–39 of GPA (ImmunoTech, France), anti-CD71 mAb and anti-CD15 mAb (Becton Dickinson, Mountain View, CA), anti-MHC class I W6/32 (American Type Culture Collection, Rockville, MD), and anti-adhesion molecules, anti-CD54 mAb, anti-CD58 mAb, anti-CD29 mAb, anti-CD18 mAb, and anti-CD56 mAb (Immunotech, Marseille, France). Purified mouse IgG was used as a control reagent. Fluorescent isothiocyanate-labeled (Flaβ') goat antibodies against mouse immunoglobulins were used as a second-step reagent for indirect staining. The cells were then washed in PBS and analyzed by flow cytometry (FacScan, Becton Dickinson). Two parameters were used: the percentage of fluorescent cells after autofluorescence background subtraction and the mean of fluorescence intensity of positive cells expressed in arbitrary units.

After fluorescence intensity calibration of the cytometer using quantitative fluorescent microbead standards, we used Simply Cellular™ microbeads (Becton Dickinson) to quantify the inserted glycophorin. These microbeads may be considered as “model lymphocytes” since they are approximately the size of lymphocytes. They bind mouse monoclonal antibodies (such as goat anti-mouse IgG antibodies) that are covalently bound to the microbead surface. The beads were calibrated in terms of the number of monoclonal mouse IgG molecules they bind, allowing detection of the effective fluorescent protein per cell.

We applied the same method of antibody labeling to these microbeads as the one used for cells.

Endoglycosidase Assays—Glycophorin A is composed of 60% carbohydrate by weight, with most of the sugars being 15-O-linked tetrasaccharides that are attached to serine or threonine and that have the structure shown in Structure 1. (25). A complete N-linked oligosaccharide is also present. Its structure is as shown in Structure 2. O-Glycanase (TEBU, Le Perray, France) catalyzes the hydrolysis of the Gal-GalNAc disaccharide core attached to serine or threonine residues of asialo-glycoporins. In this experiment, we used asialo-glycoporin since sialic acid was observed to inhibit the enzyme activity (26). Cells (3 × 10⁸) were mixed with 25 million/μl O-glycanase. The mixture was then incubated for 12 h at 37 °C.

Endoglycosidase F (Sigma, St. Quentin, France) cleaves the link between the two N-acetylgalactosamine residues linking the glycan moiety to the asparagine of the protein backbone (27). Cells (3 × 10⁸) were mixed with 60 million/μl of enzyme. Incubation was then conducted at 37 °C for 12 h.

These conditions were chosen to reach complete deglycosylation of susceptible asparagine- , serine-, or threonine-linked oligosaccharides. After enzyme treatment, the cells were washed, checked for staining with fluorescent lectins, and used in the cytotoxicity assay.

Checking Enzyme Treatments—From knowledge of GPA oligosaccharide structures, fluorescein isothiocyanate-conjugated lectins (Dolichos biflorus or Lens culinaris (Sigma)) were used to evaluate glycosylation patterns in control and endoglycosidase-treated cells. To evaluate the efficiency of endoglycosidase treatment, K562 cells were incubated for 15 min with L. culinaris lectin (25 μg/ml), which is known to react specifically with α-α-mannose. The cells were then washed in PBS and analyzed by flow cytometry. In the case of O-glycosylation, K562 cells were incubated for 15 min with D. biflorus lectin (50 μg/ml), which is known to react specifically with α-α-GalNAc. The cells were then washed in PBS and analyzed by flow cytometry. These conditions were chosen after testing various lectin concentrations to demonstrate almost complete staining (>95% of positive cells) of K562 cells.

Cytotoxicity Assay—The NK cell activity of PBL from healthy donors was tested in a standard 4-h ⁵¹Cr release assay against target cells labeled with ¹⁵⁴Cr as previously described (20). Briefly, various numbers of PBL (effector cells) were mixed in triplicate with 10⁴ labeled target cells in microtiter plates. After 4 h at 37 °C, 100 μl of the supernatants were counted in a γ-counter. The percentage of cell-mediated lysis was calculated as follows: % cell-mediated lysis = (cpmexp - cpmspont) / cpmmax × 100. cpmmax was determined by counting an aliquot of labeled target cells, and cpmspont (spontaneous release) by counting the supernatant from wells without effectors. Cytotoxicity calculated from individual effector cells/target cell curves is expressed as lytic units (LU); 10⁷ effector cells. One LUₜ is defined as the number of lymphoid cells required to lyse 25% of 10⁴ target cells under the assay conditions used.

Target Cell Binding Assay—PBL were depleted of CD3+ cells by using anti-CD3 reactive magnetic microbeads (Immunotech). The percentage of effectors conjugated to target cells was determined using the procedure of Grimm and Bonavida (28). Briefly, 100 μl (10⁷/ml) of cells from suspension of the effectors and target cell populations in culture medium + 10% fetal calf serum were mixed in centrifuge tubes. The tubes were placed in a water bath for 5 min at 30 °C. The cells were then centrifuged for 5 min at 400 × g at room temperature to promote conjugate formation. Then the pellet was resuspended 10 times with a micropipette. A small drop of this suspension was removed, and the percentage of the conjugates was determined in a hemocytometer.

RESULTS

Electroinsertion of Glycophorin A into the Membrane of K562 Cells—When K562 control cells were examined with our anti-GPA mAb, −10% of the cells were weakly positive (−1000 molecules of GPA/cell). Electroinsertion of GPA into the plasma membrane was mediated by submitting a GPA/K562 cell mixture to an electric field pulse (one pulse of 7-m duration at 0.6 kV/cm). Electrical field application allowed us to insert the GPA, detected by anti-GPA monoclonal antibody, on >90% of the K562 cells. However, a relatively large percentage (44 ± 5% for 89 μM GPA; n = 3) of fluorescent cells was detected even in the absence of electric field and for weak field intensities. In this cell population, the number of stained GPA molecules was always ~10⁶/cell. With an electrical pulse, the number of detectable glycophorin molecules/fluorescent cell rose with the increase in glycophorin concentration in medium to reach ~6 × 10⁶ molecules in the cell membrane when there was 89 μM GPA in the pulsing medium (Fig. 1A).

We previously demonstrated using Chinese hamster ovary cells that electroporation mediates a stable insertion of GPA into the cell membrane (24). To determine the stability of
the interaction between GPA and pulsed or non-pulsed K562 cells, the GPA molecules were stained by mAb at 0, 24, and 48 h after pulsing. The percentage of fluorescent cells strongly decreased when the cells were not electropulsed and cultured for 24 and 48 h (27 ± 4 and 6 ± 1%, respectively, versus 41 ± 5% at 0 h), whereas it was stable at 24 and 48 h after electropulsation (83 ± 6 and 79 ± 6%, respectively, versus 89 ± 5% directly after the pulse). In addition, the number of bound GPA molecules/cell decreased from 12,000 to 2500 after 1 day of non-pulsed cell culture and only decreased by a 2-fold factor every 24 h after electropulsation. Since the growth rate of K562 cells is one doubling/24 h, the above results indicate that (i) the GPA molecules bound to the cell membrane (termed electroinserted GPA) were shared between the daughter cells (Fig. 1B) and (ii) the GPA-cell membrane interaction is stable after pulsing. Consequently, the nature of the interaction with the membrane of electropulsed cells is likely to be different from that of the control cell membrane.

Electroinserted Glycophorin A Decreases K562 Cell Susceptibility to Attack by NK Cells—Fig. 2 (A1 and A2) shows a representative experiment of the effect of glycophorin insertion on susceptibility to NK cell-mediated lysis. The sensitivity of K562 cells to NK cell attack increased when the cells were pulsed without GPA (Fig. 2A1), whereas the presence of ~10^4 GPA molecules at the surface of 40% of the non-electropulsed cells did not alter susceptibility to lysis. Considering that the electropulsed cells were the effective target control, glycophorin had an inhibiting effect at all concentrations tested, and the resistance to NK cell lysis increased with the number of glycophorin molecules on the cell surface (Fig. 2A2).

NK cell-mediated cytotoxicity, calculated from individual effector cell/target cell curves, was cumulated and expressed as lytic units (LU50) and calculated from the three experiments described above (mean ± S.D.). K562 cells were electropulsed in the absence (P-K562) or presence (GPA^-) of 89 µM GPA or were incubated without pulsing in pulsing buffer in the absence (K562) or presence (+GPA) of GPA.
The spontaneous lysis of target cells induced by natural killer cell activity is accomplished in two main distinguishable steps: binding between target and effector cells and post-binding events leading to target cell destruction. To examine the possibility that the decreased lysis was due to a defect in the first step of binding, direct conjugate-forming cell assays were performed after CD3-depletion in electropulsed K562 cells. Table I illustrates that the GPA+ K562 cells were as efficient in binding NK cells as the control samples.

Effect of the Glycophorin Insertion on the Expression of Surface Antigens—To determine whether electron insertion of GPA into K562 cell membranes triggers a modulation of the expression of negative or positive signals for NK cells, CD71, CD15, and MHC class I antigens and certain adhesion molecules were stained by specific mAbs. No significant difference was observed between GPA+ cells and the control samples (Table II). Similar results were obtained when the cell positivity to mAbs was measured as mean fluorescence intensity (data not shown). These results indicate that the resistance to NK cell attack induced by glycophorin insertion into the membrane depends neither on the triggering of MHC class I antigen expression nor on the modulation of the expression of other molecules. In addition, they demonstrate that insertion of GPA on the membrane does not interfere with accessibility of mAbs to natural epitopes of the cell surface.

Effect of the Glycosylated Structure on NK Cell-Mediated Lysis—It has been reported that cell-surface sialic acid may contribute to the development of NK cell resistance directly or by masking the target structure(s) to NK cells (6, 10). As GPA is a highly sialylated protein, we tested the hypothesis that the resistance induced by GPA insertion could be correlated to a simple contribution from the sialic acid. Asialoglycoprotein was electroninserted into K562 cells. Fig. 3 shows that the same number of asialoglycoprotein and GPA molecules induced the same resistance of target cells to NK cell-mediated cytotoxicity.

We were also interested in determining whether changes, other than sialic acid, in glycosylation of the inserted glycophorin would alter the resistance of GPA+ cells to NK cell-mediated lysis. After GPA electroninsertion into the cell membrane, N- and O-glycosylation were eliminated by enzymatic treatment as indicated under "Materials and Methods." A comparative study of lectin binding to enzyme-treated and -untreated K562 cells (GPA+ and K562) revealed a significant reduction of binding after treatment of GPA+ cells, suggesting a decrease of N-linked (Fig. 4A) or O-linked (Fig. 4B) oligosaccharides, whereas the enzyme treatment did not affect the glycosylation of control cells. The effect of enzyme treatments on the susceptibility of K562 cells to NK cell-mediated lysis was then determined while fluorescein-labeled lectins were used in parallel to control deglycosylation on the target cells. K562 cells treated with endoglycosidase F or O-glycanase were as sensitive to NK cell lysis as the control samples. GPA+ cells treated with the

### Table I

| Target     | Exp. 1 | Exp. 2 |
|------------|--------|--------|
| K562       | 57 ± 3 | 40 ± 4 |
| P-K562     | 57 ± 7 | 37 ± 3 |
| GPA+ K562  | 55 ± 2 | 40 ± 6 |

### Table II

| Antigen  | K562 | P-K562 | GPA+ K562 |
|----------|------|--------|-----------|
| CD71     | 95.7 ± 2.3 | 95.8 ± 2.6 | 96.1 ± 3.1 |
| CD15     | 19.6 ± 2.1 | 18.5 ± 2.5 | 21.5 ± 3.2 |
| MHC Class I | 4.6 ± 0.6 | 5.1 ± 0.5 | 4.9 ± 0.8 |
| CD29     | 98.7 ± 1.2 | 97.2 ± 1.1 | 98.9 ± 0.9 |
| CD54 (ICAM-1) | 91.1 ± 5.6 | 891.1 ± 4.9 | 931.1 ± 6.2 |
| CD58 (LFA3) | 96.3 ± 3.2 | 98.2 ± 1.6 | 98.2 ± 1.6 |
| CD18     | 15.0 ± 3.2 | 14.1 ± 2.5 | 18.3 ± 4.1 |
| CD56     | 1.9 ± 0.5 | 2.9 ± 0.2 | 2.2 ± 0.9 |

*ICAM-1, intercellular adhesion molecule 1; LFA3, lymphocyte function-associated antigen 3.*

![Table.png](image.png)
Fig. 4. Control of enzyme treatments. K562 cells with electroinserted GPA (GPA+) (6.2 × 10⁴ ± 0.4 × 10⁴ molecules/cell) and those without (K562) were treated (+ Enzyme) or not (− Enzyme) with endoglycosidase (37 °C, 12 h). After endoglycosidase F treatment, the cells were washed and then stained with fluorescein-conjugated L. culinaris lectin (50 μg/ml, 15 min) (A). After O-glycanase treatment, cells were stained with fluorescein-conjugated D. biflorus lectin (25 μg/ml, 15 min) (B). Cells were analyzed by flow cytometry. Results are means ± S.D. of three separate experiments. AU, arbitrary units.

Fig. 5. Effect of glycosidases on K562 cell sensitivity to NK cell-mediated lysis. K562 cells (10⁶) were subjected to electropulsation (one pulse of 7-ms duration at 0.6 kV/cm) in the presence (69 μM) (● and ○) or absence (○) of GPA or were incubated without pulsing in pulsing buffer (○ and □). K562 cells (□) and K562 cells with electroinserted GPA (○) were treated with O-glycanase (5.4 × 10⁴ GPA molecules/cell) (A) or endoglycosidase F (5.9 × 10⁴ GPA molecules/cell) (B). Samples were then tested in the ⁵¹Cr cytotoxicity assay. A and B are results (S.D. < 10% of the mean of triplicates) from two separate experiments performed with effector cells from two different donors. Each is representative of three separate experiments using PBL from different donors.

NK cell attack was observed with −3 × 10⁴ electroinserted molecules/cell (i.e. 1 GPA molecule/2 × 10⁹ phospholipids). Consequently, the resistance to NK cell-mediated lysis can be effective when the GPA number is higher than a threshold value of between 10⁴ and 3 × 10⁴ molecules. On the other hand, the target resistance induced by electroinserted GPA may be attributed, at least partly, to the stabilization of the membrane. Indeed, a GPA molecule incorporated into experimental bilayers interacts with −500–1000 phospholipids (29, 30), which could alter the membrane stability. However, this possibility seems somewhat unlikely since (i) the interaction between GPA and phospholipids would involve only 5–10% of the membrane phospholipids; (ii) GPA electroinserted into Chinese hamster ovary cell membrane showed a free lateral diffusion with a diffusion coefficient in agreement with what would be expected for an intrinsic protein embedded in a viable cell membrane (23); and (iii) removal of N-linked oligosaccharide moieties suppresses the resistance. This relationship between the resistance to NK cell-mediated lysis and the inserted glycoprotein A level is in accordance with previous results that showed a reduced sensitivity to NK cell lysis of (i) K562 cells differentiated in vitro by drugs that increased the levels of GPA on the cell surface (10, 21) and (ii) a K562 cell clone expressing a very high number of GPA molecules (21). It is important to stress that the presence of −6 × 10⁴ glycoprotein molecules on the cell surface does not confer total resistance to NK cell lysis. This can be explained by the intervention of several NK cell subsets using different mechanisms to lyse K562 cells in chromium assay or by the choice of each effector cell between different recognition strategies. Moreover, the resistance to NK cells might only be partial because GPA+ cells maintain malignant features, i.e. positive signals for NK cells such as the absence of MHC class I antigens and/or the presence of ligands able to activate NK cells such as CD71 and CD15 antigens or some carbohydrate determinants (3–9, 16).

In our system, the protective effect of GPA might be due to masking (i) of cellular adhesion molecules involved in NK cell mechanisms and/or (ii) of putative epitopes able to deliver an activating signal to NK cells. Both explanations are unlikely because we demonstrated that GPA electroinsertion did not alter the accessibility of cell adhesion molecules (CD18, CD29, CD54, CD56, and CD58), CD71, and CD15 by mAbs. In addition, no consistent reduction of target cell binding to NK cells was recorded in the experiments using GPA+ cells. However, it is well known that conjugate formation measures integrin binding and represents an early stage of binding that is followed by a cellular reorientation and firm adhesion that probably uses other molecules. Consequently, GPA might modulate a limiting step at the level of the membrane molecular interactions involved in post-conjugating mechanisms, such as in the stabilization of binding and/or in the lethal hit (e.g. it is quite possible that GPA blocks perforin adherence).

GPA is a sialoglycoprotein made up of 131 amino acid residues with no disulfide bonds and is composed of 60% carbohydrate by weight. The sugar chains contain −45% sialic acid by weight (25). As mentioned above, several works have shown that the level of sialic acid on target cells may modulate their sensitivity to NK cell-mediated lysis, e.g. neuraminidase medi-
ates an increase of the susceptibility to NK cell lysis (10). However, in agreement with previous data (20), we have demonstrated that sialic acid is not an essential residue in the inhibition of NK cell-mediated lysis due to inserted GPA.

On the contrary, this work indicates a role for the N-linked oligosaccharide of GPA in NK cell activity modulation due to endoglycosidase F, which eliminates the N-linked oligosaccharide structure, completely reversing the resistance to NK cells. However, in agreement with previous data (20), we have demonstrated that sialic acid is not an essential residue in the biosynthesis of new sugar chains for the membrane molecules, whereas these mechanisms cannot act on electroinserted GPA. The GPA molecule contains 15 O-linked tetrasaccharides and a single complex N-linked oligosaccharide (13 monosaccharide residues). Several works have emphasized the importance of carbohydrate molecules in target cell-effector cell interactions (17). According to the structures and the location of the extra sugar residues added to a pentasaccharide common core, all the N-linked sugar chains are classified into three subgroups (31): (i) complex-type sugar chains, (ii) high mannosic-type sugar chains, and (iii) hybrid-type sugar chains. Recently, by using N-glycan processing inhibitors, it has been demonstrated that the presence of high mannose-type glycans on K562 cells correlates with increased binding of effectors and a greater susceptibility to lysis. The high mannose-type glycans can influence the NK cell-target cell interaction at the level of the adhesion molecules (32). However, the N-linked sugar chain of GPA belongs to the complex-type sugar chain, and its presence on the target cell surface decreases the susceptibility to NK cells without altering the conjugate formation. Thus, two types of sugar chain with strong differences in their structure can modulate the NK cell activity in opposite directions.

As mentioned above, NK cell receptors have been identified that bind MHC class I molecules and inhibit natural killer cell activation (14, 15), and it is possible that carbohydrates of the MHC glycoprotein participate in the inhibition of NK-mediated cytotoxicity (15). On the other hand, it has recently been shown in mice that members of the type II transmembrane lectin family are preferentially expressed on NK cells and can deliver either positive (NK-R1 protein) (33) or negative (ly49) signals to the effector cell (34). In addition, NK-R1 binds a diversity of oligosaccharides that activate NK cells and cytotoxicity (16). In humans, the same type of membrane proteins (named NKG2 proteins and with carbohydrate-binding external domains) was also detected on NK cells (34). Their ligands and their effect on natural killing are unknown (34). It is possible that sugar residues of the N-linked sugar chain, shared by several glycoproteins, fulfill the role of ligand molecule for these putative NK cell receptors or for other unknown molecules characterized by lectin activity. From this point of view, sugar residues of the N-linked oligosaccharide of GPA could be a ligand for a putative receptor that delivers a negative signal to NK cells.

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REFERENCES

1. Trinchieri, G. (1992) in The Natural Killer Cell (Lewis, C. E., and McGee, J. O’D., eds) pp. 42–67, IRL Press, Oxford
2. Arora, D. J. S., House, M., J. ustrawicz, D. M., and Mandeville, R. J. (1984) J. Virol. 52, 839–845
3. Alarcon, R., and Fresno, M. (1985) J. Immunol. 134, 1286–1291
4. Lazarus, A. H., and Baines, M. G. (1985) Cell. Immunol. 96, 255–266
5. Perl, A., Looney, R. J., Ryan, D. H., and Abraham, G. N. (1986) J. Immunol. 136, 4714–4720
6. Yogeshwaran, G., Grönborg, A., Hansson, M., Dalianis, T., Kiessling, R., and Welsh, R. M. (1981) Int. J. Cancer 28, 517–526
7. Ando, I., Hoon, D. S. B., Suzuki, Y., Sanxton, R. E., Golub, S. H., and Iriee, R. F. (1987) Int. J. Cancer 40, 12–17
8. Zarcone, D., Tilden, A. B., Friedman, H. M., and Grossi, C. E. (1987) Cancer Res. 47, 2674–2682
9. Harris, D. T., Jasso-Friedman, L., and Evans, D. L. (1994) Scand. J. Immunol. 39, 73–78
10. Werkmeister, J. A., Pross, H. F., and Roder, J. C. (1983) Int. J. Cancer 32, 71–78
11. Storkus, W. J., Howell, D. N., Salter, R. D., Dawson, J. R., and Cresswell, P. (1987) J. Immunol. 138, 1657–1659
12. Quillet, A., Presse, F., Marchiol-Fournigault, C., Harel-Bellan, A., Benbunan, M., Ploegh, H., and Fradelizi, D. (1988) J. Immunol. 141, 17–20
13. Storkus, W. J., Alexander, J., Payne, J. A., Cresswell, P., and Dawson, J. R. (1989) J. Immunol. 143, 3853–3857
14. Moretta, L., Ciccone, E., Moretta, A., Höglund, C., Öhlén, P., and Kärre, K. (1992) Immunol. Today 13, 300–305
15. Kärre, K. (1995) Science 267, 978–979
16. Bezouska, K., Yuan, C.-T., O’Brien, J., Childs, R. A., Chai, W., Lawson, A. M., Drbal, K., Fiserova, A., Papisi, M., and Feizi, T. (1995) Nature 372, 150–151
17. McCoy, J. P., Jr., and Chambers, W. H. (1991) Glycobiology 1, 321–328
18. Quillet-Mary, A., Cavarec, L., Kermaarec, N., Marchiol-Fournigault, C., Gil, M. L., Conjeaud, H., and Fradelizi, D. (1991) Int. J. Cancer 47, 473–479
19. Pataroyo, M. (1991) Clin. Immunol. Immunopathol. 60, 333–348
20. Benoist, H., Madue, C., Tremouet, C., Cariel, J., and Desplaces, A. (1988) Int. J. Cancer 42, 299–304
21. Benoist, H., Jody, P., Broglie, C., Jannesson, P., Idone, O., Dufier, J., and Desplaces, A. (1992) Immunol. Lett. 34, 45–56
22. Zlotnik, M., Tosi, P. F., Mourinme, Y., Lazarte, J., Sneed, L., Volsky, D. J., and Nicolai, C. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 4409–4413
23. El Ouagari, K., Gabriel, B., Benoist, H., and Teissié, J. (1993) Biochim. Biophys. Acta 1151, 105–109
24. El Ouagari, K., Benoist, H., Sio, S., and Teissié, J. (1994) Eur. J. Biochem. 219, 1031–1039
25. Dill, K., Hu, S., Berman, E., Pavla, A. A., and Lacombe, J. M. (1990) J. Protein Chem. 9, 129–136
26. Uemoto, J., Bhavanadan, V. P., and Davidson, E. A. (1977) J. Biol. Chem. 252, 8609–8614
27. Eldar, J. H., and Alexandre, S. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 4540–4544
28. Grimm, E., and Bonavida, B. (1979) J. Immunol. 123, 2861–2869
29. Duyku, R. A., Mendesohn, R., Casal, H. L., and Mantsch, H. H. (1983) Biochemistry 22, 1170–1177
30. Kapitza, H. G., Rüppel, D. A., Galla, H. G., and Sackmann, E. (1984) Proc. Natl. Acad. Sci. U.S.A. 129, 105–109
31. Trinchieri, G. (1992) in The Natural Killer Cell (Lewis, C. E., and McGee, J. O’D., eds) pp. 42–67, IRL Press, Oxford
32. Ahrens, P. B. (1993) J. Immunol. Today 150–151
33. Giorda, R., Rudert, W. A., Vanassori, C., Chambers, W. H., Hiserodt, J. C., and Trucco, M. (1990) Science 249, 1288–1300
34. Yokoyama, W. M. (1995) Curr. Opin. Immunol. 7, 110–120