Glycoconjugate Journal (1992) **9**:198–203

Surface glycoprotein of human natural killer cells recognized by wheat germ agglutinin

KENJI HARADA, HIROSHI YAMANE, YASUYUKI IMAI, TSUTOMU TSUJI, SATOSHI TOYOSHIMA and TOSHIAKI OSAWA*

Division of Chemical Toxicology and Immunochemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Received 13 December 1991

We analyzed surface glycoproteins of human natural killer (NK) cells by utilizing lectins. Among the lectins tested, wheat germ agglutinin (WGA) was found to bind preferentially to CD16(Leu11)-positive lymphocytes as determined by two-colour flow cytometry. Analysis of glycoproteins in the lysate prepared from NK cells with sodium dodecyl sulfate (SDS) gel electrophoresis followed by Western blotting and ^{125}I labeled WGA staining revealed that a glycoprotein with an M_r of 65 kDa was strongly bound to the lectin, but no corresponding glycoprotein was detected in the lysate of T lymphocytes. This glycoprotein (GP65) gave several spots in the pI range 4.1–4.6 on 2-dimensional gel electrophoresis. Sialidase treatment of GP65 resulted in a single spot on the 2-dimensional gel, suggesting that GP65 is heterogeneous in the degree of sialylation. GP65 was shown to be exposed on the cell surface, since it was radiolabeled with ^{125}I by the lactoperoxidase-catalyzed method. We next isolated GP65 from human peripheral blood lymphocytes by a combination of chromatography on a cation-exchange column and a WGA–agarose column and preparative SDS gel electrophoresis. It is suggested that GP65 is a novel surface glycoprotein on human NK cells.

Keywords: Sialoglycoprotein, natural killer (NK) cells, wheat germ agglutinin

Natural killer (NK) cells constitute a small population of lymphocytes, and morphologically belong to the large granular lymphocyte fraction. Their cytotoxic activity against certain tumor cells and virus-infected cells has been extensively characterized [1–4]. A number of investigations has indicated that surface molecules on NK cells, such as LFA-1 (CD11a/CD18) [5, 6], T-200 (CD45) [7], CD57 [8] and Fcγ receptor (CD16) [9], play important roles in NK cell functions. It has also been shown that each population of lymphocytes expresses specific carbohydrate chains on their cell surface. We have characterized cell surface glycoconjugates of lymphocyte subpopulations by using lectins, which recognize and bind to certain types of carbohydrate chains on the cell surface, and found that several lectins are quite effective tools for their separation; e.g. *Dolichos biflorus* lectin for the separation of mouse cytotoxic T cells [10, 11], and lentil lectin and soybean agglutinin for the separation of human and mouse lymphokine-activated killer (LAK) cells, respectively [12, 13]. In the course of these studies, it became apparent that some lectins can be used for the enrichment of NK activity by utilizing the lectin-binding characteristics of NK cells. We therefore screened many lectins for capability of enrichment of NK activity, and found that wheat germ agglutinin (WGA) could preferentially bind to a lymphocyte population which bears CD16 antigen, and displays high NK activity. We report the characterization of a surface glycoprotein on human NK cells which has high affinity for WGA, and its isolation.

Materials and methods

Antibodies, lectins and enzymes

Anti-CD3 (Leu4) and anti-CD16 (Leu11a) antibodies were purchased from Becton Dickinson (Mountain View, CA). Wheat germ agglutinin (WGA) and its biotinylated derivative and conjugate to agarose are the products of Honen Corp. (Tokyo, Japan). Phycoerythrin (PE)- and FITC-conjugated streptavidin were purchased from Becton Dickinson and Vector Laboratories (Burlingame, CA), respectively. Streptavidin conjugated with alkaline phosphatase was from Zymed Laboratories, Inc. (South San Francisco, CA). Sialidase from *Arthrobacter ureafaciens* is a product of Nacalai Tesque (Kyoto, Japan).

NK cell preparation

Human peripheral blood lymphocytes (PBL) were isolated from healthy volunteers by Ficoll-Hypaque gradient

* To whom correspondence should be addressed.

centrifugation, and subjected to 2-aminoethylisothiouronium bromide (AET)-SRBC rosette formation [14–16]. The nonrosette fraction was collected and then applied to a Nylon wool column. Cells were eluted with warm RPMI 1640 medium containing 10% fetal bovine serum after incubation for 30 min at 37 °C. This fraction was used as an NK-rich fraction for some experiments. The NK-rich fraction was further purified with Percoll (Pharmacia, Uppsala, Sweden) discontinuous density centrifugation [17]. The enrichment of NK cells in this fraction was confirmed by flow cytometric analysis with anti-CD16 and anti-CD3 antibodies, and by cytotoxic assay against human erythroleukemia cell line K562. The T-lymphocyte fraction was also obtained from the AET-rosette-forming cells by hypotonic lysis as already described [12].

Flow cytometric analysis

For two-colour staining, 1×10^6 cells in 200 μl PBS–BSA (10 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl, 0.1% bovine serum albumin and 0.1% NaN_3) were incubated first with FITC-labeled anti-CD16 antibody on ice for 30 min. The cells were washed twice with PBS–BSA, and incubated on ice for 30 min in the presence of biotinylated WGA (10 μg ml^{-1}). After the cells were washed twice with PBS–BSA, they were stained with PE-streptavidin (10 μg ml^{-1}) as a second step red label. The labeled cells were washed twice, then resuspended in 1 ml PBS–BSA, and analyzed with an FCS-1 flow cytometer (Japan Spectroscopic Co., Tokyo, Japan).

SDS-polyacrylamide gel electrophoresis (PAGE), Western blotting and lectin staining

SDS-PAGE and Western blotting were performed as described previously [18]. Briefly, cells (2×10^6) were extracted with 0.1 ml 10 mM PBS containing 0.5% Nonidet P-40 (NP-40), 1 mM EDTA, 1 mM *N*-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride (PMSF), 100 kU ml^{-1} aprotinin, 7 μg ml^{-1} pepstatin and 2.5 μg ml^{-1} leupeptin (lysis buffer) for 60 min at 0 °C, and centrifuged at 100,000 × **g** for 10 min to remove insoluble materials. The supernatant was then separated by 4–20% polyacrylamide gradient gel (Daiichi Chemicals, Tokyo, Japan) under reducing conditions according to the system of Laemmli [19], and electroblotted onto a PVDF membrane (Immobilon, Millipore, Bedford, MA) with a Milliblot semi-dry electroblotting system (Millipore). The blotted membrane was soaked first in PBS containing 3% BSA at 4 °C overnight to avoid nonspecific staining, and then incubated with a biotinylated lectin in PBS containing 0.1% Tween 20 (PBS–Tween) at room temperature for 2 h with gentle shaking. After washing the membrane with PBS–Tween four times, it was incubated with a solution of alkaline phosphatase-conjugated streptavidin (1/1000 dilution) at room temperature for 2 h with gentle shaking. The membrane was then washed four times with PBS–Tween and bands were visualized with an alkaline phosphatase substrate kit (Vector Laboratories).

Two-dimensional electrophoresis was performed as described by O'Farrell [20].

Radioiodination of cell surface proteins

Radioiodination of intact cells with ^{125}I was carried out by the lactoperoxidase-catalyzed method as described previously [18]. Briefly, 1×10^7 cells were incubated (20 °C, 30 min) with 0.5 mCi $Na^{125}I$ (NEN Du Pont, Boston, MA) in the presence of 20 μg lactoperoxidase and 200 mU glucose oxidase in 1 ml PBS, pH 7.4, containing 20 mM glucose. The labeled cell suspension was washed four times with chilled PBS containing 1 mM PMSF.

Isolation of GP65 from human peripheral blood lymphocytes (PBL)

Human PBL were used as a starting material for the isolation of GP65. All procedures were performed at 4 °C unless otherwise stated. PBL (1×10^9 cells) were extracted with 10 ml PBS containing 0.5% NP-40, 1 mM EDTA, 2 mM PMSF, and 2.5 μg ml^{-1} leupeptin for 1 h at 0 °C. After the insoluble materials were removed by centrifugation at 30 000 × **g** for 20 min, the supernatant was dialyzed against 0.1 M sodium acetate buffer, pH 5.0. The cell extract was then applied to a column (1.2 cm × 3.5 cm) of SP-Toyopearl (Tosoh Corp., Tokyo, Japan) equilibrated with 0.1 M sodium acetate buffer, pH 5.0, and the column was eluted with the same buffer. The flow-through fraction, which contained GP65, was immediately dialyzed against PBS containing 0.1% NP-40, and then applied to a WGA–agarose column (0.6 cm × 2.0 cm). The column was first washed with PBS and the bound glycoproteins were eluted with the same buffer containing 0.3 M*N*-acetylglucosamine. The fraction bound to the column was next subjected to preparative SDS-PAGE. The band corresponding to GP65 was cut out and the gel piece was homogenized. The glycoprotein was extracted with 10 mM Tris-HCl, pH 7.4, containing 0.5% SDS.

Results

Flow cytometric analysis

We screened many kinds of lectins, including wheat germ agglutinin (WGA), concanavalin A (Con A), *Lens culinaris* agglutinin (LCA), *Bauhinia purpurea* agglutinin (BPA), *Helix pomatia* agglutinin (HPA) and *Wistaria floribunda* agglutinin (WFA) for their reactivity with a CD16 (Leu11)-positive lymphocyte population by means of two-colour flow cytometry using FITC-labeled anti-CD16 antibody and biotinylated lectins with PE-labeled streptavidin. Among the lectins tested, WGA was found to stain a cell population expressing CD16 antigen, which is a surface marker of human NK cells (Fig. 1). No other lectins had a correlation with anti-CD16 antibody in the

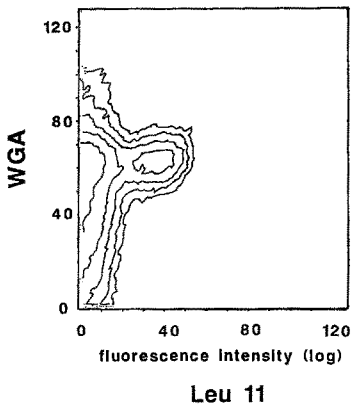

Figure 1. Two-color flow cytometric analysis with anti-CD16 (Leu11) and WGA. The NK-rich fraction (1×10^6 cells) was incubated first with FITC-labeled anti-CD16, washed with PBS/BSA, and then incubated with biotinyl WGA. After the cells were washed with PBS/BSA, they were stained with PE-streptavidin as a second step red label.

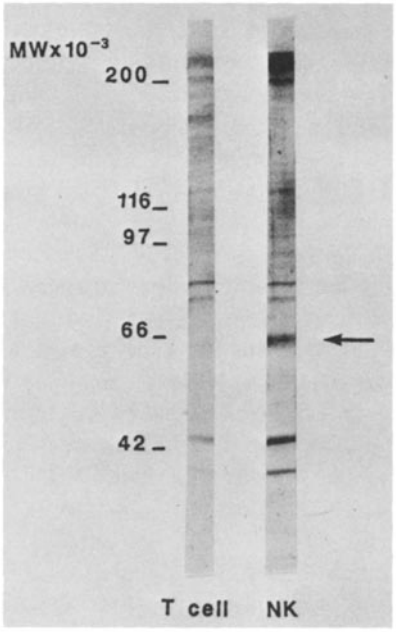

Figure 2. Electrophoretic analysis of glycoproteins from NK cells and T lymphocytes. Cell extract of NK cells or T lymphocytes (2×10^5 cells) was subjected to SDS-PAGE (4–20% polyacrylamide gradient gel) followed by Western blotting to a PVDF membrane. The blotted membrane was then stained with biotinyl WGA and alkaline phosphatase-conjugated streptavidin as described in the Materials and methods section. GP65 is indicated by an arrow.

reactivity with the cells. However, the CD3-positive or sIg-positive cell fraction was also stained with WGA but only weakly (data not shown). This result suggests that the surface of NK cells is more abundant in carbohydrate chains which are recognizable by WGA than that of T or B lymphocytes.

A glycoprotein recognized by WGA

To characterize glycoproteins recognized by WGA, the lysate of NK cell was prepared with a buffer containing 0.5% NP-40 and analyzed with SDS-PAGE followed by Western blotting and WGA staining. Figure 2 shows the electrophoretic profile of NK cells and T lymphocytes. Several major bands were observed in both lanes; however, protein bands derived from NK cells were more intensely stained than those from T cells. Although NK cells and T lymphocytes shared most glycoprotein bands on the WGA-stained blot, a band with M_r 65 kDa was specifically detected in the lane of NK cells. We tentatively designated this glycoprotein GP65 and analyzed it by 2-dimensional electrophoresis. Since a preliminary experiment showed the acidic nature of GP65, a rather low pH range (3–6) was chosen in the first dimension. When the Western blotted membrane was stained with WGA, GP65 gave seven closely linked spots in a pI range between 4.1 and 4.6, as indicated by an arrow in Fig. 3. Another group of faint spots was observed in a slightly lower molecular weight and slightly more basic region. These minor spots were also detected in the lysate from T cells. However, no spots corresponding to GP65 could be detected in the T cell lysate. To clarify whether the GP65 molecule is absent from T cells or GP65 on T cells has less affinity for WGA due to the different glycosylation, we next stained the gel with the silver staining procedure after 2-dimensional electrophoresis. As shown in

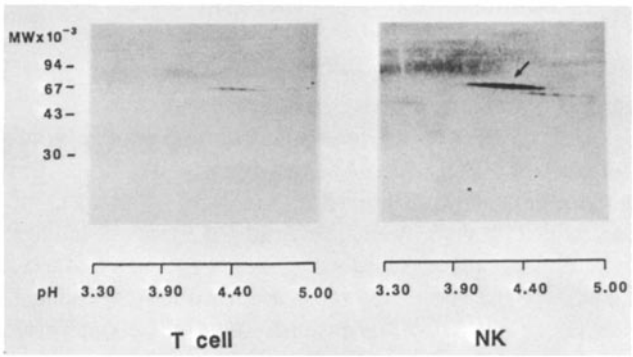

Figure 3. Two-dimensional electrophoresis of glycoproteins from NK cells and T lymphocytes. Cell extracts were subjected to 2-dimensional polyacrylamide gel electrophoresis as described by O'Farrell [20]. Western blotting and lectin staining with WGA were performed as described in the Materials and methods section. GP65 is indicated by an arrow.

Fig. 4, GP65 was detected in NK cells but not in T cells, indicating that T cells lacked GP65.

GP65 was heterogeneous in its pI, as shown by 2-dimensional electrophoresis. To elucidate whether this heterogeneity is ascribed to the difference in the glycosylation of GP65 or not, we examined the effect of

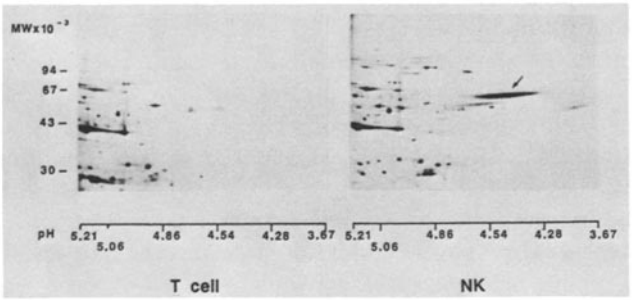

Figure 4. Silver staining of GP65 separated by 2-dimensional electrophoresis. Cell extract of NK cells or T lymphocytes was separated by 2-dimensional gel electrophoresis and stained by the silver staining procedure. GP65 is indicated by an arrow.

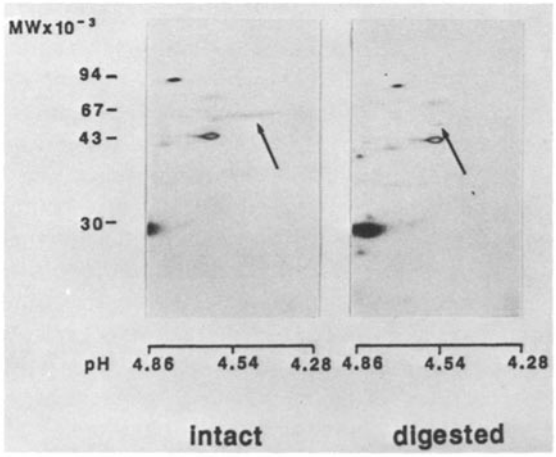

Figure 5. Effect of sialidase digestion on the electrophoretic profile of GP65. NK cell extract was incubated for 30 min at 37 °C in the presence (right) or absence (left) of sialidase (*Arthrobacter ureafaciens*, 0.1 U) and separated by 2-dimensional polyacrylamide gel electrophoresis. The gel was stained by the silver staining procedure.

sialidase treatment on its electrophoretic profile. GP65 from sialidase-treated NK cell lysate gave a single spot corresponding to the most basic one of GP65 from the untreated NK cells, when the gel was stained by the silver staining method (Fig. 5). The result suggests that GP65 is heterogeneous in the degree of sialylation but that the polypeptide portion is apparently homogeneous. The sialic acid residues in GP65 seemed to be essential for its interaction with WGA, since GP65 from the sialidase-treated NK cell lysate was not stained with WGA (data not shown).

We next carried out the surface labeling of NK cells with ^{125}I by the lactoperoxidase method. The labeled cells were extracted with a buffer containing NP-40 and the extract was applied to a WGA–agarose column. The glycoproteins bound to a WGA–agarose column were eluted with a buffer

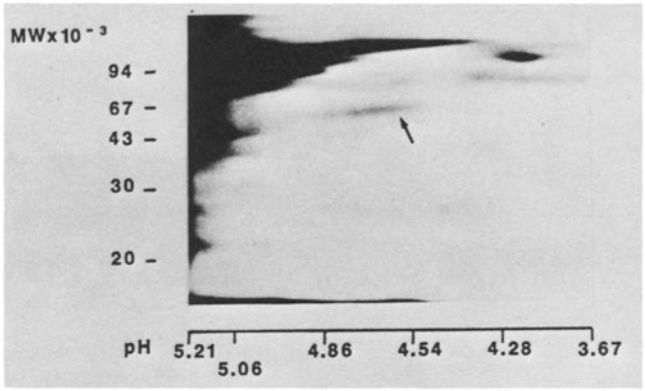

Figure 6. Autoradiography of radioiodinated proteins separated on 2-dimensional polyacrylamide gel electrophoresis. An NP-40 extract of NK cells labeled with ^{125}I by the lactoperoxidase-catalyzed method was subjected to WGA–agarose column chromatography, and the bound materials were analyzed by 2-dimensional electrophoresis and detected by autoradiography.

containing *N*-acetylglucosamine and separated by 2-dimensional electrophoresis. As shown in Fig. 6, GP65 was labeled with ^{125}I, indicating that it is located on the surface of NK cells.

Isolation of GP65

Since GP65 was shown to have a low pI range (4.1–4.6), ion exchange chromatography was selected for its separation. We first carried out the absorption test by using an NP-40 extract of human PBL as a source of GP65 and SP-Toyopearl as an adsorbent, in order to determine the appropriate condition for the cation-exchange chromatography. The NP-40 extract was incubated with SP-Toyopearl at various pH (4.0–7.0), and the proteins remaining in the supernatant were analyzed by SDS-PAGE. The supernatant obtained after the incubation at lower pH (e.g., pH 4.5 or 5) contained a reduced number of glycoprotein bands including a somewhat diffuse band at M_r 65 kDa, when the separated glycoproteins were stained with WGA (data not shown). We therefore performed the cation-exchange chromatography on a SP-Toyopearl column at pH 5.0. The flow-through fraction from a SP-Toyopearl column was then applied to a WGA–agarose column (Fig. 7). The bound glycoproteins were eluted with a buffer containing 0.3 M *N*-acetylglucosamine. The analysis of the bound fraction with SDS-PAGE showed that this fraction contained GP65 as well as some impurities (Fig. 8, lane 3). The final purification was achieved by subjecting the fraction to preparative SDS-PAGE. The sample gave a single band on SDS-PAGE when the gel was stained by either a silver staining method or WGA (Fig. 8, lanes 4 and 5). About 0.5 μg GP65 was obtained from 1×10^9 cells.

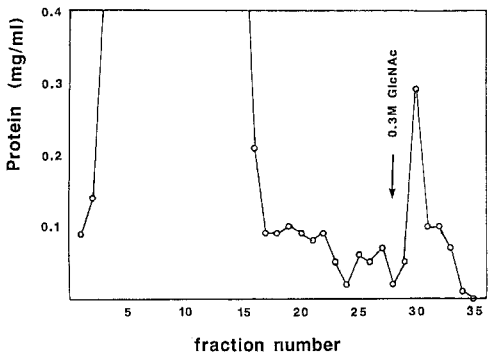

Figure 7. Affinity chromatography on a WGA–agarose column. The flow-through fraction from a SP-Toyopearl column was applied to a WGA–agarose column (0.6 × 2.0 cm). The column was washed with PBS containing 0.1% NP-40, and then the bound glycoproteins were eluted with the same buffer containing 0.3 M *N*-acetylglucosamine (GlcNAc). Fractions of 0.75 ml were collected and assayed for proteins by the BCA method (Pierce, Rockford, IL).

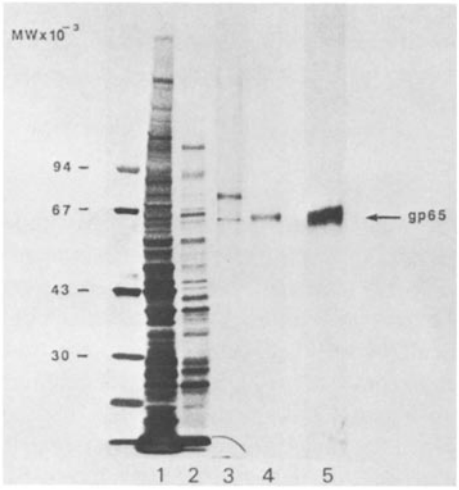

Figure 8. Electrophoretic analysis of each step for the purification of GP65: lanes 1–4, silver staining; lane 5, WGA staining after blotting to a PVDF membrane. Lane 1, cell lysate; lane 2, flow-through fraction from a SP-Toyopearl column; lane 3, fraction bound to and eluted specifically from a WGA–agarose column; lanes 4 and 5, purified GP65.

Discussion

Several recent studies have suggested that glycoproteins on the cell surface constitute a great number of surface markers of immunocytes at various differentiation stages and with various functions. We and other investigators characterized glycoproteins of lymphocyte cell membranes and also developed the procedure for the separation of lymphocyte subpopulation by using lectins. For instance, Reisner and Sharon [21] established a method for the separation of murine thymocytes at different differentiation stages by using peanut agglutinin (PNA). Their group also demonstrated that subpopulations of murine splenocytes were separated by selective agglutination with soybean agglutinin (SBA) and PNA [22, 23]. Kimura *et al.* [24] and Yamazaki *et al.* [10] reported that *Vicia villosa* agglutinin (VVA) and *Dolichos biflorus* agglutinin (DBA) have high affinity for murine cytotoxic T lymphocytes (CTL). Okada *et al.* [11] also reported that DBA could discriminate murine CTL with different cytotoxic activity *in vitro* and *in vivo*. Maruyama *et al.* [12] and Takano *et al.* [13] demonstrated that lentil lectin (LCA) and soybean agglutinin (SBA) separated subpopulations of human and murine lymphokine-activated killer (LAK) cells, respectively. In the present study, we screened many kinds of lectins for their reactivity with human NK cells, and found that WGA had high affinity for the CD16-positive lymphocyte population. The protocol for the enrichment of human NK cells using WGA will be published elsewhere. We also identified a glycoprotein with M_r of 65 kDa (GP65) on the surface of human NK cells, which is, however, absent on T lymphocytes. GP65 contains heterogeneous numbers of sialic acid residues, and gave seven linked spots with different pI on 2-dimensional electrophoresis. WGA was reported to have high affinity for sialic acid-containing carbohydrate chains which are clustered on a polypeptide backbone [25]. The sialidase treatment of GP65 indeed abolished its reactivity with WGA, indicating that the sialic acid residues are essential for the interaction between WGA and GP65.

Surface markers of human NK cells have been less characterized than other lymphocyte populations, probably because they constitute a small population of lymphocytes. However, some molecules were reported to be preferentially expressed on NK cell surface: e.g., CD16 (Leu11), CD11a and 11b (LFA-1 and Mac-1 α chain, respectively), CD18 (LFA-1 or Mac-1 β chain), CD56 (NKH-1) and CD57 (HNK-1) [5–9, 26, 27]. GP65 has a similar M_r to CD16 antigen (50–65 kDa). We confirmed, however, that GP65 is distinct from CD16 antigen, since GP65 was not immunoprecitated with anti-CD16 antibody. One of the characteristics of GP65 is its acidic nature (pI 4.1–4.6). This glycoprotein may be a possible surface marker of human NK cells. However, more precise distribution of GP65 should be clarified.

In this study, we also isolated GP65 from human PBL. Since it was apparent that GP65 had a low pI and high affinity for WGA, we employed cation-exchange chromatography on SP-Toyopearl and affinity chromatography on WGA–agarose for its purification. GP65 was purified to apparent homogeneity by a combination of the chromatography on these columns and preparative SDS-PAGE. The isolation of GP65 in a large quantity is in progress in our laboratory to characterize the detailed chemical nature of GP65 and its functional roles in NK cell-mediated cytotoxicity.

References

1. Timonen T, Ortaldo JR, Herberman RB (1981) *J Exp Med* **153**:569–82.
2. Lanier LL, Phillips JH, Hackett Jr J, Tutt M, Kumar V (1986) *J Immunol* **137**:2735–9.
3. Herberman RB, Reynolds CW, Ortaldo JR (1986) *Annu Rev Immunol* **4**:651–80.
4. Trinchieri G (1989) *Adv Immunol* **47**:187–376.
5. Hall RE (1985) *Mol Immunol* **22**:757–64.
6. Hall RE, Schall RP, Black LA (1985) *Mol Immunol* **22**:765–73.
7. Newman W, Fast LD, Rose LM (1983) *J Immunol* **131**:1742–7.
8. Abo T, Balch CM (1981) *J Immunol* **127**:1024–9.
9. Lanier LL, Ruitenberg JJ, Phillips JH (1988) *J Immunol* **141**:3478–85.
10. Yamazaki T, Imai Y, Oguchi Y, Nakano T, Osawa T (1983) *Carbohydrate Res* **120**:269–81.
11. Okada T, Ezawa K, Imai Y, Osawa T (1986) *Cancer Res* **46**:5611–7.
12. Maruyama T, Imai Y, Harada K, Okada T, Takano M, Ikeda Y, Toda G, Oka H, Osawa T (1990) *J Biol Response Mod* **9**:378–86.
13. Takano M, Okada T, Maruyama T, Harada K, Imai Y, Osawa T (1990) *Jap J Cancer Res* **80**:1228–37.
14. Pellegrino MA, Ferrone S, Dierich MP, Reisfeld RA (1974) *Clin Immunol Immunopathol* **3**:324–33.
15. Saxon A, Feldhaus JL, Robbins RA (1976) *J Immunol Methods* **12**:285–8.
16. Abrams SI, Brahmi Z (1988) *J Immunol* **140**:2090–5.
17. Timonen T, Saksela E (1980) *J Immunol Methods* **36**:285–91.
18. Yamashita Y, Imai Y, Osawa T (1989) *Mol Immunol* **26**:905–13.
19. Laemmli UK (1970) *Nature* **227**:680–5.
20. O'Farrell PH (1975) *J Biol Chem* **250**:4007–21.
21. Reisner Y, Sharon N (1984) *Methods Enzymol* **108**:168–79.
22. Reisner Y, Ravid A, Sharon N (1976) *Biochem Biophys Res Commun* **72**:1585–91.
23. Reisner Y, Itzicovitch L, Meshorer A, Sharon N (1978) *Proc Natl Acad Sci USA* **75**:2933–6.
24. Kimura A, Wigzell H, Holmquist G, Ersson B, Carlsson P (1979) *J Exp Med* **149**:473–84.
25. Bhavanandan VP, Katlic AW (1979) *J Biol Chem* **254**:4000–8.
26. Hořejší V, Bažil V (1988) *Biochem J* **253**:1–26.
27. Knapp W, Rieben P, Dörken B, Schmidt RE, Stein H, Kr. v. d. Borne AEG (1989) *Immunol Today* **10**:253–8.