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Immunocytochemical localization of CDw60 antigens on human peripheral T cells

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Abstract

About 25–35% of human T cells display the CDw60 ganglioside (9-*O*-acetyl-GD3) antigen at the cell surface [E.P. Rieber, in W. Knapp, B. Dörken, W.R. Gilks, E.P. Rieber, R.E. Schmidt, H. Stein, A.E.G.K. von dem Borne (Eds.), *Leucocyte Typing IV*, Oxford University, Oxford, 1989, p. 361.]. Other leucocytes do not express this antigen on the cell surface. This led us to investigate its presence by flow cytometry and immunoelectron microscopy (IEM). Flow cytometric analysis of isolated peripheral T cells showed 26% of the cell population to have the CDw60 antigen expressed on the cell surface whereas 74% did not. Similarly, IEM analysis of 262 random T cells by the preembedding immunogold labeling technique revealed CDw60 surface expression to be tetrapartite: (a) the majority of 63.7% of the T cells did not show any surface associated gold label; (b) 19.5% were of low CDw60 surface exposition, corresponding to a linear density of 0.05–2.0 gold markers per μm; (c) about 13.4% showed a medium surface exposition with a linear density of 2.1–4.5 gold markers per μm; and (d) a high exposition, ranging from 4.6 to 9.0 gold markers per μm, was seen at 3.4% of the T cells. From postembedding label experiments, which additionally make access to the antigen localized within the cytoplasm, it was found that nearly all T cells contained low levels of intracellular CDw60. Most of it was found to be associated with the cytoplasmic membrane or vesicles, derived from the Golgi. Immunogold conjugates associated with the cytoplasmic membrane showed a linear density up to 0.6 gold markers per μm. The asymmetric expression of the CDw60 antigen on human T cells and its occurrence in nearly all T cells suggests that its surface presentation is tightly regulated.

Keywords: CDw60; Peripheral T lymphocytes; Immunoelectron microscopy; Energy-filtered transmission electronmicroscopy

1. Introduction

CDw60 monoclonal antibodies recognize the ganglioside 9-*O*-acetyl-GD3, Neu5, $9Ac_2(\alpha2\rightarrow8)$ Neu5Ac $(\alpha2\text{-}3)$ Gal $(\beta1\rightarrow4)$ Glc$\beta1\rightarrow1'$Cer, and similar gangliosides containing terminally 9-O-acetylated disialosyl groups [1]. The CDw60 epitope is located in a distal position of the ganglioside carbohydrate chain and thus seems to be predestined for interactions with ligands of other cells. For instance, it is known that the influenza C virus utilizes specifically 9-O-acetylated sialic acids to invade host cells [2]. Although the presence of CDw60 antigens has been demonstrated in the lipid extracts of all leucocyte populations, their surface expression on white blood cells was restricted to a subpopulation of T cells

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[3]. The CDw60 positive cells are found in the CD4 positive as well as in the CD8 positive T cell population. Rieber and Rank [4] have shown that the expression of CDw60 in CD8 positive T cells is associated with their ability to provide B-cell help. There are also reports suggesting a role of CDw60 in T cell activation and in the pathogenesis of autoimmune diseases [5,6]. Whereas the CDw60 carbohydrate epitope has recently been identified as a constituent of several cellular glycosphingolipids (GSLs) [7], its presence on glycoproteins of immune cells could not be proven directly. However, it was shown that some glycoproteins of lymphocytes are recognized by the influenza C virus which suggests that these also contain 9-O-acetylated *N*-acetylneuraminic acids and therefore possibly express the CDw60 epitope [8]. The carrier of CDw60 GSL antigens on human T cells is the ganglioside 9-*O*-acetyl-GD3 [7]. This antigen was detected in the lipid extracts of nearly all leucocytes [7] but its surface expression is limited to a subpopulation of T cells. Since the discovery of 9-*O*-acetyl-GD3 as a tumour-associated antigen on melanoma cells, several monoclonal antibodies recognizing this molecule have been described in the last decade [9–12]. An involvement of carbohydrates with 9-*O*-acetylneuraminic acid residues has been suggested for some processes in developmental biology [11,13]. In one report, the antigen detected with mAb 3G5 (CDw60) on human T cells was reported to increase concomitantly with the age of the person [14].

In the present study, we investigated the surface and subcellular localization of CDw60 in the whole population of peripheral human T cells. By immunoelectron microscopy a large number of T cells were classified with respect to the surface expression of CDw60. We found (a) that all T cells contain the CDw60 antigen intracellularly irrespective of its surface expression and (b) as far as the surface expression is concerned, there exist four populations which are characterized by (i) the lack of the CDw60 antigen; (ii) a low; (iii) a medium and (iv) a high expression of the antigen.

2. Results

The use of monoclonal antibodies specific for the CDw60 surface marker of peripheral T lymphocytes, made it possible to localize and quantify this antigen. Two independent methods were used to study the distribution of the antigen at the cell's surface, i.e., targeting all CDw60 antigen molecules expressed and oriented to the outer cell surface by (a) flow cytometry and (b) IEM by preembedding labeling. In the latter technique an antigen or other biopolymer which is exposed on the surface of a cell or tissue is labeled with an electron dense marker, usually a protein-gold conjugate, before the sample is fixed, embedded in the resin monomer and finally polymerized. The labeling process runs parallel with other labeling techniques, e.g., immune fluorescence techniques, under similar physiological conditions. In a second technique 'postembedding labeling' or 'postembedding IEM', the original sample is first chemically fixed and processed for resin embedding and polymerization before the immuno-tools, i.e., primary antibodies and gold-conjugates, come into play. Application of these tools is done with ultrathin-sectioned samples, i.e., at a surface of an internal matrix, for example the cytoplasm of single cells or tissues, with an antigen or biomolecule to be detected. Under unphysiological (waterless) conditions.

Detection of CDw60 on the cell surface.—T cells were freshly isolated from buffy coats and processed for flow cytometry and preembedding IEM as described in Section 4. By incubation of the cells with the monoclonal antibody under saturating conditions it was ensured that most of the surface-exposed antigens were identified by the antibody. Flow cytometric analysis was done with FITC-conjugated goat anti-mouse IgM. CDw60-specific fluorescent label was found with 26% of the T cells population, indicated as region M1 in Fig. 1, relative to the isotype control, indicated by the dotted line.

Immunogold conjugates, specific to the μ-chain of the primary antibody, were used as electron dense markers for quantitative detection of CDw60. In order to get a clear insight into the modulation of the antigen expression,

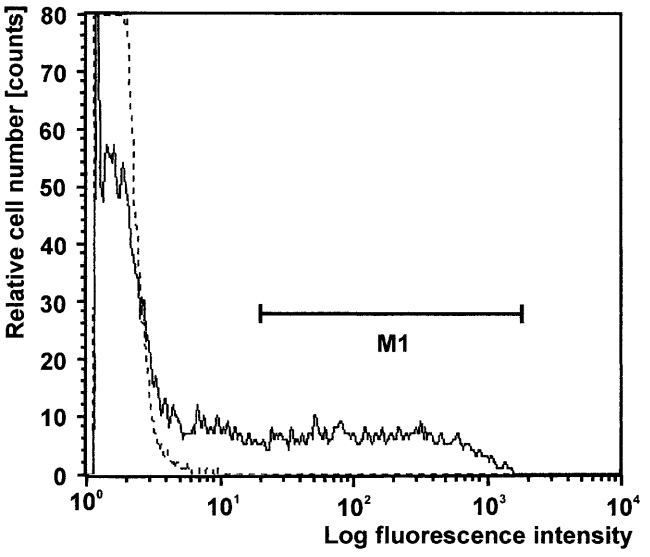

Fig. 1. Flow cytometric analysis of peripheral T lymphocytes after staining with CDw60 antigen. M1 indicates CDw60 positive cells, the dotted line represents the isotype control.

representative statistical analysis was done with 400 nm ultrathin sections. These sections had a thickness four times as thick as those used in conventional electron microscopy. Because of the high amount of inelastically scattered electrons, which occur due to the interactions of beam electrons with outer shell valence electrons of the sample atoms there is a loss in resolution and significant blurr of image details. These inelastically scattered electrons were thus eliminated with the aid of an energy filtered transmission electron microscope (EFTEM). These energy-filtered images, showed the 10 nm gold markers in detail throughout the whole thickness of the sections and thus a large section volume and in consequence a broad area of the cell surface could be analysed. In this way, sufficient label signal could be detected, even when the surface expression of the CDw60 antigen was rather low. All peripheral gold markers per cell cross-section were quantified and the linear densities were calculated as described below.

A relative frequency distribution of CDw60 labeled cells over the linear density was calculated on random T cells. Of the whole cell population ($N = 262$) analyzed, 167 individuals, i.e., 63.7%, did not show any electron dense surface marker (Fig. 2(a)). A total of 51 cells (19.5%) had a linear density of 0.05–2.0 gold markers per μm and represent a low surface expression of CDw60. 35 cells (13.4%) in the range from 2.1 to 4.5 markers per μm showed a medium surface expression (Fig. 2(b)), and the residual part of the population, i.e., nine cells (3.4%), ranging from 4.6 to 9.0 gold markers per μm, indicated high surface expression of the antigen (Fig. 2(c)). From the graph in Fig. 4(a) there exist four maxima, the first at 0.0, the second at 0.5, the third at 2.5, according to no, low and medium surface presentation of CDw60, and a fourth of high surface expression at 5 (Au/μm) linear density. These IEM preembedding label experiments confirmed and extended cytofluorimetric results (Fig. 1) and demonstrated a differentiated expression of CDw60 in human T cells [3].

Intracellular localization of CDw60.—The relative high concentration of CDw60 antigens in the lipid extract of human T cells [7] suggested that a portion of the antigen might occur intracellularly. Thus the question arose as to how an intracellular CDw60 expression and distribution relative to the external would look like. By postembedding labeling of ultrathin sections of Lowicryl embedded T cells, we looked for the intracellular CDw60 antigen distribution (Fig. 3(a)). As can be seen from the survey view of a representative cell, the overall cellular antigen expression density at the cell periphery is rather low (Fig. 3(a): arrowheads). The linear density of CDw60 antigen on the cytoplasmic membrane was analyzed, but because of the spanning radius of the primary IgM antibody, no discrimination could be made between extra- versus intracellular orientation of the antigen and thus only the localization of the antigen with respect to the cytoplasmic membrane could be managed. The amount of CDw60 localized by postembedding IEM showed a significant lower linear density, i.e., from 0.0 to 1.35 gold particles per μm relative to preembedding IEM, including three maxima at 0.15, 0.6 and 1.05 gold marker per μm (Fig. 4(b)). This low label density was mainly based on the unphysiological conditions of the postembedding labeling process (see above) and the reduced antigen presentation of the cross-sectioned cytoplasmic membrane on the section surface.

In spite of these limitations of postembedding IEM it could be shown that nearly all

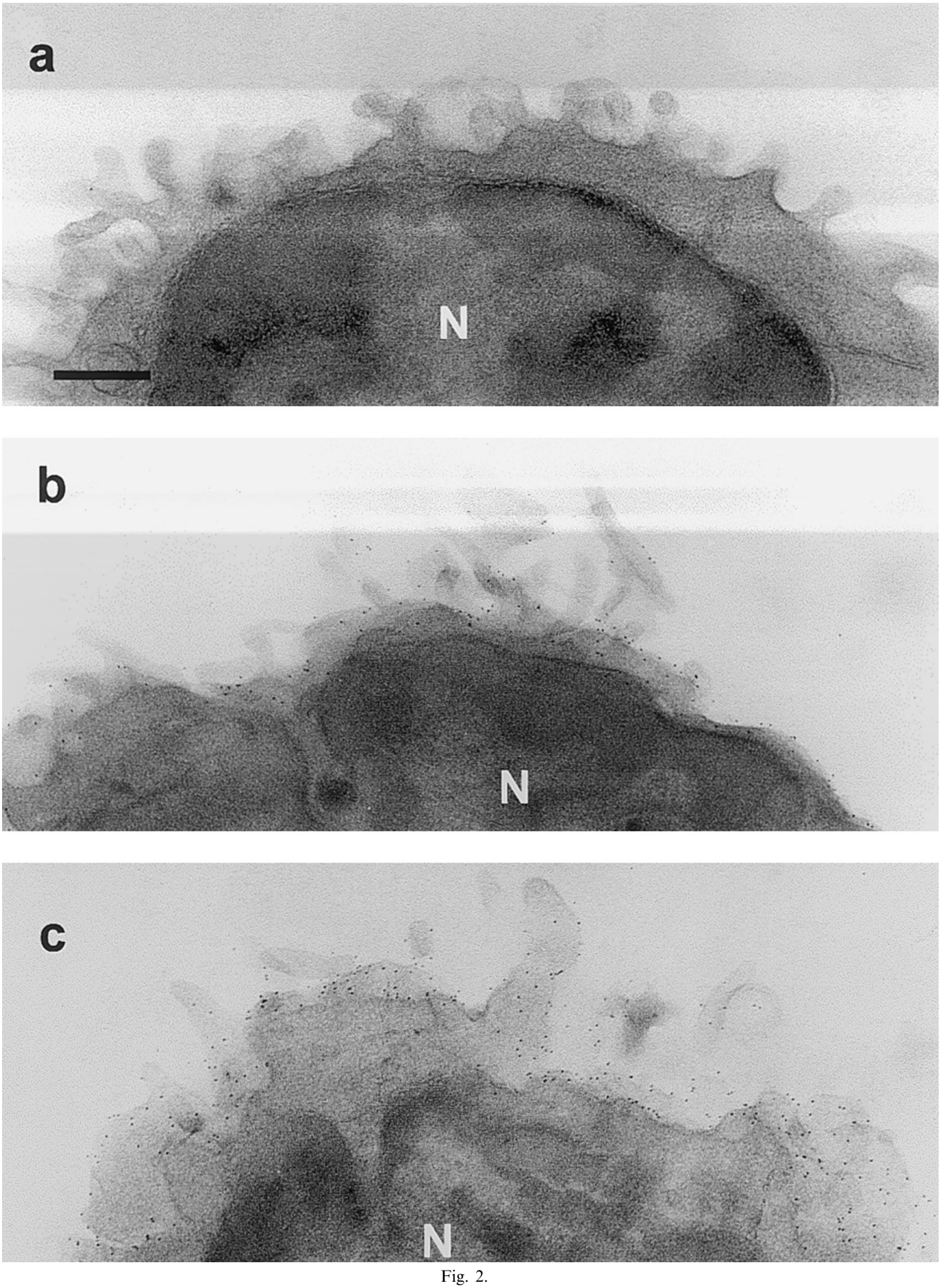

Fig. 2.

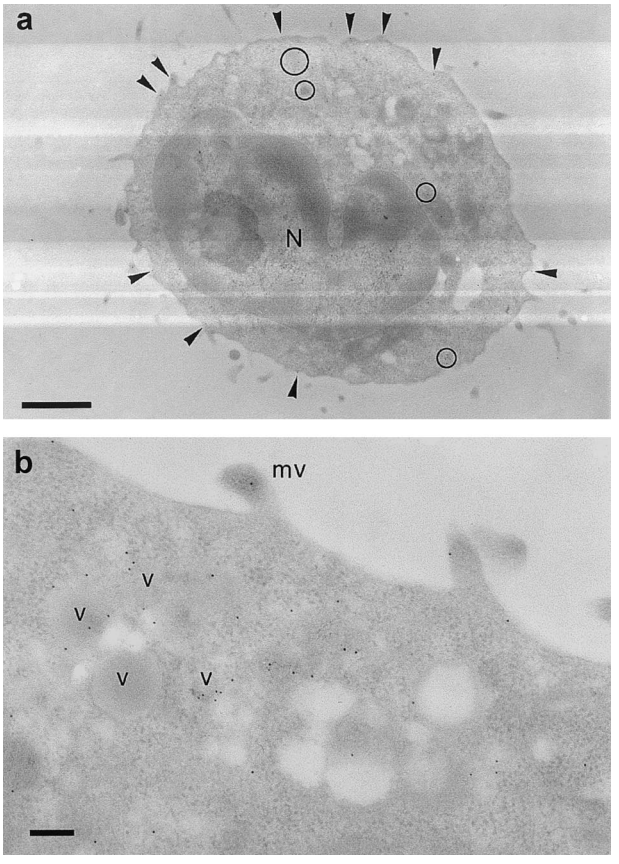

Fig. 3. Intracellular detection of the CDw60 antigen by postembedding localization with monoclonal IgM M-T32 anti-CDw60. (a) Survey of a typical T lymphocyte showing low level expression of the antigen and localization at the cytoplasmic membrane (arrowheads). Intracellular clusters (circles) are associated with vesicles. (b) Detailed view of the intracellular antigen distribution. Mainly vesicles with electron dense to transparent content (v) and microvilli (mv) are labeled. Bars: (a) 1 μm; (b) 200 nm.

peripheral T lymphocytes contained intracellularly deposited CDw60 antigen (see Fig. 3(a)). This observation significantly contrasts with antigen presentation on the external cell surface, as shown by preembedding IEM and flow cytometry experiments. From Fig. 3(a), CDw60 appears to be restricted to the Golgi of the lymphocytes, mainly seen as clusters of electron dense particles (Fig. 3(a): circles); apart from unspecific ribosome-associated gold markers most were bound to vesicles (Fig. 3(b)).

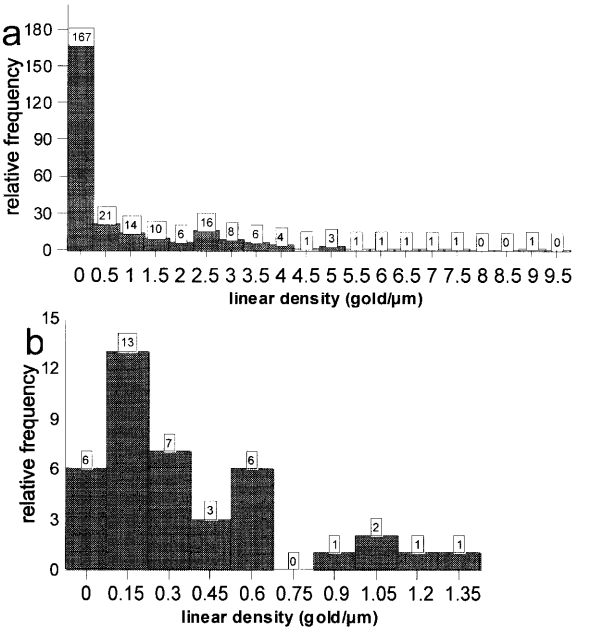

Fig. 4. Diagrammatic distribution of CDw60 antigen by pre- and postembedding labeling. (a) Frequency distribution of cells versus linear densities of gold markers of the surface exposed antigen (preembedding label); (b) frequency distribution diagram of membrane-associated CDw60 (postembedding label).

3. Discussion

The present work analyzed the expression of the CDw60 antigen in peripheral human T cells and its intra- and extracellular distribution by immunocytochemical methods. 9-*O*-Acetyl GD3 was first found in melanomas as a tumour-associated antigen [9]. We realized later that the T cell antigen CDw60 [3] was identical with 9-*O*-acetyl GD3 [1] and that its presence in leucocytes was not restricted to T cells because Cdw60 was found in the lipid extracts of all leucocyte populations [7]. This is in contrast to the general assumption of glycosphingolipids being located at the outer cell surface. However, several examples of intracellularly occurring GSLs have been recently described. They were found associated with intracellular organelles [15], secretory

Fig. 2. Preembedding label of peripheral T lymphocytes with monoclonal IgM M-T32 anti-CDw60, detected with 10 nm immunogold, specific to the μ-chain of the mouse IgM. Electron spectroscopic imaging was done on 400 nm ultrathin sections at an electron energy loss of 50 eV, set for contrast tuning. (a) Cell periphery of a T lymphocyte, typical a non-expressor. (b) Periphery of a medium expressor with a standardized linear density of 2.9 gold/μm; and (c) a high expressor at 7.3 gold/μm (N = nucleus). Bar: 500 nm.

granules of mast cells [16] and in the Golgi complex of canine epithelial cells [17]. Further examples are their association with the chromaffin granule membrane of bovine adrenal medulla [18], with the endoplasmic reticulum of rat liver cell [19], rat sciatic nerve [20] and rat brain [21]. Similarly, ganglioside protein complexes have been found in human fibroblasts [22] and an association with intermediate filaments has been reported [23,24]. In two studies it was shown that 80–90% of the main glycosphingolipids of neutrophils were located inside the cell [25,26]. This result encouraged us to analyse the distribution of a selected glycosphingolipid antigen, namely CDw60, in human T cells at the individual cellular level by preembedding IEM. Here, it was found that the external cell surface presentation of the antigen is distinctly differentiated, since low, medium and high CDw60 expression of about 33% of peripheral lymphocytes of a human cell population has been observed (see the clustering of label densities of preembedding labeled cell in the graph Fig. 4(a)). These findings were corroberated by flow cytometric data, which showed 26% of the T cell population to carry the CDw60 antigen, and thus extend the results of Ref. [3].

We observed that all T cells contained about the same level of CDw60 antigens intracellularly, irrespective of their surface expression. The finding of medium and high surface expression of CDw60 relative to the non-exposing cells and the fact of its general intracellular presence is indicative of its regulated surface presentation. Present studies are on the way to answer the question whether the CDw60 positive T cells interact with lectin-like molecules. The mere activation of T cells seems not to increase the amount of CDw60 on the surface of T cells [27]. However, B-cells, being normally CDw60 negative, show this antigen after activation [28].

4. Experimental

Antibodies.—The mouse monoclonal IgM antibody M-T32 (CDw60), originally raised against T cells from patients suffering from chronic lymphocytic T cell leukemia [29], was a kind gift from E.P. Rieber, Technical University of Dresden.

Isolation of peripheral T cells.—T cells were isolated from buffy coats by separation on Ficoll-Paque (Amersham-Pharmacia, Freiburg, Germany) and passage through nylon wool followed by water-lysis of erythrocytes, according to Julius et al. [30].

Flow cytometry.—Aliquots of the M-T32-treated cells were labeled with FITC-conjugated goat anti-mouse IgM (Dianova, Hamburg, Germany). Control cells were treated with IgM isotype control antibody (CD15, Becton Dickinson, Heidelberg, Germany). Incubation and washing was performed as described for preembedding labeling. The cells were subjected to flow cytometry with a FACScan flow cytometer (Becton-Dickinson, Heidelberg, Germany).

Immunoelectron microscopy.—(a) Preembedding label with mouse monoclonal IgM antibody M-T32 (CDw60): Isolated T cells were suspended in PBS, pH 7.2 + 2% (v/v) FCS + 0.02% (w/v) NaN_3 (FACS buffer). Fc-receptors were blocked by addition of human IgG (100 μg/mL, 10 min, 0 °C). After washing with FACS buffer, the cells were incubated with monoclonal IgM M-T32 (20 μg/mL) for 30 min at 0 °C. After washing three times with FACS buffer, sedimented T cells were labeled with anti μ-chain specific immunogold$_{10nm}$ conjugate (goat anti-mouse IgM) for 60 min at 0 °C. As a control a T cell aliquot was incubated with FACS buffer without primary antibody in parallel and treated with the anti-μ goldconjugate.The immunogold labeled cells were washed three times with FACS buffer. The final sediment was resuspended in PBS, containing 2% glutardialdehyde and fixed overnight at 4 °C. After fixation the cells were immobilized in 2% (w/v) agar, buffered with 30 mM veronal-acetate, pH 7.5 containing 5% (w/v) sucrose. After one washing of the immobilized cells for 20 min at rt, they were postfixed in 2% (w/v) OsO_4, buffered with veronal-acetate, pH 7.5 containing 5% (w/v) sucrose at 4 °C overnight. The cells were dehydrated by a $(CH_3)_2CO$ series and finally embedded in epoxy resin [31]. Ultrathin sections of 400 nm were cut with a Reichert Ultracut S (Leica, Bensheim, Germany) and were picked up with Cu-grids (300 mesh).

(b) Postembedding labeling with mouse monoclonal IgM antibody M-T32 (CDw60): Isolated peripheral T cells were fixed with 4% (w/v) paraformaldehyde-0.1% (v/v) glutardialdehyde

in 50 mM Na-phosphate, pH 7.5. After washing once for 20 min on ice in PBS, the cells were immobilized in 2% (w/v) agar in PBS and dehydrated in a $(CH_3)_2CO$ series. Cells were embedded in Lowicryl K4M at − 35 °C [32]. Ultrathin sections were picked up on Formvar-coated Ni-grids (300 mesh) and were blocked with 1% (w/v) ovalbumin for 5 min at 20 °C. They were incubated with monoclonal IgM MT-32 (CDw60, 0.8 μg/mL) overnight at 4 °C, washed with PBS, pH 7.2 and blocked with 1% (w/v) ovalbumin for 5 min at 20 °C. The primary antibody was labeled with immunogold$_{10nm}$ conjugate anti-μ (mouse) for 15 min, washed with 0.01% (v/v) Tween 20–PBS, pH 7.2 for 5 min, followed by five washing steps with PBS, 5 min each, and once with water, finally followed by poststaining with 4% (w/v) uranylacetate, pH 4.5. As a control for label specificity and checking for background binding of the immunogold gold conjugate the primary antibody was omitted.

Electron microscopy was done with a CEM 902 energy-filtered transmission electron microscope (Zeiss, Oberkochen, Germany) at 80 kV acceleration voltage in the ESI-imaging mode. The electron energy loss was adjusted from 0 to 50 eV with a slit width of 15 eV. Primary magnification ranged from × 4400 to × 20,000.

Statistical evaluation of membrane-bound antigen.—For evaluation of surface-exposed CDw60-antigen of the whole peripheral T cell population ultrathin sections of 400 nm thickness were used. Thus low antigen concentrations could be detected. All peripheral immunogold targets per cell were counted and the medium circumference of the corresponding cells was measured. The linear density was calculated as the number of goldconjugates per μm and this value then was standardized to the general ultrathin section thickness of 120 nm. The standard deviation of the average value of all T cell circumferences was used as class width for the linear density distribution profile.

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