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ORIGINAL ARTICLE

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Diagnostic relevance of Langerin detection in cells from bronchoalveolar lavage of patients with pulmonary Langerhans cell histiocytosis, sarcoidosis and idiopathic pulmonary fibrosis

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Abstract The diagnosis of pulmonary Langerhans cell histiocytosis might be refined by demonstrating reliability of a new cell marker, i.e., Langerin (CD207), used on bronchoalveolar lavage fluid. For this purpose, we collected material from patients with this disease and also with sarcoidosis and idiopathic pulmonary fibrosis as controls. In addition to the immunocytochemical detection of Langerin, we examined the expression profiles of CD1a and the macrophage tandem-repeat mannose receptor (CD206). To test accessibility of Langerin, a C-type lectin, for mannosides, we employed reverse lectin histochemistry using mannose-containing neoglycoproteins. The analysis revealed a significantly increased percentage of CD1a- and Langerin-positive cells in pulmonary Langerhans cell histiocytosis in comparison with both other studied diseases. No expression of the 175-kDa mannose-binding lectin (CD206) in Langerhans cells was observed. Evidently, binding sites on the cells were not accessible for the mannose-containing neoglycoligand. These results provide evidence for the usefulness of Langerin-directed immuno- and glycohistochemical monitoring of bronchoalveolar lavage fluid in the diagnosis of pulmonary Langerhans cell histiocytosis.

Keywords Fibrosis · Histiocytosis · Immunohistochemistry · Langerin (CD207) · Lectin · Neoglycoprotein · Sarcoidosis

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Introduction

The definition of new cell markers by the development of monoclonal antibodies provides a means to refine current diagnostic procedures. Following this first step on the way to proving diagnostic usefulness, the tool under study should next be tested with clinical material. In this study, we have focused on pulmonary Langerhans cell histiocytosis (PLCH). This disease belongs to the Langerhans cell histiocytosis family. The patient ratio between males and females is approximately equal [8, 18]. The etiology of the disease is not fully known, but it is notable that the majority of patients have a tobacco smoking history [2, 3, 12]. The clonal character of Langerhans cells (LC) in PLCH suggests a manifestation of the disease with tumorous character [8]. However, a reactive nature of PLCH is also documented [8, 18]. No characteristic clinical symptoms are attributed to the disease, and its diagnosis is, thus, based on the combination of clinical, radiological and laboratory investigations [18]. Among them, the cytological analysis of bronchoalveolar lavage (BAL) is recommended, with the percentage of Langerhans cells higher than 5% being an important factor for PLCH detection [1, 13]. At present, the apparent quantity of reports employing immunocytochemical monitoring of cells in BAL underscores that it is warranted to develop a reliable screening for this purpose. In this study, we introduce a recently described cell marker for this purpose and validate the application of the procedure by demonstrating marked differences in LC presence in PLCH,

sarcoidosis and idiopathic pulmonary fibrosis (IPF). In addition to monitoring CD1a detection, an antibody against Langerin (CD207), a protein specific for LC in skin and in epithelia, such as lung bronchiolae, with properties of a mannose-reactive C-type lectin, was tested in this study [16, 17]. To address the further question whether the carbohydrate recognition domain of Langerin is accessible for ligand, we selected an α-mannoside-presenting neoglycoprotein as a probe. By concomitantly monitoring the presence of the 175-kDa macrophage mannose receptor (MR; CD206), an internal quality control of cell-type specificity was established.

Materials and methods

Three groups of patients with PLCH (three females, five males, mean age 36±17 years), sarcoidosis (five females, three males, mean age 42±10 years) and IPF (four females, six males, mean age 49±17 years) were included in this study. All patients with PLCH were characterized with tobacco smoking history. The percentage of cell types in BAL fluid collected from patients was evaluated after conventional cytological staining [mean±SD (%), macrophages: 71±10, neutrophils: 4±3, eosinophils: 2±2, lymphocytes: 24±12] to prove usual interstitial pneumonia pattern in combination with clinical criteria of the American Thoracic and European Respiration Societies (2000). The diagnosis was verified histopathologically from biopsy in all cases. BAL was performed according to a standard procedure. Cytospin specimens were prepared, and the cells were fixed with paraformaldehyde. Any antigen-independent binding of antibody, for example, via Fc fragments, was blocked using non-immune swine serum (Dako, Brno, Czech Republic). CD1a was detected using the monoclonal antibody obtained from Immunotech (Prague, Czech Republic) and Langerin using the DCGM4 monoclonal antibody [16]. MR was detected using a goat polyclonal antibody (TNO-PG, Leiden, The Netherlands). Swine anti-mouse antiserum labeled with fluorescein isothiocyanate (ALSEVA, Prague, Czech Republic) and tetramethylrhodamine isothiocyanate (TRITC)-labeled donkey anti-goat antiserum (Santa Cruz, Santa Cruz, CA, USA) were used as second-step antibodies. Accessibility of the carbohydrate-binding site for exogenous ligands was probed using biotinylated neoglycoprotein with covalently attached α-D-mannopyranoside derivatives [5, 6]. TRITC-labeled ExtrAvidin (Sigma-Aldrich, Prague, Czech Republic) was used as the second-step reagent. Non-immune goat serum and mice monoclonal antibodies of the same isotype without reactivity for LC were used as controls of the specificity of the immunohistochemical reaction. Competitive inhibition of neoglycoprotein binding by D-mannose (Sigma-Aldrich, Prague, Czech Republic) was used to ascertain the specificity of the reverse lectin histochemical reaction. The double-labeling reaction at the single-cell level was performed in several specimens according to an optimized protocol [4]. Several specimens were also subject to counterstaining with 4′-6′-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Prague, Czech Republic).

A fluorescence microscope Optiphot-2 (Nikon, Prague, Czech Republic) equipped with suitable filterblocks, CCD camera and computer-assisted image analysis system (LUCIA, Laboratory Imaging, Prague, Czech Republic) were used for data collection, storage and analysis. The individual data sets (minimally 500 cells/patient) were statistically analyzed. In detail, with respect to the number of cases, the Shapiro-Wilk's test was used for testing of distribution. Where a derivation from a monitored distribution was detected, the Mann-Whitney test was used for comparison. For calculating of the correlation between two variables, the Spearman correlation coefficient (r), which determines the extent to which values of two variables are "proportional" to each other, was instrumental.

Results and discussion

When performing the immunocytochemical protocol, any antigen-independent staining reaction, for example, using kit reagents, was first excluded. Under these conditions, Langerin (CD207) detection was consistent, reliable and reproducible (Fig. 1). Likewise, CD1a expression was visible. To address the question on the diagnostic value of this procedure, we monitored cell populations originating from patients with PLCH, sarcoidosis and IPF. BAL of patients suffering with PLCH contained a significantly higher number of CD1a- and Langerin-positive cells than BAL of patients with sarcoidosis and IPF (Fig. 2). No apparent differences between the number of CD1a- and Langerin-positive cells in patients with sarcoidosis or IPF were observed. As shown in detail in Fig. 2, Langerin detection improved the P value to 0.000533 for distinguishing between PLCH and sarcoidosis. Interestingly, the numbers of CD1a- and Langerin-positive cells in all tested cases were almost identical ($P<0.0001$, r=0.976), as shown in Fig. 3. This result indicates the high specificity of Langerin detection. Regarding diagnostic assessment, literature data defined a level of more than 5% of LC in BAL as borderline for the diagnosis of PLCH [1, 13]. Two PLCH patients of our group had a percentage of LC of 4%. The clearly increased LC level warrants extending

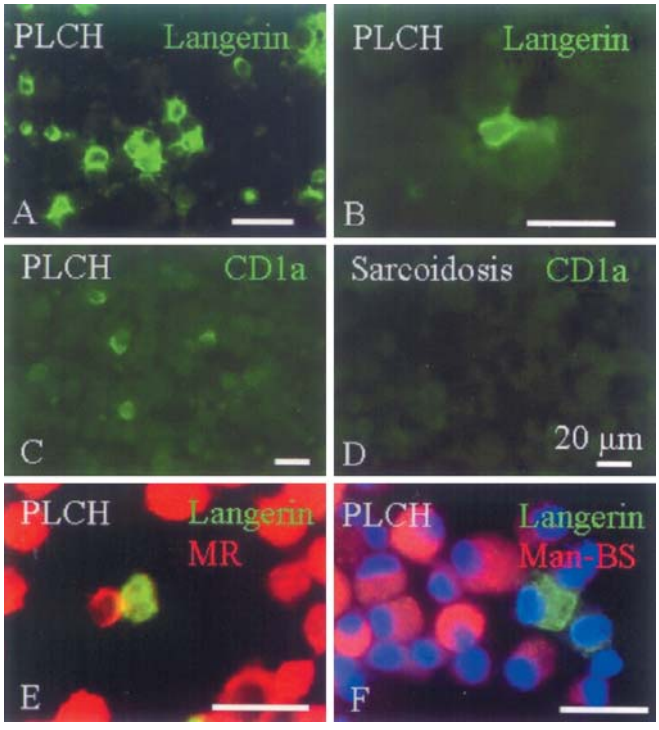

Fig. 1 Representative figures illustrating detection of Langerin (CD207) (*green*, **A**, **B**, **E**, **F**), CD1a (*green*, **C**, **D**), the 175-kDa tandem-repeat C-type mannose receptor (CD206) (*red*, MR, **E**) and mannose-binding sites (*red*, Man-BS, **F**) in cells of bronchoalveolar lavage (BAL) obtained from patients with pulmonary Langerhans cell histiocytosis (PLCH) (**A**, **B**, **E**, **F**) and sarcoidosis (**C**, **D**). Nuclei in **F** are counterstained with 4′-6′-diamidino-2-phenylindole; *bar* is 20 μm

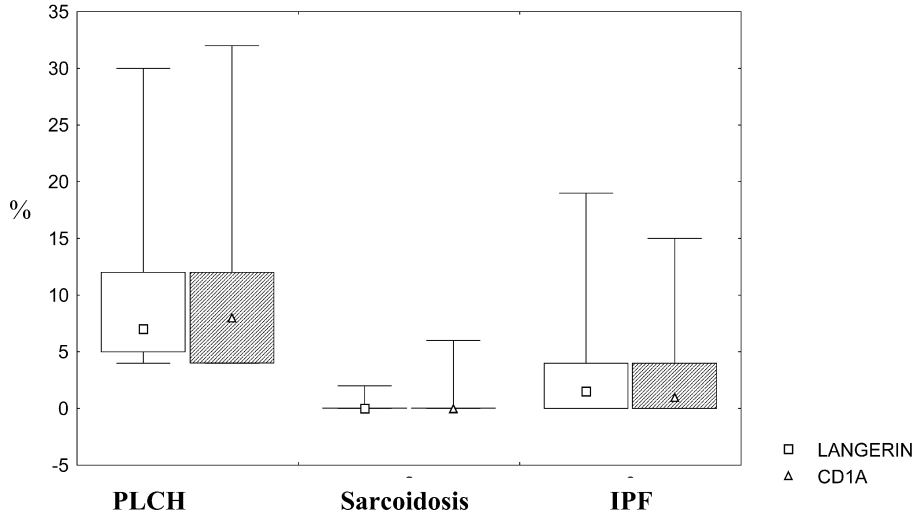

Fig. 2 Difference of the percentage of the number of Langerin- or CD1a-positive Langerhans cells in patients suffering from pulmonary Langerhans cell histiocytosis (PLCH), sarcoidosis and idiopathic pulmonary fibrosis (IPF). The data are statistically evaluated using the Mann-Whitney test. Each value gives the median. Minimal and maximal values are shown by *bars* and 75% of the data lie within the boundaries of the box. In the case of Langerin expression, the difference between pulmonary Langerhans cell histiocytosis and sarcoidosis is significant at the level of P=0.000533 and between pulmonary Langerhans cell histiocytosis and idiopathic pulmonary fibrosis at the level of P=0.016. In the case of CD1a expression, the difference between pulmonary Langerhans cell histiocytosis and sarcoidosis is significant at the level of P=0.002145 and between pulmonary Langerhans cell histiocytosis and idiopathic pulmonary fibrosis at the level of P=0.0147. No significant differences between sarcoidosis and idiopathic pulmonary fibrosis were detected

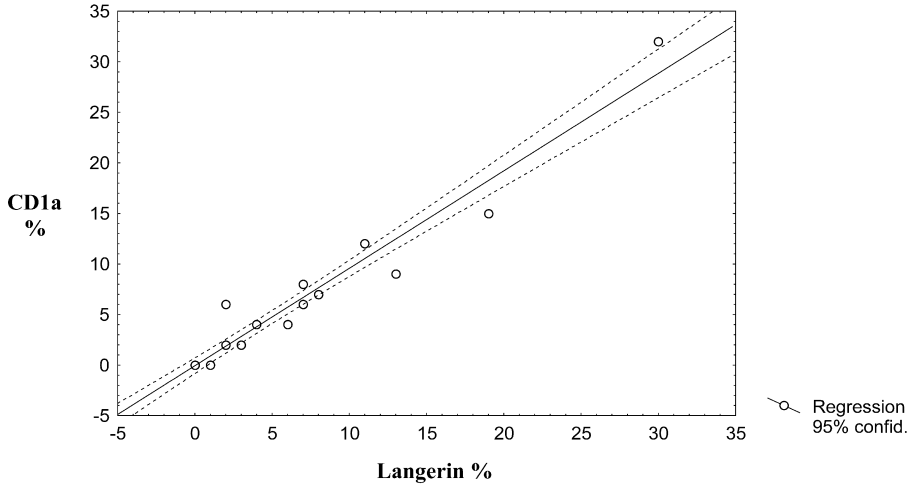

Fig. 3 The percentage of CD1a- and Langerin-positive cells in pulmonary Langerhans cell histiocytosis were almost identical, as was shown by regression analysis using the two data sets yielding a linear relationship at P=0.0001. Since values obtained from two patients were the same, the number of individual observations shown here is 14

diagnostic procedures to clarify clinical manifestations. As required for a valid control, no cells expressing CD1a and Langerin were positive for the presence of the MR. This observation is in complete accord with the fact that, in contrast to macrophages and dendritic cells of the non-Langerhans type, Langerhans cells do not express this lectin [10, 14]. Similar to Langerin, a protein that controls formation of Birbeck granules and is assumed to be important for antigen uptake and presentation, this molecule has lectin properties for mannosides [11, 16, 17]. Using a synthetic class of probe (neoglycoprotein), we, thus, examined whether the CD207-positive cells harbored accessible receptor sites (that is, sites not blocked by endogenous ligands). As a positive control, Langerin- and CD1a-negative cells (predominantly macrophages) specifically bond this glycohistochemical marker, whereas no reactivity was observed for the Langerin- and CD1a-positive cell population (Fig. 1). In this context, it is interesting to refer to previous data that LC infiltrating malignant tumors also expressed Langerin and did not bind the mannose-containing neoglycoprotein in contrast to LC present in normal epithelia, which were reactive with the marker [11]. LC expressing Langerin in Langerhans cell histiocytosis lesions from sites other than the lung are described as having properties of immature LC [7], being able to recognize antigen in contrast to the

PLCH LC that were characterized as mature [15], and, therefore, the uptake of antigen by these cells could be limited. Changes related to the maturation of LC [9], thus, can be relevant for explaining this difference in binding-site accessibility. In conclusion, the immunocytochemical monitoring of BAL focusing on Langerin as new LC marker has proven its usefulness for the diagnosis of patients suffering from PLCH.

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