Glycobiology Vol. 3 no. 1 pp. 69–76, 1993

Cell surface glycoconjugates as possible target structures for human natural killer cells: evidence against the involvement of glycolipids and N-linked carbohydrate chains

Hans Voshol[1], Hub F.J.Dullens[2], Willem Den Otter[2] and Johannes F.G.Vliegenthart[1,3]

[1]Utrecht University, Bijvoet Center, Department of Bio-Organic Chemistry, PO Box 80075, 3508 TB Utrecht and [2]Utrecht University, Department of Pathology, PO Box 85500, 3508 GA Utrecht, The Netherlands

[3]To whom correspondence should be addressed

Natural killer (NK) cells can spontaneously kill various malignant cells, but the susceptibility towards NK cells differs greatly among different types of tumour cells. The molecules, which are recognized by NK cells, have not yet been identified, but there is ample evidence that target cell surface glycoconjugates are involved in the interaction with NK cells. In this report, we show that the recognition of K562 target cells by human NK cells depends on the presence of protein-bound determinants, implying that glycolipids are not the primary target structures on K562 cells. The NK susceptibility of K562 cells was not altered by enzymic removal of various cell surface carbohydrates or oligosaccharides, mostly related to N-linked carbohydrate chains. Treatment of K562 cells with 1-deoxynojirimycin and 1-deoxymannojirimycin, inhibitors of *N*-glycan processing, resulted in drastic alterations in the carbohydrate phenotype of the cell surface, as could be shown by flow cytometric analysis of the lectin-binding properties of the cells. Despite these clear changes in *N*-glycosylation, the NK susceptibility of K562 cells remained unaffected. Summarizing, the results described in this report show that potential target structures for NK cells are protein bound, but the involvement of a specific (N-linked) carbohydrate determinant in the interaction between NK cells and target cells could not be established.

Key words: cell adhesion molecules/cell–cell interaction/cell surface glycoconjugates/natural killer cells/target structures

Introduction

Human natural killer (NK) cells are lymphoid cells with $CD3^-$, $CD16/56^+$ phenotype, which comprise 10–15% of human peripheral blood lymphocytes (Trinchieri, 1989; Robertson and Ritz, 1990). Probably the most clearly established physiological function of NK cells is their antiviral activity (Welsh, 1986; Biron *et al.*, 1989). Furthermore, NK cells have attracted much attention because of their ability to mediate spontaneous, non-major histocompatibility complex (MHC)-restricted cytotoxicity against various malignant cells *in vitro* (reviewed by Den Otter, 1986, 1987; Herberman, 1986; Trinchieri, 1989).

NK cell-mediated cytotoxicity consists of a sequence of distinct steps (Herberman, 1986): (i) recognition and binding of the target cell; (ii) activation and rearrangement of intracellular granules; (iii) release of cytotoxic factors; (iv) interaction of cytolytica with the target cell; (v) target cell lysis.

Evidently, the recognition of target cell surface structures is crucial to the regulation of NK cell-mediated cytotoxicity. The initial binding step is of particular interest because NK cells appear to be able to discriminate between normal and transformed cells. One of the reasons to propose a role for target cell surface glycoconjugates in the recognition process is the fact that essentially all tumour cells have an altered cell surface carbohydrate composition compared to their non-transformed counterparts. The most common features of these alterations, which can be present in both glycolipids and glycoproteins (Hakomori, 1989), are a higher degree of branching of asparagine-linked oligosaccharides (Dennis *et al.*, 1987), higher levels of sialylation (Smets and Van Beek, 1984) and $\alpha(1\rightarrow3)$ fucosylation (Foster *et al.*, 1991). The experimental evidence regarding the involvement of cell surface glycoconjugates and their carbohydrate chains in certain stages of NK cell-mediated cytotoxicity against tumour cells has been reviewed recently (McCoy and Chambers, 1991).

There is still some controversy over whether the NK target structures reside in target cell surface glycolipids or glycoproteins, or in a combination of both. Apparently, the molecules which are responsible for NK–target interaction are sensitive to proteases (Hiserodt *et al.*, 1983), but attempts to isolate the corresponding glycoproteins or glycopeptides from target cells are not conclusive yet (Roder *et al.*, 1979; Decker *et al.*, 1984; Henkart *et al.*, 1986). On the other hand, it has also been reported that the glycolipid GM2 could function as a target molecule for NK cells (Ando *et al.*, 1987; Grayson and Ladisch, 1992).

The correlation between cell surface glycosylation of target cells and their NK susceptibility has been studied with glycosylation variants. These variants can be obtained with various approaches, two of which will be considered here: (i) treatment with exo- or endoglycosidases and (ii) inhibitors of *N*-glycosylation and/or *N*-glycan processing. The rather rigid specificity of most glycosidases, and the fact that accessibility for these enzymes to native cell surface glycoconjugates is often restricted, impose limitations on this approach. On the other hand, it seems likely that molecules, which are involved in cell–cell interaction, are well exposed on the cell surface and thereby accessible to enzymes. So far, the application of glycosidases has been largely limited to experiments with sialidase, which have provided indications that the sialic acid content of target cell membranes is inversely correlated with the NK susceptibility of target cells (Yogeeswaran *et al.*, 1982; Werkmeister *et al.*, 1983; Rooney and Munro, 1984). Because *N*-glycosylation of proteins follows a strictly ordered biosynthetic pathway, it can be relatively easily blocked by compounds which interfere in an early stage of the process (Fuhrmann *et al.*, 1985; Elbein, 1987). Tunicamycin, which completely prevents *N*-glycosylation, and swainsonine, a blocker of *N*-glycan processing, have been applied to modify the surface glycosylation of NK target cells. However, the

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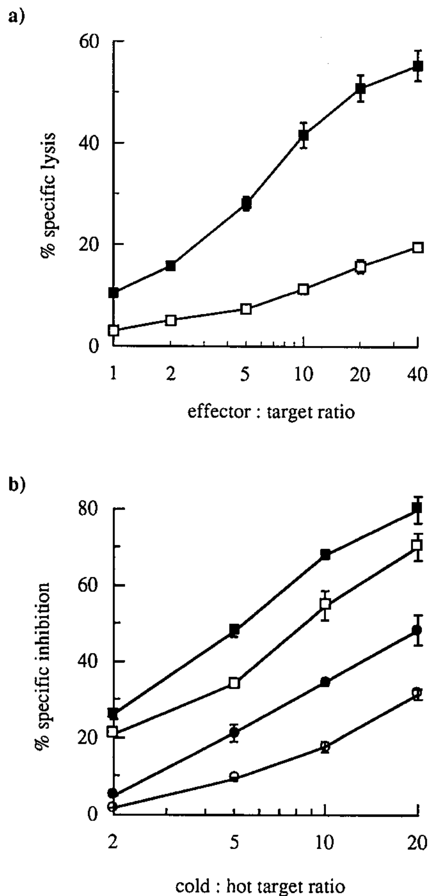

Fig. 1. The effect of trypsin treatment on NK-mediated lysis of and NK-mediated binding to K562 cells. **Panel a**: K562 cells were treated with 1 mg/ml trypsin as described. The lysis of trypsin-treated (□) and control cells (■) by NK cells was tested in a standard chromium release assay. **Panel b**: K562 cells were trypsinized at different trypsin concentrations and subsequently tested in a cold target inhibition assay. In this assay, various amounts of unlabelled (cold) target cells, which have undergone different treatments, are added to a mixture of 10^5 NK cells and 10^4 ^{51}Cr-labelled (hot) K562 cells. Depicted in this figure is the specific inhibition of chromium release, caused by the addition of untreated (■), 0.01 mg/ml trypsin-treated (□), 0.1 mg/ml trypsin-treated (●), or 1 mg/ml trypsin-treated (○) cold target cells. Bars indicate the SD between triplicate determinations.

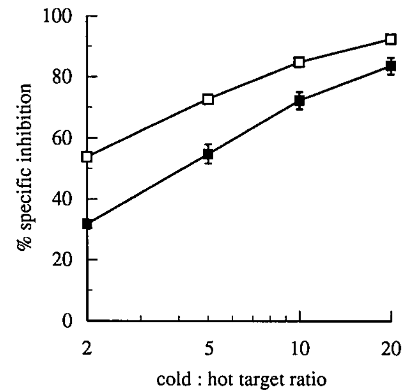

Fig. 2. Cold target inhibition test with sialidase-treated K562 cells. K562 cells were treated with a mixture of the sialidases from *V.cholerae* and *A.ureafaciens* as described, and tested in a cold target inhibition assay. In this assay, various amounts of unlabelled (cold) target cells, which have undergone different treatments, are added to a mixture of 10^5 NK cells and 10^4 ^{51}Cr-labelled (hot) K562 cells. Depicted in this figure is the specific inhibition of chromium release, caused by the addition of untreated (■) or sialidase-treated (□) cold target cells. Bars indicate the SD between triplicate determinations.

effects of these inhibitors on the recognition of target cells by NK cells were not straightforward (reviewed by McCoy and Chambers, 1991).

Application of monoclonal antibodies against leukocyte cell surface molecules has provided interesting data regarding glycoproteins on the effector and target cell surfaces, which could be involved in NK–target interaction. Several of the 'classical' adhesion molecule–ligand pairs (Springer, 1990), e.g. CD2 and LFA-1 on the effectors and LFA-3 and ICAM-1 on the targets, seem to be involved in the conjugation process because antibodies against these molecules block binding of NK cells to target cells (Timonen *et al.*, 1988; Robertson *et al.*, 1990). Unfortunately, in almost all cases the exact nature of the epitope to which these antibodies are directed is not known. Therefore, (bio-)chemical characterization of the adhesion molecules will be required to provide evidence on the precise molecular nature of the structures and of the interactions, which are crucial to the process of NK-mediated cytotoxicity.

In this paper, we report on the progress of our investigations concerning the involvement of target cell surface glycoconjugates in the recognition of target cells by NK cells, using human NK cells and their model target cell line K562 (Trinchieri, 1989).

Results

The effect of trypsinization of K562 cells on their interaction with NK cells

After treatment with 1 mg/ml of trypsin, K562 cells are almost refractory to killing by NK cells (Figure 1a). At effector to target (E/T) ratios of 20–40:1, lysis of trypsinized cells is comparable to that of untreated cells at 10 times lower E/T ratios. This seems to be primarily due to a drastic reduction in binding of effector cells to target cells, as the ability of trypsin-treated K562 cells to act as cold target inhibitors for untreated cells is reduced, even at much lower trypsin concentrations (Figure 1b). The cold target inhibition of cells, treated with 1 mg/ml trypsin, at a cold/hot target ratio of 20:1 is similar to that of untreated cells at 2:1. From both assays depicted in Figure 1, it follows that up to ~90% of the NK target structures are removed by trypsin. This observation shows that NK target structures are bound to cell surface proteins and not lipids.

Cell surface sialic acid and NK susceptibility

As mentioned before, there are several reports indicating that the level of cell surface sialic acid of target cells is inversely correlated with their NK susceptibility. In a number of experiments, we also observed that K562 cells were consistently recognized and lysed more effectively by NK cells after treatment with sialidase from *Arthrobacter ureafaciens* or from *Vibrio cholerae*. However, the differences between control and sialidase-treated cells were relatively small. Finally, K562 cells were treated with a mixture of both sialidases. This approach resulted in a clear increase in cold target inhibition compared to control cells (Figure 2). To determine whether the

increased effect on target cell recognition by NK cells was due to improved removal of sialic acid from the cell surface by the enzyme mixture, the periodate/borotritiide method (Gahmberg and Andersson, 1977) was used to radiolabel cell surface sialic acid. Remarkably, there appeared to be only minor quantitative differences in the specific release of label, which was always ~30%, with either of the sialidases or the combination of both.

One of the explanations for the increased interaction between NK cells and target cells after sialidase treatment could be that sugars, which were originally masked by sialic acid, are now recognized. One of the obvious candidates for such a sugar, particularly in desialylated N-linked glycoproteins, is galactose. The exposure of galactose residues by sialidase could be demonstrated by using the galactose oxidase/borotritiide radiolabelling method (Gahmberg *et al.*, 1976). When K562 cells were treated with sialidase prior to the labelling procedure, incorporation of tritium into galactose residues was 8–10 times higher than in cells treated with galactose oxidase alone.

To determine the importance of the exposure of new terminal galactoses, the effect of removing these residues on the recognition of target cells by NK cells was studied. After sialidase treatment, K562 cells were subjected to digestion by green coffee bean α-galactosidase or by β-galactosidase from jack beans or bovine testes. Control experiments confirmed that β-galactosidase released about 10–20% of the radiolabel incorporated into K562 membranes by galactose oxidase, whereas α-galactosidase did not release any label. However, neither of these treatments influenced the recognition (measured as cold target inhibition) of K562 cells by NK cells, whether or not the target cells were pretreated with sialidase (data not shown).

Furthermore, we tested the possible involvement of poly-*N*-acetyllactosamine glycans on K562 cell surface glycoconjugates in the interaction with NK cells. These experiments showed that although endo-β-galactosidase from *Bacteroides fragilis* was capable of releasing 10–15% of the radiolabel in either sialic acid or galactose residues from K562 membranes, the enzyme did not alter the NK susceptibility of the target cells.

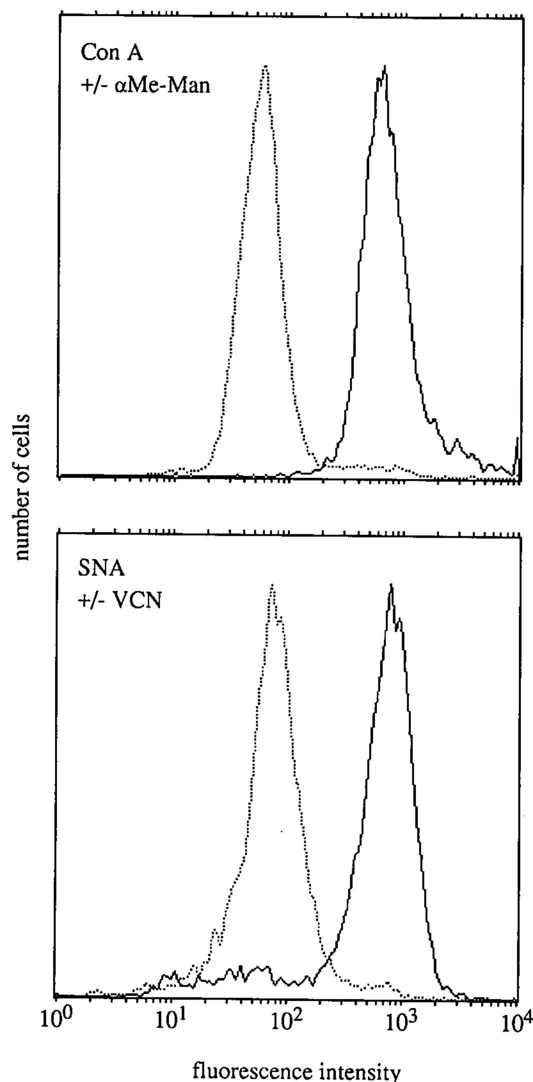

Fig. 3. Flow cytometric analysis of the lectin-binding properties of K562 cells. **Upper panel**: K562 cells were incubated with biotinylated Con A, without (solid line) or with (dotted line) 5 mM α-methylmannoside (αMe-Man). **Lower panel**: K562 cells were incubated with biotinylated SNA without (solid line) or with (dotted line) pretreatment with sialidase. Subsequently, cells were stained with FITC–avidin and analysed by flow cytometry.

Modification of K562 cell surface N-glycosylation

Attempts were made to remove the entire N-linked carbohydrate chains with the enzyme *N*-glycanase (EC 3.2.2.18, PNGase F). Radiolabelling experiments confirmed that some *N*-glycosidic material (5–10% of the radiolabelled sialic acid) was released, but this was without measurable influence on the recognition by NK cells in the cold target assay (data not shown).

Before trying to influence the expression of *N*-glycosylated NK target structures on K562 cells with the glycosidase inhibitors 1-deoxymannojirimycin (DMM) and 1-deoxynojirimycin (DNM), a suitable method to monitor the changes in the glycosylation induced by these inhibitors had to be developed. Initially, we applied a flow cytometric method for this purpose, in which staining of cells with the lectins concanavalin A (Con A, from *Canavalia ensiformis*) (as a marker for 'inhibited' high-mannose chains) and the lectin from *Sambucus nigra* (SNA) (as a marker for 'mature' hybrid and complex chains) was analysed. In Figure 3, it is shown by flow cytometry that both lectins specifically stain K562 cell surface glycoconjugates. Control cells give a homogeneous staining pattern with Con A. Addition of α-methylmannoside, a ligand for Con A, largely abolishes the binding to this lectin (Figure 3, upper panel). Likewise, the binding of SNA to K562 cells is diminished when cells are pretreated with sialidase (Figure 3, lower panel).

For the actual treatment of K562 cells with DMM and DNM, a specific protocol was followed, starting with the removal of NK target structures with trypsin (cf. Figure 1). When K562 cells are recultured after trypsinization, they recover much of their NK susceptibility almost completely within 24 h, as measured by cold target inhibition (Figure 4). By reculturing with or without inhibitors, the influence of these compounds on the recovery of NK target structures can be tested.

To get an idea of the maximal perturbation of *N*-glycan processing, which could be achieved with the inhibitors without affecting cellular viability, K562 cells were trypsinized and recultured for 24 h in the presence of both 1 mM DMM and 5 mM DNM. After this reculture period, cells were harvested and tested for their binding to Con A and SNA by flow cytometry. During the experiments, the lectin PHA-L (leukoagglutinin from *Phaseolus vulgaris*) was included in these tests

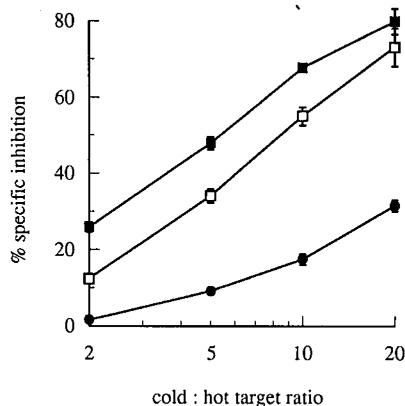

Fig. 4. Recovery of the NK susceptibility of K562 cells after trypsinization. K562 cells were trypsinized with 1 mg/ml trypsin, washed and recultured as described. After 24 h, cells were harvested and tested in a cold target inhibition assay. In this assay, various amounts of unlabelled (cold) target cells, which have undergone different treatments, are added to a mixture of 10^5 NK cells and 10^4 ^{51}Cr-labelled (hot) K562 cells. Depicted in this figure is the specific inhibition of chromium release, caused by the addition of cold target cells, which received the following treatments: no treatment (■), trypsinized with 24 h reculture (□), or trypsinized without reculture (●). Bars indicate the SD between triplicate determinations.

because it proved to be a better marker for 'mature' carbohydrate chains than SNA [see below and Dennis *et al.* (1987)]. Treatment with inhibitors during the reculture period had no measurable influence on the number of cells or their viability, compared to cells which were trypsinized and recultured without inhibitors.

The lectin-binding properties of K562 cells in the different stages of this experiment are illustrated in Figure 5 and compiled in Table I. The binding characteristics of K562 cells with lectins Con A and PHA-L before and after treatment with DMM and DNM clearly demonstrate the expected alterations in cell surface glycosylation. The elevation in the expression of high-mannose-type carbohydrate chains causes a significant increase in binding to Con A (Figure 5, upper panel), whereas the strong decrease in PHA-L binding reflects the diminished occurrence of branched, complex structures (Figure 5, lower panel).

A quantitative analysis of the lectin-binding experiments (Table I) shows that trypsinization of K562 cells influences staining with Con A and PHA-L in a comparable way, whereas the effect on staining with SNA is smaller (trypsin-0 values). After the recovery period of 24 h (trypsin-24 values), values for all the lectins are back at ~80% of the control. In quantitative terms, the most striking observation is the fact that treatment with DMM and DNM reduces the expression of PHA-L ligands, complex branched oligosaccharides, by almost 90%. Opposite to this, Con A binding increases 2- to 3-fold. The influence of DMM/DNM on the overall expression of SNA ligands—$\alpha(2\rightarrow6)$ linked sialic acid—is smaller. Nevertheless, a significant decrease in SNA binding is apparent, which is consistent with a diminished expression of 'mature' complex and/or hybrid carbohydrates. Finally, the results from an experiment in which DMM and DNM were used separately indicate that DMM-treated cells bind more Con A than their DNM-treated counterparts (Table I).

From the lectin staining characteristics in Figure 5 and Table I, it is evident that the cell surface carbohydrate composition of K562 cells is drastically altered by treatment with DMM and DNM. Nevertheless, cold target assays with these cells showed

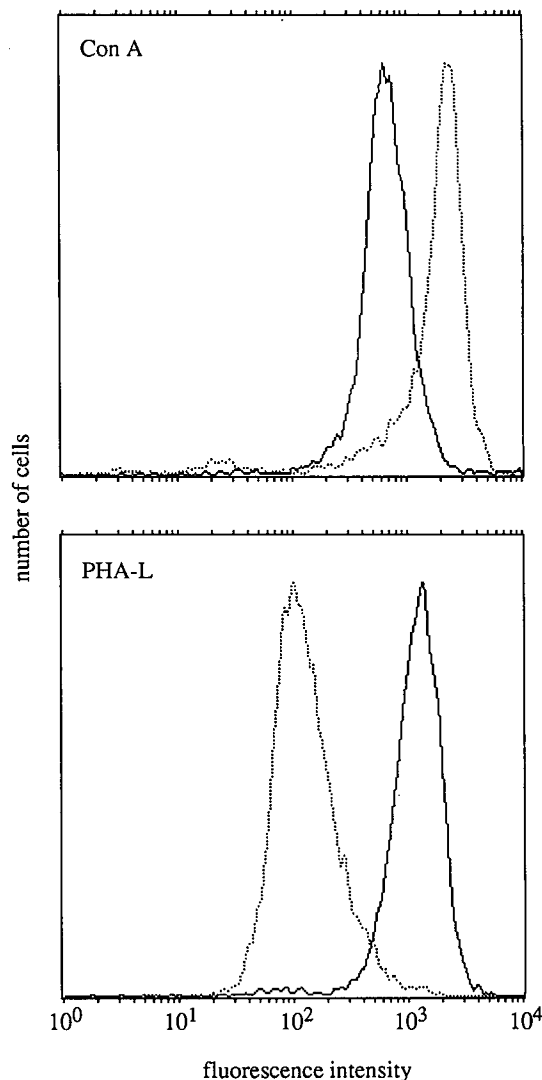

Fig. 5. Flow cytometric analysis of the lectin-binding properties of K562 cells after treatment with DMM and DNM. Control K562 cells (solid lines) and cells which were trypsinized and recultured for 24 h in the presence of 1 mM DMM and 5 mM DNM (dotted lines) were incubated with the biotinylated lectins Con A (**upper panel**) and PHA-L (**lower panel**), stained with FITC–avidin and analysed by flow cytometry.

that the expression of NK target structures was not affected (Figure 6). For the sake of clarity, only the experiment where both inhibitors were added simultaneously is shown. When DMM and DNM were used separately, similar results were obtained. In the post-binding stage, no effect on NK-mediated cytotoxicity could be observed either, because DMM/DNM-treated targets did not differ from untreated cells in sensitivity to lysis by NK cells (data not shown).

Discussion

In this paper, it is shown that the recognition of K562 target cells by NK cells depends on the presence of protein-bound determinants. Removal of these structures by trypsinization results in an almost complete abolition of binding (Figure 1b) and of lysis (Figure 1a) of target cells. The regeneration of target structures after trypsinization proceeded to ~80% in 24 h (Figure 4), which is in close correlation with the overall

Table I. Quantitative comparison of lectin staining characteristics of K562 cells during treatment with *N*-glycosylation inhibitors

Treatment	Relative fluorescence intensity[a]		
	Con A	PHA-L	SNA
Control	100	100	100
Trypsin-0	36±4	47±1	71±17
Trypsin-24	78±13	82±13	78±5
DMM/DNM	239±5	13±1	56±5
DMM	272[b]		
DNM	152[b]		

K562 cells were trypsinized as described and recultured for 24 h in medium (trypsin-24) or in medium containing 1 mM DMM and 5 mM DNM (DMM/DNM), or in medium with 1 mM DMM (DMM) or medium with 5 mM DNM (DNM). After the reculture period, cells were harvested, stained with Con A, PHA-L or SNA, and analysed by flow cytometry. Untreated cells (control) and freshly trypsinized cells (trypsin-0) were included as controls. For a quantitative comparison, logarithmic fluorescence patterns were converted to linear values, which represent the mean fluorescence intensity. Values are given as mean ± SD of two (PHA-L and SNA) or three (Con A) independent determinations.
[a]Fluorescence intensity of control K562 cells set at 100 for each lectin.
[b]Single determination.

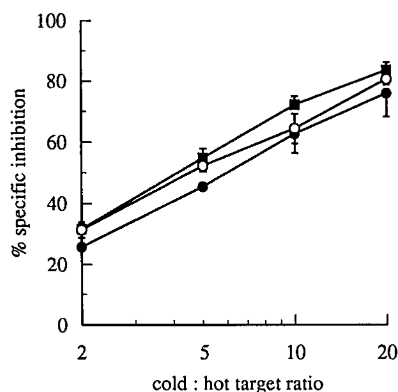

Fig. 6. The effect of DMM/DNM treatment of K562 cells on the recognition by NK cells. K562 cells were trypsinized as described and recultured in culture medium with or without DMM/DNM. After 24 h, cells were harvested and tested in a cold target inhibition assay. In this assay, various amounts of unlabelled (cold) target cells, which have undergone different treatments, are added to a mixture of 10^5 NK cells and 10^4 ^{51}Cr-labelled (hot) K562 cells. Depicted in this figure is the specific inhibition of chromium release, caused by the addition of cold target cells, which received the following treatments: no treatment (■), trypsinized and recultured in medium (●), and trypsinized and recultured in medium with 1 mM DMM and 5 mM DNM (○). Bars indicate the SD between triplicate determinations.

regeneration of cell surface glycoproteins as measured by the lectin-binding properties of the cells (Table I). The observation that the removal of NK target structures by trypsin is dose dependent over a wide range of trypsin concentrations suggests that the recognition of target cells is not restricted to only peripheral determinants. If that were the case, target structures would probably be more sensitive to trypsinization and therefore their removal would not show such a broad dose dependency (Figure 1b).

Contrary to the concept of protein-bound determinants, several studies report that the ganglioside GM2, isolated from malignant (Ando *et al.*, 1987) or normal (Grayson and Ladisch, 1992) tissue, specifically inhibits both NK-mediated binding to and NK-mediated lysis of K562 cells. Although in these reports the structure of the glycolipid is not considered in detail, the observation of inhibition by GM2 is very interesting because it is obvious that the carbohydrate part of the ganglioside should be responsible for its specificity. The terminal carbohydrate structure of GM2, GalNAcβ(1→4)(NeuAcα(2→3))Gal β, is equivalent to the Sd[a] blood group determinant (Blanchard *et al.*, 1983). The possibility that this structure, or its essential elements, could function as recognition points as part of glycoprotein-bound carbohydrate chains is currently under investigation in our laboratory.

Our experiments concerning sialic acid or poly-*N*-acetyllactosamine chains (see below), whose expression is considered to display tumour-associated changes (Smets and Van Beek, 1984; Dennis *et al.*, 1987), have not provided evidence for the involvement of these structures in NK-mediated cytotoxicity. For sialic acid, the opposite appears to be true. Our results (Figure 2) indicate that this molecule functions as an 'anti-adhesion molecule'. Consistent, but relatively small, increases in NK susceptibility of target cells after sialidase treatment have been described before (Yogeeswaran *et al.*, 1982; Werkmeister *et al.*, 1983; Rooney and Munro, 1984). Apparently, the amount of cell surface sialic acid, which can be cleaved off by virtue of its accessibility to the enzyme, is limited. Probably, the application of two different sialidases is more efficient than the use of one enzyme because together they have a broader substrate repertoire on the cell surface. That is the most obvious explanation for the increased effect of the mixture on NK susceptibility because we could not detect a higher release of sialic acid with the periodate/borotritiide labelling method. The 'protective' effect of sialic acid against the action of effector cells could relate to the finding that in some (animal) tumour cells a correlation has been found between cell surface sialylation and metastatic capacity (Yogeeswaran and Salk, 1981; Passaniti and Hart, 1988).

Removal of sialic acid can influence recognition in different ways. By exposing sugars, which were originally masked, sialidase can create new terminal carbohydrate determinants that could mediate binding with NK cells. Alternatively, removal of the negatively charged sialic acid molecules can cause a decrease in the repulsive electrostatic forces between target and effector cell, thereby facilitating conjugation. In accordance with earlier findings with other target cells (Rooney and Munro, 1984), the experiments with different galactosidases show that unmasking of galactose residues, as in Asn-linked complex and hybrid glycans, is probably not the reason why removal of sialic acid increases the NK susceptibility of target cells. α-Galactosidase was included in these experiments because of recent reports of the occurrence of terminal α-galactose residues on malignant human cells (Castronovo *et al.*, 1989).

In haematopoietic cells, the expression of poly-*N*-acetyllactosamine glycans on glycoconjugates is associated with the (malignant) differentiation stage of the cells (Fukuda, 1985). However, although the endo-β-galactosidase experiments showed the presence of this type of structure on the K562 cells, a role in target–effector interaction could not be demonstrated.

In principle, the biological significance of N-linked carbohydrate chains of glycoproteins can be investigated relatively easily because of the availability of enzymes which cleave their common core structure (Damm *et al.*, 1987) and of inhibitors which interfere in their biosynthetic pathway (Fuhrmann *et al.*, 1985; Elbein, 1987). Because these enzymes, e.g. PNGase F, have to cleave close to the protein backbone to release N-linked carbohydrate chains, they are usually only fully active on

denatured substrates. Therefore, the lack of influence of PNGase F digestion on the interaction between NK cells and target cells does not provide conclusive evidence as to whether or not N-linked carbohydrate chains are involved in this process.

K562 cells restore their NK susceptibility rapidly during reculture after trypsinization (Figure 4). Apparently, the biosynthetic machinery is not damaged by the proteolytic treatment. During the 24 h reculture period after trypsinization, we observed practically no increase in the number of cells, but after this period normal cell growth was restored.

Reculture of K562 cells in the presence of DMM and DNM leads to the expected changes in the cell surface carbohydrate composition, as indicated by the alterations in Con A and PHA-L binding (Figure 5 and Table I). Both the elevated expression of high-mannose (detected with Con A) and the disappearance of branched complex (detected with PHA-L) N-linked carbohydrate chains are nicely demonstrated. This is in accordance with earlier findings that the expression of normally developed hybrid and complex structures on K562 cells is almost completely blocked after treatment with 1 mM DMM (Neefjes *et al.*, 1988). Furthermore, our results (Table I) seem to confirm the observation that DMM is a more effective inhibitor in cell culture than DNM (Tan *et al.*, 1991), even though the latter is used at a higher concentration. However, it should be emphasized that the quantitative differences between DMM- and DNM-treated cells could, in part, be due to differences in affinity towards Con A between the resulting carbohydrate structures.

Apparently, FACS analysis of the binding of lectins to K562 cells provides a useful technique to detect (inhibitor-mediated) changes in the cell surface carbohydrate composition. Compared to other methods, e.g. metabolic labelling or structural analysis of purified glycoproteins (Tan *et al.*, 1991), the FACS technique has the advantage of specificity and representativity for the cell surface. Metabolic labelling is usually not confined to the cell membrane (Schachter, 1978; Baumann and Doyle, 1982). The isolation and subsequent analysis of one specific protein (Tan *et al.*, 1991) provides detailed data, but it is a quite laborious method, which does not necessarily reflect the overall changes in cell surface glycosylation.

The main reason for the rather small variation in SNA staining (Table I) is probably the ubiquitous occurrence of $\alpha(2\rightarrow6)$-linked sialic acid in O-linked carbohydrate chains and glycolipids, which are only partly affected by trypsin and not at all by DMM/DNM. Swainsonine and tunicamycin have been previously applied to alter target cell susceptibility towards NK cell-mediated cytotoxicity by modifying *N*-glycosylation. The conflicting results of these studies [reviewed by McCoy and Chambers (1991)] show that these compounds are less suitable to study biological effects of the modification of *N*-glycosylation. Tunicamycin is highly toxic to cells and can already interfere with protein synthesis at low levels (Elbein, 1987). Furthermore, the complete prevention of the attachment of N-linked carbohydrate chains to proteins by tunicamycin is very likely to have effects on the folding of the protein. Processing inhibitors like DMM and DNM have a much smaller influence on the protein part of glycoproteins. Therefore, they are less toxic (Neefjes *et al.*, 1988; Tan *et al.*, 1991) and more suitable to study the carbohydrate part specifically. The effects of swainsonine on the structure of *N*-glycans are less far reaching (Fuhrmann *et al.*, 1985) and, consequently, less likely to have much influence on bioactivity.

In our view, there are several implications for the fact that a variety of modifications of the cell surface carbohydrate phenotype lacked any influence on the recognition of K562 target cells by NK cells. Particularly, the potential involvement of N-linked glycans seems questionable, regarding the fact that neither the presence of common terminal structures nor normal processing is essential. A further consequence of the DMM/DNM experiments is that apparently the trafficking of the target structures to the cell membrane is not altered when full processing of *N*-glycans is blocked. Similar observations with DMM and DNM have been made in the related system with lymphokine-activated killer cells as effector cells (Chong *et al.*, 1988). Therefore, there could be analogies in target cell recognition between NK cells and LAK cells, despite the differences in target cell repertoire (Rosenberg and Lotze, 1986).

In this paper, we have not considered the possible role of effector cell surface glycoconjugates in NK target interaction. In a recent report (Asada *et al.*, 1991), it was shown that the leukocyte cell surface glycoprotein LFA-1, a member of the integrin family, which is involved in natural killing by human large granular lymphocytes (Timonen *et al.*, 1988), contains sialyl Le^x. Since this carbohydrate determinant is a ligand for several adhesion molecules (Foxall *et al.*, 1992), it could be involved in the interaction between NK cells and target cells. On the other hand, it must be noted that LFA-1 occurs abundantly on all leukocytes (Trinchieri, 1989). Therefore, this integrin cannot be crucial for target cell recognition by NK cells, but it probably plays an accessory role in binding of target cells.

Summarizing, our results have not yet provided an obvious candidate target structure for human NK cells. Instead, we have found evidence against the involvement of glycolipids and N-linked carbohydrates of glycoproteins. However, more detailed information than can be obtained with modification of cell surface glycosylation will be necessary to get a complete picture of the involvement of glycoconjugates in NK cell-mediated cytotoxicity against malignant cells.

Materials and methods

Reagents

Enzymes were purchased from Boehringer Mannheim (Mannheim, Germany) unless stated otherwise. DMM and SNA–biotin were from Boehringer Mannheim. Con A–biotin, PHA-L–biotin and fluorescein isothiocyanate (FITC)–avidin were from Sigma (St Louis, MO). DNM was a kind gift of Dr L.A.G.M.Van Den Broek of Organon International (Oss, The Netherlands).

Cell culture

Cell culture media and additives, including heat-inactivated fetal calf serum (FCS), were purchased from Life Technologies (Gaithersburg, MD). The human erythromyeloid leukaemia cell line K562 (ICN-Flow, Irvine, UK) was kept in continuous culture in RPMI 1640, supplemented with 10% FCS and 2 mM L-glutamine (culture medium). K562 cells were free of mycoplasma. Throughout the experiments, the effect of all treatments on the viability of cells was checked by trypan blue dye exclusion at the highest reagent concentrations used, and found to be >95% unless stated otherwise.

Effector cells

Human $CD3^-$, $CD16/56^+$ natural killer cells were isolated from peripheral blood by a negative selection protocol, based on the method of Cosentino and Cathcart (1987). Briefly, peripheral blood mononuclear cells, obtained by Ficoll centrifugation, were enriched for NK cells by incubation on nylon wool to remove monocytes and B cells, and subsequent panning with anti-CD3 (anti-Leu-4, Becton Dickinson, Mountain View, CA) to remove T cells. The phenotype of the effector cells was determined by flow cytometric analysis

(see below), after staining with a Simultest anti-T cell/anti-NK cell reagent (Becton Dickinson, anti-Leu-4 FITC, anti-Leu-11c phycoerythrin (PE), anti-Leu-19 PE) according to the manufacturers' instructions. The resulting effector cell population contained 61±11% $CD3^-$, $CD16/56^+$ NK cells. Peripheral blood was obtained from the Red Cross Bloodbank Utrecht; in a number of experiments, the mononuclear cell fraction from peripheral blood was kindly provided by the Department of Pulmonary Disease of the University Hospital, Utrecht.

Enzymatic modifications of target cells

Exposed proteins on the K562 cell surface were removed by proteolytic treatment with trypsin (EC 3.4.21.4). K562 cells (5–20 × 10^6 cells/ml) were incubated with trypsin during 20 min at 37°C, with enzyme concentrations up to 1 mg/ml in phosphate-buffered saline (PBS; 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, 8 g/l NaCl, 0.2 g/l KCl, pH 7.2) with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. In most experiments, 20–40 μg/ml of DNase I (EC 3.1.21.1) were included to avoid cell clumping, due to DNA released from damaged cells. DNase I treatment did not influence the NK susceptibility of K562 cells, measured as either lysis or cold target inhibition. The viability of K562 cells was slightly affected by protease treatment, but remained consistently >90%.

Vibrio cholerae (VCN) or *A.ureafaciens* (AUN) sialidase treatment of K562 cells was performed by incubating 5 × 10^6 cells with 25–100 mU of enzyme in 1 ml of PBS (VCN, supplemented with 1 mM $CaCl_2$) for 30 min at 37°C.

Green coffee bean α-galactosidase (Sigma, EC 3.2.1.22) and β-galactosidase (EC 3.2.1.23) from jack beans (Sigma) or bovine testes were used to remove terminal galactose residues from K562 cells. Usually, 5 × 10^6 cells/ml of PBS/0.1% bovine serum albumin (BSA) (pH 6.0) were incubated with 100 mU of enzyme for 30 min at 37°C. The two β-galactosidases have different specificities towards β(1→4) and β(1→3) linkages (Madiyalakan *et al.*, 1984). Enzyme activities of α- and β-galactosidase were regularly tested with *p*-nitrophenyl-α-galactose and *p*-nitrophenyl-β-galactose, respectively.

Poly-*N*-acetyllactosamine chains on K562 cell surfaces were digested with endo-β-galactosidase (EC 3.2.1.103) from *B.fragilis* (cf. Viitala and Finne, 1984). K562 cells (5 × 10^6/ml) in PBS/0.1% BSA (pH 6.0) were treated with 10 mU of enzyme for 30 min at 37°C.

Cell surface labelling

To establish the presence of certain carbohydrates on the cell surface of K562 cells and to follow the enzymic removal of these structures, two carbohydrate-directed cell surface radiolabelling methods were used. Sialic acid residues were specifically labelled with the periodate/borotritiide method (Gahmberg and Andersson, 1977). The galactose oxidase/borotritiide method (Gahmberg *et al.*, 1976) was used to specifically label galactose moieties.

Modification of cell surface N-glycosylation

Compared to their O-linked counterparts, N-linked carbohydrate chains can be modified or even cleaved off rather easily because of their common core structure and ordered biosynthetic pathway (Fuhrmann *et al.*, 1985). Digestion of intact K562 cells with PNGase F, an enzyme that specifically releases entire N-linked carbohydrate chains (Damm *et al.*, 1987), was performed by incubating 5 × 10^6 cells in 1 ml of PBS (supplemented with 1 mM EDTA) with 2–4 U of PNGase F for 2–4 h at 37°C. Secondly, *N*-glycosylation of K562 cells was altered by treatment with DMM and DNM, inhibitors of the processing of N-linked carbohydrate chains. K562 cells were trypsinized with 1 mg/ml trypsin as described above and washed three times with medium to remove trypsin. Subsequently, cells were resuspended at 1–2 × 10^6/ml in culture medium, supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin, and recultured at 37°C and 5% CO_2. Inhibitors were used at a concentration of 1 mM (DMM) or 5 mM (DNM). After the appropriate period of reculture, cells were collected by centrifugation, washed three times and used in cytotoxicity assays or FACS analyses.

Flow cytometry

The cell surface N-glycosylation of K562 cells was analysed on the basis of binding properties of the cells with the lectins from Con A, SNA and PHA-L. Con A is specific for mannose-containing carbohydrate chains (Goldstein and Hayes, 1978), whereas PHA-L binds to complex tri- and tetraantennary structures containing the extra β(1→6)-linked branch (Dennis *et al.*, 1987) and SNA to α(2→6)-linked sialic acid (Shibuya *et al.*, 1987). In each experiment, 5000 cells were analysed by flow cytometry on a FACScan flow cytometer (Becton Dickinson). To determine the lectin-binding properties of the K562 cells, 10^6 cells were washed and resuspended in 50 μl PBS/BSA (PBS with 0.5% bovine serum albumin and 0.05 % NaN_3), containing 20 μg/ml of the biotinylated lectin, and incubated for 30 min on ice. After two washes with PBS/BSA, cells were stained in 50 μl PBS/BSA with 10 μg/ml of FITC–avidin for 30 min on ice, washed twice, resuspended in 0.5 ml PBS/BSA and analysed. Aspecific binding of FITC–avidin was negligible. Serial dilutions of the biotinylated lectins and FITC–avidin showed that, under the above-mentioned conditions, saturated staining of K562 cells was obtained.

Cytotoxicity assays

Two types of chromium release assays were used, which will be referred to as standard and cold target assay, respectively.

In the standard chromium release assay, the lysis of target cells at different E/T ratios is determined. This assay can therefore be seen as a measure for the net result of the complete cytolytic cascade (see Introduction). Target cells (10^6) were labelled with 3.7 MBq $Na_2{}^{51}CrO_4$ in sodium chloride solution (Amersham International, Amersham, UK) at 37°C for 1 h. After removal of excess ^{51}Cr by two washes with medium, cells were preincubated in excess medium at 37°C and 5% CO_2 for 30 min, washed once, resuspended at 10^5/ml and used as targets. Effector cells and 10^4 ^{51}Cr-labelled target cells were mixed at various E/T ratios in 200 μl medium and plated in 96 well round-bottom microtitre plates.

In the cold target assay, various amounts of unlabelled (cold) target cells are added to a system consisting of fixed numbers of effector cells and labelled (hot) target cells. The cold cells compete with the hot cells for the effector cells, leading to a reduction in chromium release. This reduction depends on the relative affinity of the effector cells for the cold and hot targets. This assay is indicative for the expression of molecules on the target cells which are involved in the interaction between NK cells and their targets. For the cold target assays, modified unlabelled target cells were mixed with 10^4 labelled K562 cells and 10^5 effector cells at different cold to hot target ratios in a total volume of 200 μl and plated as above. Prior to incubation, plates were centrifuged for 5 min at 100 *g* to facilitate cell-to-cell contact. After a 4 h incubation at 37°C and 5% CO_2, plates were again centrifuged and 100 μl of the supernatants were collected and counted in a gamma counter. Specific cytotoxicity was calculated using the equation:

$$\%\ \text{specific lysis} = \frac{\text{c.p.m. test} - \text{c.p.m. spontaneous}}{\text{c.p.m. maximal} - \text{c.p.m. spontaneous}} \times 100\%$$

where c.p.m. spontaneous and c.p.m. maximal are determined in the presence of medium alone and 1% sodium dodecyl sulphate (SDS), respectively. Spontaneous release was always <10% of maximal release. The efficiency of cells to act as cold target inhibitors is expressed as:

$$\%\ \text{specific inhibition} = \frac{\%\ \text{lysis without cold targets} - \%\ \text{lysis with cold targets}}{\%\ \text{lysis without cold targets}} \times 100\%$$

Results from cytotoxicity assays are presented as representative examples from a series of at least three similar experiments. Individual experiments were always performed in triplicate.

Acknowledgements

The authors would like to thank Dr L.A.G.M.Van Den Broek of Organon International for his kind gift of DNM and the Department of Pulmonary Disease, University Hospital, Utrecht, for the generous supply of peripheral blood mononuclear cells. This investigation was supported by grant UUKC 88-14 from the Dutch Cancer Society. The Department of Pathology was supported by donations of the Maurits & Anna de Kock Stichting and the Nijbakker-Morra Stichting.

Abbreviations

AUN, *Arthrobacter ureafaciens* sialidase; BSA, bovine serum albumin; Con A, lectin from *Canavalia ensiformis*; DMM, 1-deoxymannojirimycin; DNM, 1-deoxynojirimycin; FITC, fluorescein isothiocyanate; MHC, major histocompatibility complex; NK, natural killer; PBS, phosphate-buffered saline; PE, phycoerythrin; PHA-L, leukoagglutinin from *Phaseolus vulgaris*; PNGase F, *N*-glycanase; SDS, sodium dodecyl sulphate; SNA, lectin from *Sambucus nigra*; VCN, *Vibrio cholerae* sialidase.

References

Ando,I., Hoon,D.S.B., Suzuki,Y., Sarton,R.E., Golub,S.H. and Irie,R.F. (1987) Ganglioside GM2 on the K562 cell line is recognized as a target structure by human natural killer cells. *Int. J. Cancer*, **40**, 12–17.

Asada,M., Furukawa,K., Kantor,C., Gahmberg,C.G. and Kobata,A. (1991) Structural study of the sugar chains of human leucocyte cell adhesion molecules CD11/CD18. *Biochemistry*, **30**, 1561–1571.

Baumann,H. and Doyle,D. (1982) Turnover of plasma membrane glycoproteins and glycolipids (hepatoma as a model). In Horowitz,M.I. (ed.), *The Glycoconjugates*. Academic Press, New York, Vol. **IVB**, pp. 105–154.

Biron,C.A., Byron,K.S. and Sullivan,J.L. (1989) Severe herpesvirus infections in an adolescent without natural killer cells. *N. Engl. J. Med.*, **320**, 1731–1733.

Blanchard,D., Cartron,J-P., Fournet,B., Montreuil,J., Van Halbeek,H. and Vliegenthart,J.F.G. (1983) Primary structure of the oligosaccharide determinant of blood-group Cad specificity. *J. Biol. Chem.*, **258**, 7691–7695.

Castronovo,V., Colin,C., Parent,B., Fiodart,J.-M., Lambotte,R. and Mahieu,P. (1989) Possible role of human natural anti-Gal antibodies in the natural antitumor defense system. *J. Natl. Cancer Inst.*, **81**, 212–216.

Chong,A.S.-F., Hersh,E.M. and Grimes,W.J. (1988) Blocking of lymphokine activated killer (LAK) cell mediated cytotoxicity by cell-sized beads bearing tumor cell proteins. *J. Immunol.*, **141**, 4418–4424.

Cosentino,L.M. and Cathcart,M.K. (1987) A multi-step isolation scheme for obtaining CD 16^+ human natural killer cells. *J. Immunol. Methods*, **103**, 195–204.

Damm,J.B.L., Kamerling,J.P., Van Dedem,G.W.K. and Vliegenthart,J.F.G. (1987) A general strategy for the isolation of carbohydrate chains from *N*-, *O*-glycoproteins and its application to human chorionic gonadotrophin. *Glycoconjugate J.*, **4**, 129–144.

Decker,J.M., Hinson,A. and Ades,E.W. (1984) Inhibition of human NK cell cytotoxicity against K562 cells with glycopeptides from K562 plasma membranes. *J. Clin. Lab. Immunol.*, **15**, 137–143.

Dennis,J.W., Laferté,S., Waghorne,C., Breitman,M.L. and Kerbel,R.S. (1987) β(1-6) branching of Asn-linked oligosaccharides is directly associated with metastasis. *Science*, **236**, 582–585.

Den Otter,W. (1986) Immune surveillance and natural resistance: an evaluation. *Cancer Immunol. Immunother.*, **21**, 85–92.

Den Otter,W. (1987) Evaluation of *in vivo* tumoricidal effector mechanisms. *Res. Monogr. Immunol.*, **11**, 109–123.

Elbein,A.D. (1987) Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annu. Rev. Biochem.*, **56**, 497–534.

Foster,C.S., Gillies,D.R.B. and Glick,M.C. (1991) Purification and characterization of GDP-L-Fuc-*N*-acetyl-β-D-glucosaminide α(1→3) fucosyltransferase from human neuroblastoma cells. *J. Biol. Chem.*, **266**, 3526–3531.

Foxall,C., Watson,S.R., Dowbenko,D., Fennie,C., Lasky,L.A., Kiso,M., Hasegawa,A., Asa,D. and Brandley,B.K. (1992) The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl Lewis^x oligosaccharide. *J. Cell Biol.*, **117**, 895–902.

Fuhrmann,U., Bause,F. and Ploegh,H. (1985) Inhibitors of oligosaccharide processing. *Biochim. Biophys. Acta*, **825**, 95–110.

Fukuda,M. (1985) Cell surface glycoconjugates as onco-differentiation markers in hematopoietic cells. *Biochim. Biophys. Acta*, **780**, 119–150.

Gahmberg,C.G. and Andersson,L.C. (1977) Selective radioactive labeling of cell surface sialoglycoproteins by periodate-tritiated borohydride. *J. Biol. Chem.*, **252**, 5888–5894.

Gahmberg,C.G., Häyry,P. and Andersson,L.C. (1976) Characterization of surface glycoproteins of mouse lymphoid cells. *J. Cell Biol.*, **68**, 642–653.

Goldstein,I.J. and Hayes,C.E. (1978) The lectins: carbohydrate binding proteins of plants and animals. *Adv. Carbohydr. Chem. Biochem.*, **35**, 127–340.

Grayson,G. and Ladisch,S. (1992) Immunosuppression by human gangliosides, II carbohydrate structure and inhibition of human NK activity. *Cell. Immunol.*, **139**, 18–29.

Hakomori,S. (1989) Aberrant glycosylation in tumors and tumor-associated carbohydrate antigens. *Adv. Cancer Res.*, **52**, 257–331.

Henkart,P.A., Lewis,J.T. and Ortaldo,J.R. (1986) Preparation of target antigens specifically recognized by human natural killer cells. *Nat. Immun. Cell Growth Regul.*, **5**, 113–126.

Herberman,R.B. (1986) Mechanism of cytotoxicity by natural killer (NK) cells. *Annu. Rev. Immunol.*, **4**, 651–680.

Hiserodt,J.C., Britvan,L. and Targan,S.R. (1983) Studies on the mechanism of the human natural killer cell lethal hit: analysis of the mechanism of protease inhibition of the lethal hit. *J. Immunol.*, **131**, 2705–2709.

Madiyalakan,R., DiCoccio,R.A. and Matta,K.L. (1984) A simple and rapid method for the purification of the β-D-galactosidase from bovine testes. *Carbohydr. Res.*, **129**, 298–302.

McCoy,J.P.,Jr and Chambers,W.H. (1991) Carbohydrates in the functions of natural killer cells. *Glycobiology*, **1**, 321–328.

Neefjes,J.J., Verkerk,J.M.H., Broxterman,H.J.G., Van Der Marel,G.A., Van Boom,J.H. and Ploegh,H.L. (1988) Recycling glycoproteins do not return to the *cis*-Golgi. *J. Cell Biol.*, **107**, 79–87.

Passaniti,A. and Hart,G.W. (1988) Cell surface sialylation and tumor metastasis. Metastatic potential of B16 melanoma variants correlates with their relative number of specific penultimate oligosaccharide structures. *J. Biol. Chem.*, **263**, 7591–7603.

Robertson,M.J. and Ritz,J. (1990) Biology and clinical relevance of natural killer cells. *Blood*, **76**, 2421–2438.

Robertson,M.J., Caligiuri,M.A., Manley,T.J., Levine,H. and Ritz,J. (1990) Human natural killer cell adhesion molecules. Differential expression after activation and participation in cytolysis. *J. Immunol.*, **145**, 3194–3201.

Roder,J.C., Rosen,A., Fenyo,E.M. and Troy,F.A. (1979) Target effector interaction in the natural killer system, isolation of target structures. *Proc. Natl. Acad. Sci. USA*, **76**, 1405–1409.

Rooney,C.M. and Munro,A.J. (1984) NK cells can recognize asialylated autologous lymphocytes and ABO-mismatched lymphocytes. *Immunology*, **51**, 193–199.

Rosenberg,S.A. and Lotze,M.T. (1986) Cancer immunotherapy using interleukin-2 and interleukin-2-activated lymphocytes. *Annu. Rev. Immunol.*, **4**, 681–709.

Schachter,H. (1978) Glycoprotein biosynthesis. In Horowitz,M.I. and Pigman,W. (eds), *The Glycoconjugates*. Academic Press, New York, Vol. **II**, pp. 88–185.

Shibuya,N., Goldstein,I.J., Broekaert,W.F., Nsimba-Lubaki,M., Peeters,B. and Peumans,W.J. (1987) The elderberry (*Sambucus nigra*) lectin recognizes the NeuAc α(2-6) Gal/GalNAc sequences. *J. Biol. Chem.*, **262**, 1596–1601.

Smets,L.A. and Van Beek,W.P. (1984) Carbohydrates of the tumor cell surface. *Biochim. Biophys. Acta*, **738**, 237–249.

Springer,T.A. (1990) Adhesion receptors of the immune system. *Nature*, **346**, 425–434.

Tan,A., Van Den Broek,L., Van Boeckel,S., Ploegh,H. and Bolscher,J. (1991) Chemical modification of the glucosidase inhibitor 1-deoxynojirimycin, structure–activity relationships. *J. Biol. Chem.*, **266**, 14504–14510.

Timonen,T., Patarroyo,M. and Gahmberg,C.G. (1988) CD11a-c/CD18 and GP-84 (LB-2) adhesion molecules on human large granular lymphocytes and their participation in natural killing. *J. Immunol.*, **141**, 1041–1046.

Trinchieri,G. (1989) Biology of natural killer cells. *Adv. Immunol.*, **47**, 187–376.

Viitala,J. and Finne,J. (1984) Specific cell-surface labeling of polyglycosyl chains in human erythrocytes and HL-60 cells using endo-β-galactosidase and galactosyltransferase. *Eur. J. Biochem.*, **138**, 393–397.

Welsh,R.M. (1986) Regulation of virus infections by natural killer cells: a review. *Nat. Immun. Cell Growth Regul.*, **5**, 169–199.

Werkmeister,J.A., Pross,H.F. and Roder,J.C. (1983) Modulation of K562 cells with sodium butyrate. Association of impaired NK susceptibility with sialic acid and analysis of other parameters. *Int. J. Cancer*, **32**, 71–78.

Yogeeswaran,G. and Salk,P.L. (1981) Metastatic potential is positively correlated with cell surface sialylation of cultured murine tumor cell lines. *Science*, **212**, 1514–1516.

Yogeeswaran,G., Fujinami,R., Kiessling,R. and Welsh,R.M. (1982) Interferon-induced alterations in sialic acid and glycoconjugates of L-929 cells. *Virology*, **121**, 363–371.

Received on September 28, 1992; accepted on November 17, 1992