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HUMAN LYMPHOCYTE MEMBRANE PROTEINS TREATED WITH NEURAMINIDASE

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Human peripheral blood lymphocytes were surface-iodinated, treated with neuraminidase from *Vibrio cholerae* and lysed with non-ionic detergent. In addition, surface membrane fractions were isolated from surface-iodinated cells in the absence of detergents and treated with neuraminidase after membrane isolation. The effect of neuraminidase treatment on the membrane proteins was studied by two-dimensional gel electrophoresis. One surface-labelled protein of 45000 molecular weight which is characterized by its association with the detergent-resistant matrix of the cells and by its specific enrichment in an isolated membrane fraction, was found to be particularly sensitive to neuraminidase treatment both of intact cells and isolated membranes. A prominent labelled protein of apparent molecular weight of 60000 is observed in the soluble fraction after neuraminidase treatment of intact cells. The analogous protein is detected when isolated membrane fractions are treated with neuraminidase.

Introduction

The structure and function of the membrane proteins of the human T lymphocyte cell surface still are largely unknown. One approach to further elucidate the T cell membrane is to vectorially label the proteins exposed at the surface with reagents which are unable to permeate the membrane [1,2]. The labelled proteins may then be analysed on two-dimensional gels [3] whose greatly improved resolution allows to identify individual components which might overlap in one-dimensional gels [4]. By this approach it has been shown that the lactoperoxidase iodinated surface membrane proteins of the human peripheral blood T lymphocyte can be fractionated by their solubility in non-ionic detergent [4]. The majority of the labelled proteins including the major histocompatibility antigens, HLA-A and B, is solubilized by, e.g., octylphenylpolyoxyethylenes [4,5], but some labelled proteins remain associated with the detergent-insoluble matrix of the cells [4,6–8]. The predominant detergent-insoluble surface-labelled protein of the human T lymphocyte is a heterogeneous protein of 45000 apparent molecular weight (the 45 kDa protein) which can be differentiated from HLA-A,B and from actin by its gel electrophoretic migration [4]. In addition, the 45 kDa protein can be specifically enriched in a membrane fraction prepared from a detergent-free cell homogenate [4]. In order to confirm the surface exposition of this protein, intact cells are enzymatically treated with neuraminidase. It is shown that the 45 kDa protein in intact cells and in isolated surface membranes is sensitive to neuraminidase treatment. A 60000 molecular weight component is detected in the soluble fraction of cells which were treated with neuraminidase after surface iodination. An analogous component is found when iso-

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Abbreviation: HLA, human lymphocyte antigen.

lated surface membrane fractions are treated with neuraminidase.

Materials and Methods

The isolation of T-cell enriched lymphocytes from human peripheral blood by Ficoll/Paque density gradient centrifugation and glass/nylon wool column chromatography, the surface labelling by lactoperoxidase, glucose oxidase and ^{125}I and the cell lysis in detergent are described elsewhere [4]. The two-dimensional gel electrophoresis was carried out according to O'Farrell [3].

For neuraminidase treatment, surface-iodinated cells ($5 \cdot 10^7$ or 10^8 cells/ml) in acetate buffer (50 mM sodium acetate, 150 mM NaCl, 9 mM $CaCl_2$, final pH 6.0) or in phosphate-buffered saline (1.8 mM KH_2PO_4, 8.2 mM $Na_2HPO_4 \cdot 2H_2O$, 137 mM NaCl, pH 7.4) were incubated with 25, 50 or 100 μl neuraminidase from *Vibrio cholerae* (Behringwerke, Marburg/Lahn, F.R.G., 1 U/ml) per ml cell suspension for 30 or 45 min at 37°C in a water bath.

In control experiments, cells were suspended in phosphate-buffered saline with 1 mM phenylmethylsulfonylfluoride, in phosphate-buffered saline containing 1 mM D-(+)-glucose, D-(+)-galactose, D-(+)-mannose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine, or in 8.2 mM $Na_2HPO_4 \cdot 2H_2O$, 1.8 mM KH_2PO_4, 27 mM NaCl containing either 200 mM galactose, glucose or mannose, and treated with neuraminidase as above. In addition, unlabelled cells were treated with neuraminidase and surface-labelled after enzyme treatment.

Cell membrane fractions from surface-iodinated cells were isolated essentially according to Monneron and D'Alayer [9] as described elsewhere [4]. Briefly, a detergent-free cell homogenate was fractionated by density gradient centrifugation; surface membrane fractions were recovered from the 0/22.5%, 22.5/35% and 35/40% sucrose interphases. The washed pellets of the isolated membrane fractions obtained from $(3-5) \cdot 10^8$ surface-labelled cells were resuspended by brief sonication (1–2 s, Branson sonifier B-30, output control 2) in 250 μl acetate buffer and preincubated for 5 min at 37°C in a water bath. 50 μl neuraminidase/ml were added and the enzyme treatment was quenched by the addition of 40 vol. ice-cold phosphate-buffered saline after 0.5, 5 and 30 min incubation at 37°C. Controls were carried out without added neuraminidase.

The membranes were then pelleted (360000 × *g*, 45 min, 4°C) and processed for gel electrophoresis [4].

Results and Discussion

Cells lysed by detergent after neuraminidase treatment

Surface-iodinated T-lymphocytes were treated with neuraminidase as outlined in the methods section, lysed in 0.5% or higher concentrations of octylphenylpolyoxyethylenes in 150 mM NaCl, 50 mM Tris, 0.02% NaN_3 (pH 7.4), fractionated in a detergent-soluble fraction and an insoluble sediment [4,5], and analysed according to O'Farrell [3] by two-dimensional gel electrophoresis and autoradiography (Figs. 1 and 2). The neuraminidase treatment causes a number of the spots to shift to more basic pH values due to the removal of terminal sialic acid residues (see, for example, HLA-A,B in Figs. 1–3). In addition to these expected results, two further effects of neuraminidase treatment were observed.

(1) A new heterogeneous protein component of apparent molecular weight 60000 (the 60 kDa protein) is detected after the enzyme treatment in the detergent-soluble fraction (Fig. 1a). There is a component at more acidic pH values and slightly lower molecular weight which might be considered as sialylated precursor of the 60 kDa protein. The slight difference in molecular weight does not exclude this possibility, since it is established that some membrane glycoproteins migrate anomalously in sodium dodecylsulfate/polyacrylamide gels (see, for example, Ref. 10). However, the sialylated precursor of the 60 kDa protein were expected to show at least an equivalent charge heterogeneity and intensity to the 60 kDa protein; these conditions are not met by this particular component. The heterogeneity of the 60 kDa protein remaining after neuraminidase treatment probably is due to incomplete sialic acid removal; in an analogous situation, it was shown that probably for steric reasons *Vibrio cholerae* neuraminidase does not act on *N*-acetylneuraminic acids

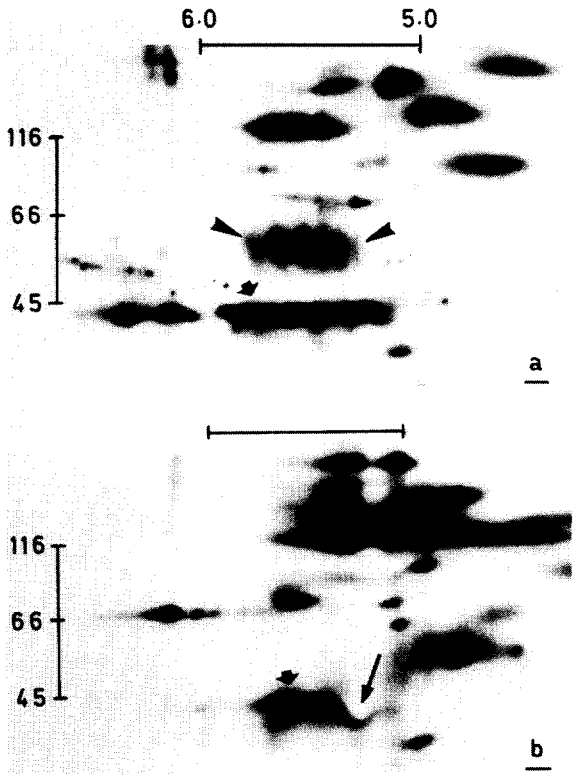

Fig. 1. Supernatant fraction of surface-iodinated lymphocytes lysed in 0.5 or 1.0% Triton X-100 buffer and sedimented at $400 \times g$ for 10 min at 4°C. (a) Cells treated with neuraminidase. Autoradiograph of a gel loaded with a sample equivalent to $5 \cdot 10^6$ cells. (b) Cells without neuraminidase treatment. Autoradiograph of a gel loaded with a sample equivalent to 10^7 cells. HLA-A and B (➧), the 60 kDa protein (▼), actin (↓). The molecular weight scale ($\times 10^{-3}$; vertical) and the pH-units of the isoelectric focusing (horizontal) are indicated.

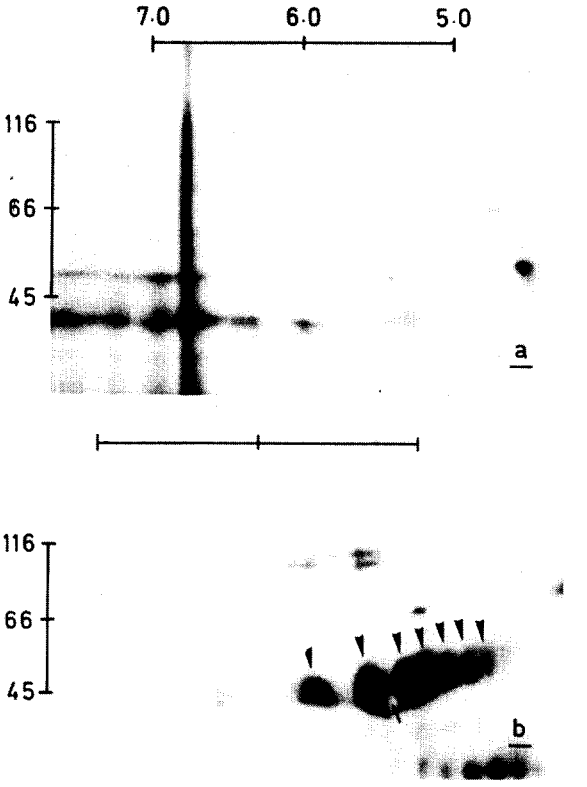

Fig. 2. Detergent-insoluble sediment ($400 \times g$, 10 min, 4°C) of surface-iodinated, lysed lymphocytes (0.5 or 1.0% Triton X-100 buffer). (a) Cells treated with neuraminidase. Autoradiograph of a gel loaded with a sample equivalent to $(3–5) \cdot 10^7$ cells. (b) Cells without neuraminidase treatment. Autoradiograph of a gel loaded with a sample equivalent to $(3–5) \cdot 10^7$ cells. Molecular weight and pH scales same as in Fig. 1. The 45 kDa protein (▼), actin (↓).

(α,2-6)-linked to *N*-acetylgalactosamine in glycophorin A [10].

(2) Neither the main detergent-insoluble protein, the 45 kDa protein, nor an immediately apparent asialo-derivative of the 45 kDa protein are detected in either Fig. 1a or Fig. 2a. The presence of 1 mM phenylmethylsulfonylfluoride during enzyme treatment and cell lysis does not affect this result. Proteolysis by contaminating serine proteases can therefore be practically excluded. Part of the 45 kDa protein may precipitate irreversibly or shift to high pH values. The exposed saccharide residues after neuraminidase treatment are typically galactose or *N*-acetylgalactosamine [10,11]. Specific receptors for these residues are known to occur in cell membranes which rapidly internalize asialo-glycoproteins [11]. In order to exclude that the asialo-45 kDa protein is binding to such receptors, the enzyme treatment and cell lysis was carried out in the presence of 1 mM galactose, *N*-acetylgalactosamine, glucose, *N*-acetylglucosamine and mannose, or 200 mM galactose, mannose or glucose; no inhibitory effect of these saccharides was detected.

Neuraminidase-treatment of isolated membrane fractions

The 45 kDa protein can be identified in surface membrane fractions isolated from detergent-free cell homogenates on sucrose density gradients

according to Monneron and D'Alayer [9]. Three membrane fractions of different densities are observed. The 45 kDa protein is the predominant protein in the two lightest fractions [4], but it is detected in lower amount also in the third fraction (Fig. 3b). It has been shown that also in the isolated membrane the 45 kDa protein is not solubilized by octylphenylpolyoxyethylenes in concentrations of up to 5.0 mg/mg total protein [4]. A parallel sample of the specimen shown in Fig. 3b was treated with neuraminidase (see Methods) and analysed by gel electrophoresis (Fig. 3a). It is concluded from Figs. 3a and 3b that the 45 kDa protein retains its sensitivity to neuraminidase in the isolated membrane; no asialo-45 kDa protein is detected after the enzyme treatment. A new heterogeneous protein component of 60000 apparent molecular weight appears after neuraminidase treatment which from the location in the gel is identical with the 60 kDa protein.

Analogous results are obtained in principle by neuraminidase treatment of the two lightest membrane fractions, but both the 60 kDa and 45 kDa proteins are detected after the enzyme treatment. When the enzyme treatment is carried out during various time periods of up to 30 min at 37°C, it can be shown that the final result of the enzymatic treatment is reached within 30 s. Preliminary electron microscopic studies show that the two lightest fractions are mainly in the form of closed vesicles and the partial sensitivity of the 45 kDa protein to neuraminidase may then be explained by inside-out vesicle formation.

The results in Figs. 3a and 3b confirm the sensitivity of the 45 kDa protein to neuraminidase treatment. Furthermore, they indicate that a reciprocal relation exists between the 45 kDa protein and the component identified with the 60 kDa protein. The discrepancy in apparent molecular weight does not allow to rule out this possibility. Membrane glycoproteins are known to migrate anomalously in the second dimension of the gel system used [10]; quite large differences in apparent molecular weight have been reported after neuraminidase treatment for glycoproteins containing *O*-linked glycosylated side chains [10]. The observed difference in apparent molecular weights then might be explained by anomalous migration of a membrane glycoprotein and its asialo derivative. However, it cannot be excluded either that the 60 kDa protein represents a protein aggregate which in part includes the asialo derivative of the 45 kDa protein, or even a dimeric form of the asialo derivative of the 45 kDa protein, which remains stable in gel electrophoresis. Analogous phenomena are known to occur with another well characterized membrane glycoprotein, glycophorin A [12]. Furthermore, a relation of the 45 kDa protein with the 60000 molecular weight component, which was considered as sialylated precursor of the 60 kDa protein, cannot be excluded.

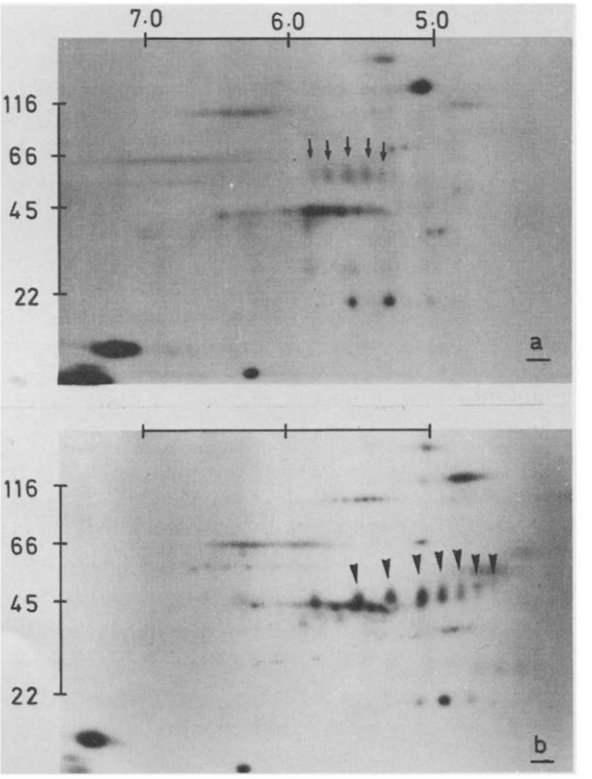

Fig. 3. Membrane fraction isolated from a homogenate of surface-iodinated cells in the absence of detergents by sucrose density gradient centrifugation. (a) Membrane pellet treated with neuraminidase after isolation. Autoradiograph of a gel loaded with $6.3 \cdot 10^4$ cpm. (b) Membrane pellet without neuraminidase treatment. Parallel sample of specimen in Fig. 3a. Autoradiograph of a gel loaded with $1.4 \cdot 10^5$ cpm. Molecular weight and pH scales same as in Fig. 1. The 45 kDa protein (▼), the 60 kDa protein (↓).

Conclusions

The results contained in Figs. 1 and 2 demonstrate that the 45 kDa protein, by the criterion of sensitivity to enzymatic treatment in intact cells, is exposed at the cell surface. Furthermore, a prominent solubilized component, the 60 kDa protein, is observed after neuraminidase treatment of cells (Fig. 1). A relation of a part of the asialo derivative of the 45 kDa protein and the 60 kDa protein cannot be excluded (Fig. 3).

The treatment of mononuclear cells with neuraminidase and galactose oxydase is known to induce extensive blastogenesis [13,14] which depends on a lymphocyte/monocyte intercellular association [15–17]. There is substantial, if indirect evidence that transient associations of receptors with the detergent-resistant cytoskeletal matrix and intermolecular interactions of receptors are functionally significant [6,7,18,19]. From preliminary data galactose oxidase after treatment of the cells with neuraminidase does not further modify the gel pattern of membrane proteins. The 45 kDa protein thus appears to be a specifically affected substrate of an enzymatic treatment which is known to induce cell surface alterations which are required for the enzymatic cell activation [13–15].

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