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The Glycosylation Profile of Interleukin-2 Activated Human Lymphocytes Correlates to their Anti-Tumor Activity

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Abstract. *Background: Natural killer cells display spontaneous, non-MHC-restricted cytotoxicity against tumour cells, which is strongly enhanced after incubation with IL-2. The molecular background of the increased anti-tumour activity of these lymphokine-activated killer cells is still only partly understood. Materials and Methods: In this paper, investigation has been made of the correlation between cell surface glycosylation and anti-tumour activity of LAK cells by stimulating peripheral blood lymphocytes with interleukin-2, in the presence of inhibitors of N- and O-glycosylation. Results: Inhibition of N- or O-glycosylation of proteins during IL-2 activation leads to a 70 - 80 % decrease in the cytolytic activity of LAK cells against K562 and Daudi tumour cells, coinciding with drastic alterations in their cell surface carbohydrate profile. Conclusion: The conclusion is drawn that there is a clear correlation between the glycosylation of LAK cell glycoproteins and their anti-tumour activity, which points to the involvement of cell surface glycoconjugates in the development of LAK activity.*

The *in vitro* culture of peripheral blood lymphocytes (PBL) with IL-2 results in the generation of lymphokine-activated killer (LAK) cells, which have an increased ability to recognize and kill tumour cells, compared to 'resting' PBL (1). LAK activity is mainly mediated by cells with the NK ($CD3^-$, $CD16/56^+$) phenotype (2). Since NK and LAK cells are phenotypically closely related, they are usually discriminated functionally, by virtue of the ability of LAK cells to kill many NK-resistant tumour cells (3). Because of their strong anti-tumour activity, LAK cells have been applied in the immunotherapy of cancer, mostly in combination with IL-2 (3, 4). However, clinical results of LAK/IL-2 immunotherapy have not fulfilled the initial expectations so far (4). Currently, administration of IL-2 alone, with induction of LAK activity *in vivo*, is the preferred modality for immunotherapy, since it is equally effective as a combined IL-2/LAK treatment (5). Insight into the target specificity of LAK cells could be of great value in understanding the differences between tumour cells in susceptibility to LAK/IL-2 therapy.

The current concept of killing of tumour cells by NK and LAK cells is a two receptor model. On one hand, the effector cells express receptors for MHC class I molecules, which, upon engagement with their counterparts on the target cells, transmit an inhibitory signal to the lytic machinery; in this way killing of 'self' cells is prevented (6). A second class of receptors on NK and LAK cells is thought to be involved in target recognition and activation of the lytic machinery. Different molecules of this second type have been reported, including the NKR-P1 family (8), which shows homology to C-type lectins, and the NK-Tr1 family (7). Target cells express tightly binding carbohydrate ligands for NKR-P1 (9), but conserved protein target molecules for natural killing have also been described (10).

The differential killing of tumour cells by NK and LAK cells depends on different parameters, concerning both effector and target cells. On the effector cell side several well-

Abbreviations: Bn-GalNAc, α-Benzyl-N-acetylgalactosamine; Con A, lectin from *Canavalia ensiformis*; DMM, 1-deoxymannojirimycin; HPA, lectin from *Helix pomatia*; PHA-L, leucoagglutinin from *Phaseolus vulgaris*; PNA, lectin from *Arachis hypogaea*; SW, swainsonine.

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known adhesion proteins appear to be involved in establishing or maintaining the contact with the target cell (11). IL-2 modulates the expression of these molecules, in particular CD2 and LFA-1, on the effector cells, both *in vitro* (11, 12) and in patients receiving IL-2 therapy (13). However, increased levels of CD2 and LFA-1 are not solely responsible for the increased cytolytic capacity of LAK cells, because CD2 and LFA-1 are not restricted to lymphocytes capable of natural killing and because the expression on target cells of their most important ligands does not correlate with NK- or LAK-susceptibility (14).

Since the induction of LAK activity in lymphocytes by IL-2 is accompanied by specific alterations in the carbohydrate phenotype of these cells (15, 16), it was decided to investigate the correlation between cytolytic activity and carbohydrate profile of human LAK cells. In this paper it is shown that blocking N- or O- glycosylation during IL-2 stimulation of NK cells results in the generation of LAK cells with decreased cytolytic capacity against NK-susceptible and particularly against NK-resistant target cells. This suggests that proper glycosylation of LAK cell proteins is necessary for the development of LAK activity.

Materials and methods

Reagents. Enzymes and 1-deoxymannojirimycin (DMM) were purchased from Boehringer Mannheim (Mannheim, FRG). α-Benzyl-*N*-acetylgalactosamine (Bn-GalNAc), swainsonine (SW) from *Rhizoctonia leguminicola*, biotinylated lectins and FITC-avidin were from Sigma (St. Louis MO, USA).

Cell culture. Cell culture media and additives were obtained from Life Technologies (Gaithersburg MD, USA). The human erythromyeloid leukaemia cell line K562 (ICN-Flow, Irvine, Scotland) was kept in continuous culture in RPMI 1640, supplemented with 10% FCS and 2 mM L-glutamine (culture medium). The Burkitt's lymphoma cell line Daudi (a gift of Dr. W.B.M. De Lau, Department of Immunology, University Hospital Utrecht) was maintained in culture medium supplemented with 25 mM Hepes buffer. Cells were mycoplasma-free.

Effector cells. Human peripheral blood mononuclear cells were isolated from peripheral blood (Red Cross Bloodbank Utrecht) by Ficoll centrifugation, and incubated on nylon wool to remove adherent cells (17). The resulting non-adherent cells consisted mainly of T cells and NK cells (17), and will be referred to as peripheral blood lymphocytes (PBL) for convenience. To obtain lymphokine-activated killer cells, PBL were incubated for 5 - 7 days in lymphocyte medium (culture medium, supplemented with 25 mM Hepes buffer, 100 IU/ml penicillin and 100 μg/ml streptomycin) with 1000 U/ml recombinant human IL-2 (Sanofi, Toulouse, France). Medium was replaced every 48 - 72 hours.

To modify the cell surface glycosylation of LAK cells, the glycosylation inhibitors DMM (2 mM), SW (12 μM), or Bn-GalNAc (2 mM) were added during the IL-2 activation period. DMM and SW interfere with two consecutive stages in the processing of N-linked carbohydrate chains, thereby preventing the development of hybrid (DMM) and lactosamine type (SW) carbohydrate chains, respectively (18). Bn-GalNAc is an inhibitor of O-glycosylation (19). Neither of these inhibitors had any effect on the viability or the recovery of LAK cells during or after the culture period.

Trypsinization and recovery of LAK cells. LAK cells (10 x 10^6 cells per ml) were trypsinized by incubation with 1 mg/ml trypsin (E.C. 3.4.21.4) in PBS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 20 - 30 minutes at 37 °C. DNase I (E.C. 3.1.21.1) was included (40 mg/ml) to avoid cell clumping. Cellular viability was slightly affected by trypsin treatment, but remained consistently > 90 %. In order to study the recovery of cytotoxic activity, trypsin was removed, and LAK cells were recultured with 1000 U/ml IL-2 for 40 hours, whether or not in the presence of 2 mM DMM, 12 μM SW, or 2 mM Bn-GalNAc.

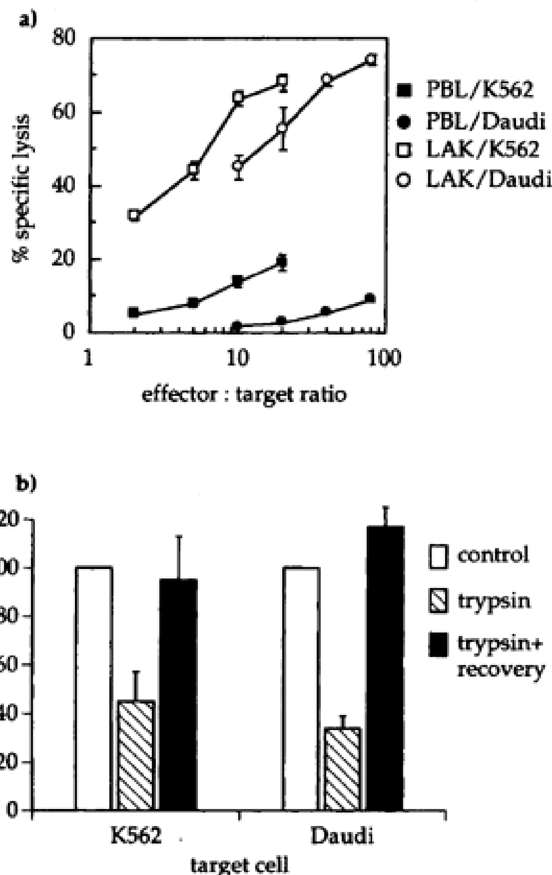

Figure 1. *Characteristics of the cytotoxicity of resting and IL-2 activated PBL. a, PBL were incubated for 6 days with (LAK) or without (PBL) IL-2 and tested for cytotoxicity against K562 and Daudi target cells. A representative example is given as mean ± S.D. b, LAK cells were trypsinised and tested for cytotoxicity before (trypsin) or after (trypsin/recovery) reculture in the presence of IL-2. Columns represent cytotoxicity relative to untreated cells (control, set at 100 % cytotoxicity). Error bars represent S.E.M. of triplicate experiments with cells from at least two donors.*

Flow cytometry. The cell surface carbohydrate profile of cells was analysed by staining with biotinylated lectins and subsequent flow cytometry as described (20). The following lectins were used: Con A from *Canavalia ensiformis*, HPA from *Helix pomatia*, PHA-L from *Phaseolus vulgaris*, and PNA from *Arachis hypogaea*. Con A and PHA-L are markers for the maturation of N-linked carbohydrate chains (20). Con A binds to 'immature' mannose-containing oligosaccharides, whereas PHA-L marks 'mature' complex tri- and tetra-antennary structures (20). HPA (19) and PNA (21) are markers for O-glycans (see also Results). In all cases, flow cytometric analysis showed that cell

Table I. *Quantitative comparison of lectin staining characteristics of PBL before and after IL-2 stimulation and treatment with glycosylation inhibitors.*

Treatment[a]	Relative fluorescence intensity[b]		
	Con A	PHA-L	PNA
control	77 ± 9	86 ± 9	n.d.
IL-2	100	100	100
IL-2 + SW	158 ± 13	24 ± 9	n.d.
IL-2 + DMM	197 ± 23	41 ± 9	n.d.
IL-2 + Bn-GalNAc	106[c]	n.d.	> 2500

[a] PBL were cultured for 6 days in medium (control), with 1000 U/ml IL-2 (IL-2), or with IL-2 and swainsonine (SW), DMM (DMM), or α-benzyl-N-acetylgalactosamine (Bn-GalNAc), as described in Materials and Methods. Subsequently, cells were harvested, stained with lectins, and analyzed by flow cytometry (24). Values represent fluorescence intensity, given as mean ± S.D. of two or more experiments with effector cells from different donors. The results in this table show that PNA binds strongly to Bn-GalNAc-treated cells. Since PNA binds preferably to the core disaccharide of O-glycans (21), a decrease in PNA binding was expected after Bn-GalNAc treatment. Instead, it increased 20 - 30 fold. The same result was found with Bn-GalNAc-treated K562 and Daudi cells. Possibly, new binding sites for PNA (e.g. on glycolipids) become exposed after truncation of O-glycans with Bn-GalNAc. In this study, binding of the lectin from Helix pomatia (cf. 19), to either LAK cells or K562 cells, was not significantly altered by Bn-GalNAc treatment.
[b] the mean fluorescence of IL-2-treated cells was set at 100;
[c] single experiment; n.d., not determined

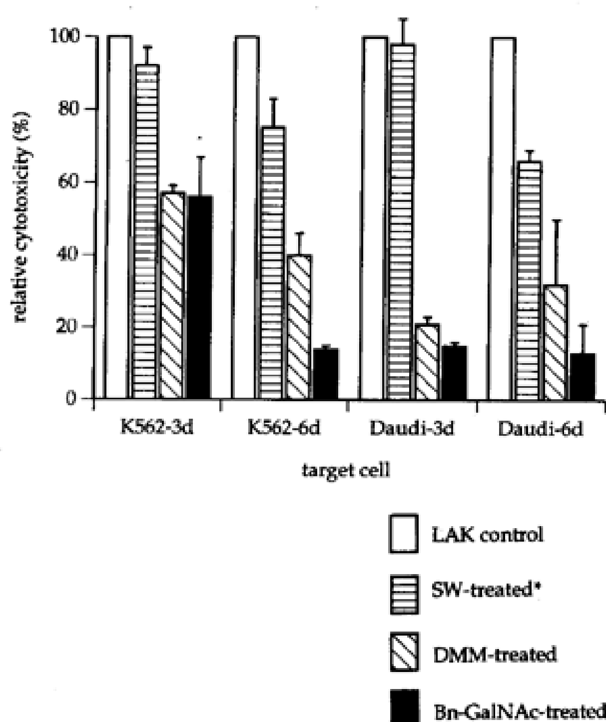

Figure 2. *The influence of glycosylation inhibitors on LAK cytotoxicity. Cytotoxicity against K562 and Daudi targets was determined of PBL, which received the following treatments: 1000 U/ml IL-2 (control), IL-2 and 12 mM swainsonine (SW), IL-2 and 2 mM DMM (DMM), or IL-2 and 2 mM Bn-GalNAc (Bn-GalNAc). Columns represent cytotoxicity after treatment for 3 days or 6 days, relative to cells treated with IL-2 alone (which were set at 100 % cytotoxicity). Error bars represent S.E.M. of triplicate experiments with cells from at least two donors.*
**Three-day cytotoxicity of SW is mean ± S.D. (n = 3) from a single donor.*

populations were homogeneously stained with the lectins. For quantitative comparison, the fluorescence patterns are represented by the weighted mean fluorescence.

Chromium release assay. A standard 4 hour chromium release assay was performed in triplicate as described (17). For quantitative comparison, cytotoxicity values at four different effector: target ratios (cf. Figure 1a) were converted into lytic units as according to Bryant *et al* (22).

Results

We tested the cytotoxicity of IL-2 activated, non-adherent human PBL with two human target cell lines, the erythroleukaemia cell line K562 and the Burkitt's lymphoma cell line Daudi. These cells differ in susceptibility to NK and LAK cells. K562 is the classical NK-sensitive target (23), which is killed by LAK cells with even higher efficiency (Figure 1a). Daudi, on the other hand, is resistant to NK cells, but is killed by LAK cells. (Figure 1a). Although the difference in NK susceptibility between these two target cells is obvious, it should be noted that in this experiment the 'resting' PBL have also been in culture for a week. This leads to some endogenous activation (17). Freshly isolated PBL are virtually incapable of killing Daudi cells (*cf.* 17).

LAK activity is reduced to about 40 % of the original level by mild trypsin treatment (Figure 1b). This suggests that the molecules on LAK cells, which are involved in the interaction with target cells, are primarily (bound to) proteins. However, there is a substantial resistance to trypsin. In this respect, there is a clear difference with the molecules on the target cells which are recognized by NK cells, since these are almost fully removed by trypsin under identical conditions (20). The partial trypsin resistance can be an indication that not the proteins themselves, but rather protein-bound carbohydrates are involved in target recognition by LAK cells, since carbohydrates often confer a certain degree of protease-resistance to their carrier proteins (24). After trypsinization, LAK cells recover their cytotoxic activity completely within 40 hours by restimulation with IL-2 (Figure 1b).

In order study the importance of LAK cell surface carbohydrates for anti-tumor activity, PBL were activated with IL-2 in the presence of the glycosylation inhibitors SW, DMM, or Bn-GalNAc. Changes in the carbohydrate profile of

LAK cells due to these treatments were monitored by flow cytometric analysis of the binding of several lectins (Table I). The 60 - 75 % decrease in binding to the lectin PHA-L after treatment with DMM and SW shows that these N-glycosylation inhibitors significantly reduce the amount of fully developed, branched carbohydrate chains. The expression of 'immature' mannose-containing structures (detected by Con A) is clearly elevated. Treatment of cells with Bn-GalNAc leads to a marked increase in staining with PNA (Table I).

Subsequent experiments revealed a remarkable influence of DMM and Bn-GalNAc on the lytic activity of LAK cells against both target cells (Figure 2). Compared to untreated cells, cytotoxicity against Daudi is reduced as much as 70 % by DMM and by more than 80 % by Bn-GalNAc after 6 days. The influence of SW on cytotoxicity is much smaller, despite its clear effect on LAK cell glycosylation (Table I). Cytotoxicity against K562 cells shows a similar pattern, although the level of inhibition is lower, particularly after 3 days. Apparently, the glycosylation inhibitors alter carbohydrate structures, which are vital for IL-2-induced cytotoxicity.

To verify these results, a different experimental approach was chosen, in which the exposure of LAK cells to the glycosylation inhibitors was reduced. LAK cells were trypsinized and recultured to study the effect of glycosylation inhibitors on the recovery of LAK activity (cf. Figure 1b). Consistent with the results presented in Figure 2, recovery is significantly affected when DMM or Bn-GalNAc is present (Figure 3). Again SW is hardly inhibitory. Lectin binding analysis of the carbohydrate profile after trypsinization and recovery showed similar results as described in Table I (data not shown).

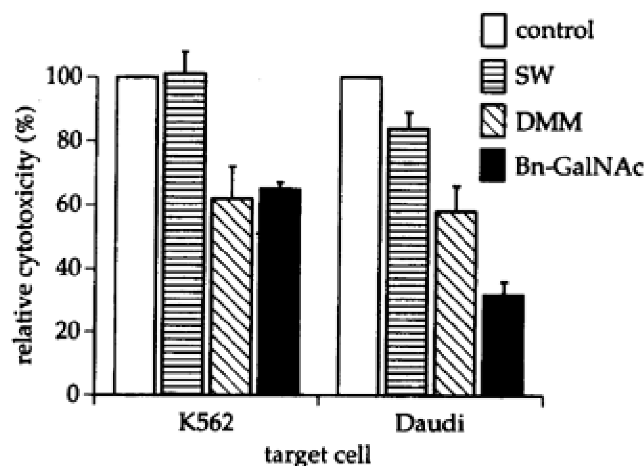

Figure 3. *The influence of glycosylation inhibitors on the recovery of LAK cytotoxicity after trypsinization. LAK cells were trypsinized and recultured for 40 h with 1000 U/ml IL-2 (control), with 1000 U/ml IL-2 and 12 mM swainsonine (SW), with 1000 U/ml IL-2 and 2 mM DMM (DMM), or with 1000 U/ml IL-2 and 2 mM Bn-GalNAc (Bn-GalNAc). Cytotoxic activity of the resulting LAK cells was tested against K562 and Daudi target cells. Columns represent cytotoxicity relative to cells recultured without inhibitors (which were set at 100 % cytotoxicity). Error bars indicate the S.E.M. of triplicate experiments with cells from at least two donors.*

Discussion

This report shows that inhibition of protein N- or O-glycosylation is accompanied by a drastic decrease in the anti-tumour activity of IL-2 activated human PBL. A clear correlation between alterations in the glycosylation profile of LAK cells and their anti-tumour activity is demonstrated in two independent models. In a previous report an enhancement of LAK activity was observed after swainsonine treatment (25). In the experiments, presented here, SW did not have a significant effect on LAK activity, either in the long term IL-2 stimulation or in the short-term trypsinization/recovery model. An explanation for the pattern of inhibition, strong with DMM and Bn-GalNAc and much weaker with SW, is that N- and O-linked carbohydrate chains often have similar terminal structures (26). These extensions are of the N-acetyllactosamine (or complex) type. Contrary to DMM and Bn-GalNAc, SW allows the expression of hybrid carbohydrate chains with an N-acetyllactosamine branch (18). Others have shown that IL-2-induced LAK activity is accompanied by up-regulation of polylactosamine carbohydrate structures (27, 28), which are characteristic representatives of the N-acetyllactosamine family. Since the results presented here show that blocking the biosynthesis of fully developed N- and O-glycans leads to a strong reduction in LAK activity, they support the idea that polylactosamine carbohydrates have a functional role in the interaction with target cells (28).

The data in this paper do not yet pinpoint the exact mechanistic role of carbohydrates in the process of LAK activity. It is not likely, however, that inhibition of glycosylation, as applied, is of direct influence on the structure of crucial glycoproteins. The N-glycosylation inhibitors SW and DMM act after the initial cotranslational attachment of N-glycans, without altering the core structure of the carbohydrates (21, 22). Therefore, these compounds probably have little effect on protein folding. The fact that LAK activity is hardly influenced by SW treatment is indicative that there are no major side-effects on protein structure, but rather that specific carbohydrates are important. Since the interference of Bn-GalNAc with O-glycosylation occurs at an even later stage (26), this compound does not affect protein structure. Although SW is the closest possible internal control, it cannot be excluded that transport of glycoproteins to the cell surface is altered after treatment with DMM or Bn-GalNAc. This is difficult to control as long as specific target receptors are only poorly identified. However, the fact that these compounds, which act

on biosynthetically unrelated events, have similar effects renders this option rather unlikely. Furthermore, there is no general effect on mediator molecules in the lytic machinery (29), since lysis of Daudi or K562 cells is not equally affected.

The alteration of cell surface glycosylation by means of inhibitors cannot be used to selectively modify specific glycoproteins. Subsequent investigations should therefore aim at defining the stage of the multistep process of target cell lysis which is affected. This may provide indications for involvement of specific glycoproteins and their carbohydrates in target cell binding or in later stages of the lytic cascade. The objective of the present study is to provide new data to initiate detailed investigations of the carbohydrate-dependent events in LAK-mediated killing of target cells.

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