Diagnosis of Pulmonary Histiocytosis X by Immunodetection of Langerhans Cells in Bronchoalveolar Lavage Fluid

SYLVIE CHOLLET, PhD, PAUL SOLER, PhD, PIERRE DOURNOVO, MD, MARIE S. RICHARD, MD, VICTOR J. FERRANS, MD, PhD, and FRANÇOISE BASSET, MD

From INSERM, Unité 82, Faculté de Médecine Xavier-Bichat, Paris, France; Clinique Physiopathologique Respiratoire, Hôpital de la Salpêtrière, Paris, France; and Pathology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland

Based on the finding that Langerhans cells and histiocytosis X cells react with the monoclonal antibody OKT6, raised against a subset of thymocytes, we used this antibody to study the cells collected by bronchoalveolar lavage (BAL) from 131 patients, including 18 with pulmonary histiocytosis X, 43 with pulmonary sarcoidosis, 67 with miscellaneous pulmonary disorders, and 3 controls. Immunofluorescence studies demonstrated the presence of OKT6-reactive cells in all patients with pulmonary histiocytosis X (mean ± SEM, 5.29% ± 1.14% of all cells in BAL fluid). Immunoelectron microscopic studies revealed that the cells labeled in these patients (n = 13) contained Langerhans granules. The number of fluorescent cells in the other 113 patients was significantly smaller (mean ± SEM, 0.20% ± 0.04% of all cells; $P < 0.001$). In the 3 control patients, in the 43 patients with sarcoidosis, and in 61 of the 67 patients with miscellaneous disorders unrelated to histiocytosis X, no cells or <1% of the total were labeled; however, in the 6 remaining patients in this miscellaneous group, 1.3 to 2.8% of all cells in BAL were labeled. In 3 of these 6 patients, imunoelectron-microscopic examination showed that the cells labeled by OKT6 had the general characteristics of Langerhans cells but lacked Langerhans granules. OKT3, OKT4, and OKT8 monoclonal antibodies did not stain histiocytosis X cells in BAL fluid. (Am J Pathol 1984, 115: 225–232)

BRONCHOALVEOLAR LAVAGE (BAL) was introduced a few years ago as a new means of studying alveolar cell populations.[1] It has proven useful in many respects, and extensive reviews[2–7] have been made of the results of studies of both the cells and the supernatant fluid obtained by lavage. The main advantages of BAL are that it provides an immediate assessment of the processes occurring in the alveolar structures and that it can be performed several times during the course of an illness. The cell populations obtained by BAL have been studied in normal subjects, smokers and nonsmokers, and patients with different pathologic conditions, mainly the interstitial lung diseases.[2–5] In many disease states the total cell count and the differential cell count in BAL fluid are altered in comparison with those in normal, nonsmoking individuals. Typical cell count patterns have been observed in several conditions, and the main cell types involved vary from one disease to another.[8,9]

Ultrastructural examination of cells collected by BAL has proven useful in the diagnosis of only a few conditions, including pulmonary alveolar proteinosis,[10] peripheral adenocarcinoma of the lung (unpublished personal observation), and pulmonary histiocytosis X.[10,11] The diagnostic feature in the latter disease is the finding of Langerhans cells (LCs), which contain characteristic cytoplasmic organelles, the LC granules.[12,13] Although ultrastructural examination of BAL cells has been very helpful in certain patients with histiocytosis X, sometimes it is time-consuming and unsuccessful in other patients with this disease; in a few patients, the negative re-

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Address reprint requests to Françoise Basset, MD, INSERM U 82, Faculté Xavier Bichat, 16 Rue Henri Huchard, 75018 Paris, France.

Table 1—Diagnoses in 67 Patients with Miscellaneous Disorders in Whom BAL Cells Were Studied by OKT6 Immunofluorescence

Diagnosis	Number of patients	Number of BALs
Viral pneumonia	1	1
Tuberculosis (or suspected)	4	4
Bronchial asthma	2	2
Silicone-induced pneumonitis	3	3
Bronchioloalveolar carcinoma	2	2
Pulmonary embolism	1	1
Pneumoconiosis	5	5
Chronic eosinophilic pneumonia	1	1
Idiopathic pulmonary fibrosis	4	4
Bullous interstitial lung disease	2	2
Undetermined interstitial lung disease	19	19
Collagen vascular disease	14	14
Behçet's syndrome	1	3
Hypersensitivity pneumonitis	3	3
Malignant histiocytosis	1	1
Lymphoma	1	1
Legionnaire's disease	3	3
Total	67	69

sults of BAL studies have been in contradiction to those of subsequent open lung biopsies demonstrating typical granulomatous lesions of histiocytosis X. Thus, it is desirable to have a more rapid and sensitive technique for the light- and electron-microscopic identification of Langerhans cells in BAL fluid. We have developed such a technique,[14] based on the use of OKT6 monoclonal antibody, after the finding of Murphy[15,16] and Fithian[17] that epidermal Langerhans cells react with this antibody. We have also tested other commercially available monoclonal antibodies in an effort to confirm the recent observation made by Murphy[18] that histiocytosis X cells (but not Langerhans cells) also react with the monoclonal antibody OKT4, raised against helper T-lymphocytes.

Materials and Methods

Patients Studied

A total of 143 BALs were performed in 131 patients, including 18 patients (total of 23 lavages) with histologically confirmed histiocytosis X, 67 patients (total of 69 lavages) with miscellaneous lung disorders, 43 patients (total of 48 lavages) with sarcoidosis, and 3 controls. A detailed list of the patients with miscellaneous disorders is given in Table 1. The method of BAL has been described in detail previously.[2]

Description of Antibodies

The monoclonal antibodies employed were from the OKT Series, Ortho Diagnostic Systems, Inc. These antibodies were generated in a mouse hybridoma system by immunization with the appropriate purified human cells. OKT6 antibody is known to react with 70% of human thymocytes, but not with circulating T-lymphocytes or monocytes.[19,20] OKT4 antibody reacts with helper T-lymphocytes,[20] OKT3 with T-lymphocytes,[20] OKT8 with suppressor T-lymphocytes,[20] OKM1 with monocytes, macrophages, and null cells, and OKIa with cells bearing HLA-DR antigen.

Indirect Immunofluorescence Staining

The cell suspension obtained by bronchoalveolar lavage was adjusted to a concentration of 10^7 cells/ml by dilution with RPMI 1640 medium (GIBCO). For each assay, 200 μl of this cell suspension was placed in a plastic test tube, and 10 μl of the undiluted antibody was added. The cells were resuspended by pipetting, incubated at 4 C for 30 minutes, and agitated every 10 minutes. The cells were then washed twice with 2 ml of phosphate-buffered saline (PBS), pH 7.4, by centrifugation (150 G for 10 minutes at 4 C). After washing, 20 μl of fluorescein-labeled goat antimouse immunoglobulin (Meloy) at a dilution of 1:20 was added to each tube. The mixture was reincubated at 4 C for another 30 minutes and washed again twice in PBS as described above. The cells were then resuspended in two drops of 30% glycerol in PBS. One drop of this suspension was placed on a slide, covered with a coverslip, and examined using a Leitz Ortholux II fluorescence microscope equipped with phase-contrast optics. For each assay, a control study was performed in which incubation with the monoclonal antibody was omitted. The percentage of fluorescein-labeled cells and of other cells was calculated on the basis of examining a total of 1000 cells.

Immunoelectron Microscopy

In 11 patients (a total of 13 samples) with histologically confirmed histiocytosis X and in 4 patients (total of 4 samples) with miscellaneous lung disorders, an aliquot of cells collected by BAL was reacted with OKT6 antibody and studied with the use of immunoelectron microscopy in addition to immunofluorescence. In two patients with histologically confirmed histiocytosis X and in 2 patients with another interstitial disorder of the lung the immunoelectron microscopy technique was performed with OKT4 monoclonal antibody as a first reagent.

For immunoelectron microscopy, the cell suspension was processed as described above, except that the second antibody was a sheep Fab'_2 antimouse immunoglobulin conjugated with horseradish peroxidase (HRP) (New England Nuclear, Boston, Mass). This

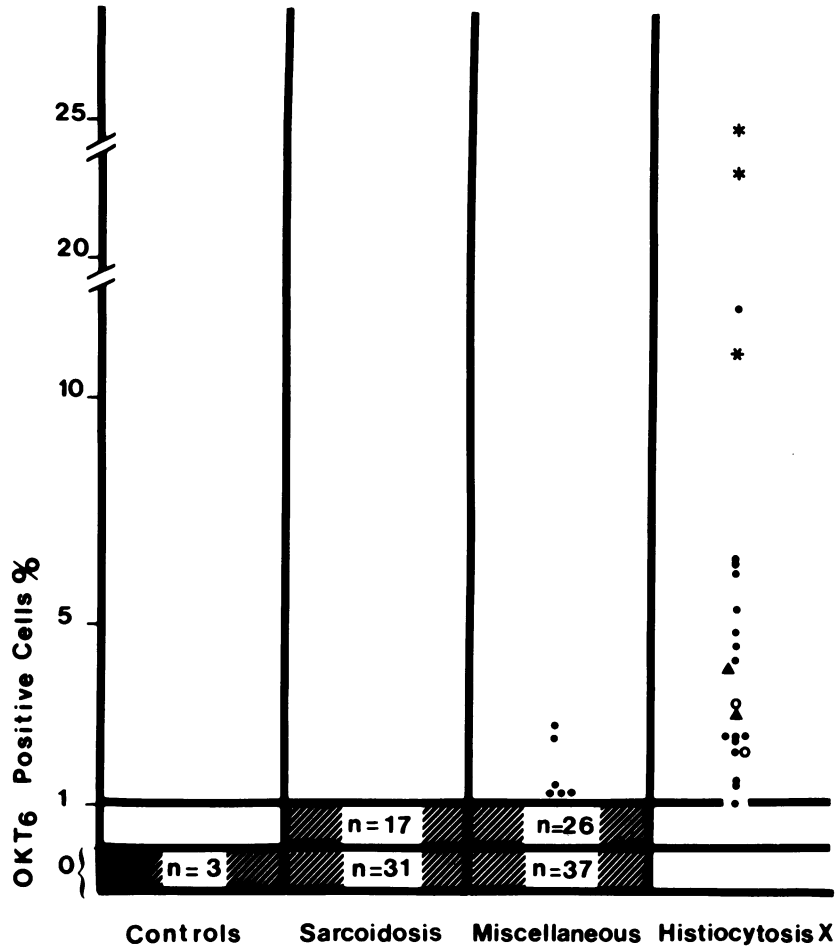


Figure 1 — Percentage of OKT6-positive cells in the BAL fluid in four different groups of individuals. Assorted signs (▲, O, *) are used for patients with more than one BAL.

antibody was used undiluted (10 μl for 2 × 10^6 cells) and was allowed to react by incubating for 60 minutes at 20 C. In 4 samples, the monoclonal OKT6 antibody was also used after 1:10 dilution. The results obtained were similar. After two rinses in PBS, the peroxidase activity was demonstrated using 3,3'-diaminobenzidine (DAB) (Sigma) and hydrogen peroxide, according to the method of Roels.[21] After 1 hour incubation with the diaminobenzidine solution in the dark, the cells were fixed in 1.25% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon. Ultrathin sections were examined in a Siemens Elmiskop 101 electron microscope without additional contrasting. Two control studies were performed, one without monoclonal antibody and another without HRP-conjugated antibody.

Results

Immunofluorescence Microscopy

In preparations reacted with OKT6 monoclonal antibody, fluorescent cells were found in each of the 18 patients with biopsy-confirmed pulmonary histiocytosis X. In this group of patients, fluorescent cells constituted between 1.8% and 25% of the total number of cells in BAL fluid (mean ± SEM, 5.29% ± 1.17%). In the other 113 patients, fluorescent cells constituted 0.2% ± 0.04% of the total number of BAL cells. This was significantly less ($P < 0.001$) than the number of fluorescent cells in patients with histiocytosis X. In the 3 control patients, in the 43 patients with sarcoidosis, and in 61 patients with miscellaneous lung disorders, fluorescent cells were absent or represented less than 1% of the cells examined; however, 6 patients with lung disorders unrelated to histiocytosis X were found to have from 1.3% to 2.8% of fluorescent cells (Figure 1). Table 2 indicates the diagnoses and the percentage of OKT6-positive cells found by immunofluorescence in these 6 patients.

The fluorescent cells varied in shape and size, and they did not all have an identical surface labeling: those that were found in abundance, in the most typical cases, were smaller than macrophages, but usually larger than lymphocytes (Figure 2). They were irregular in size and shape, often rounded or ovoid, and did not have intracellular autofluorescent granules of lipofuscin, in sharp contrast with alveolar

Table 2—Percentage of OKT6-Positive Cells in BAL Fluid From 6 Patients With Diseases Other Than Histiocytosis X

Diagnosis	Number of patients	OKT6 (+) cells
Viral pneumonia	1	1.2%
Bullous interstitial lung disease	2	1.3% and 2.8%
Idiopathic pulmonary fibrosis	1	2.5%
Behçet's syndrome	1	1.5%
Malignant histiocytosis	1	1.2%

macrophages, which were filled with such yellow to brown granules. The fluorescent surface labeling was bright and almost continuous, with extremely bright, dotted areas distributed over the whole surface. Some cells had a more irregular shape, and the labeling was continuous throughout their surface but was weaker than in more typical cells. Details of the morphology of the fluorescent cells were always examined with the use of phase-contrast microscopy: the shape and size of the cells were irregular, their nuclei often were indented, and no granular content was observed in their cytoplasm, in contrast to macrophages. Control preparations for the immunofluorescence techniques were always negative. A total of 16 BAL samples from 14 patients with pulmonary histiocytosis X were reacted with OKT4, OKT3, and OKT8 monoclonal antibodies. All the positive cells observed in these preparations were small (7–15 μ), and their surface labeling was dotted. The intensity of the fluorescence was weak with OKT3 and OKT4 and stronger with OKT8. When examined by phase-contrast microscopy, the cells appeared rounded, with a high nucleus-to-cytoplasm ratio, and were readily identifiable as lymphocytes. These characteristics remained identical regardless of the patient's diagnosis. In particular, no cells with the characteristics of Langerhans cells seemed to be labeled with OKT4 monoclonal antibody.[18] The cells labeled with OKM1 showed a continuum of sizes. The larger cells were recognizable as alveolar macrophages, the smaller ones as lymphocytes. The number of OKM1-positive cells varied greatly, particularly according to the smoking habits, regardless of the pathologic condition. With OKIa monoclonal antibody, most (approximately 90%) of the alveolar macrophages were labeled.

Immunoelectron Microscopy

OKT6 Monoclonal Antibody

In the BAL from the 13 patients with histiocytosis X studied by immunoelectron microscopic techniques, a certain number of cells, smaller than the number or cells detected by immunofluorescence in the corresponding samples, showed surface labeling by the immunoperoxidase reaction product. In 12 of these patients, at least 3% of the cells were labeled, and in 1 patient (in whom the immunofluorescence technique had shown 25% labeling), 12% of the total number of cells on the electron microscope grids were labeled by the immunoperoxidase method. The OKT6-positive staining consisted of flocculent, electron-dense aggregates of DAB reaction product (Figure 3). These aggregates were regularly distributed in a semicontinuous fashion along the outer surfaces of the plasma membranes.

Dendritic processes of the cells also showed membrane staining. All the cells with peroxidase-reactive plasma membranes also showed peroxidase activity in cisterns of rough endoplasmic reticulum (RER) as well as in the perinuclear cisterns (Figure 4). Most of the peroxidase-reactive cells contained LC granules, which were visible without additional contrasting, sometimes in great numbers. The LC granules were sometimes stained by the DAB reaction. Mitochondrial membranes (Figure 4) were labeled, because of their cytochrome oxidase content.[22]

In the small group of 3 patients with miscellaneous disorders who had a few fluorescent cells demonstrated by OKT6 antibody, and in whom the immunoperoxidase technique was also performed, a few cells coated with electron-dense DAB reaction product were observed. Although these reactive cells were similar to Langerhans cells in their general characteristics, including nuclear shape, cytoplasmic organelles and lack of phagocytosed material, they did not contain identifiable LC granules.

The surfaces of all other cells examined, including

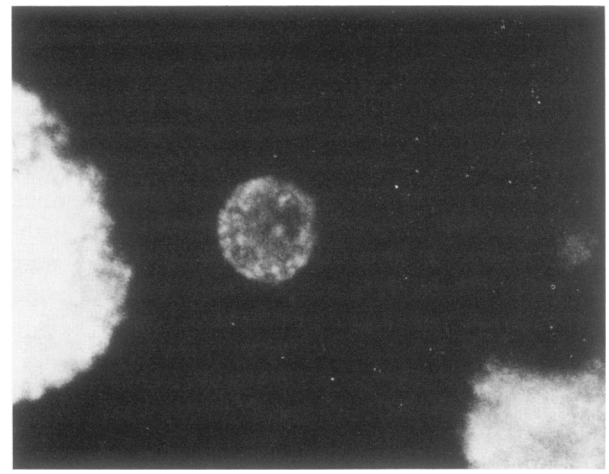


Figure 2—FITC labeling. One OKT6-reactive cell between two autofluorescent macrophages.

alveolar macrophages, lymphocytes, neutrophils, eosinophils, mast cells, and desquamated pulmonary epithelial cells did not show any peroxidase labeling. Endogenous peroxidase activity was present in specific cytoplasmic granules of neutrophils and eosinophils, as well as in lysosomes of some alveolar macrophages (exudate type alveolar macrophages).[22,23] Mast cells exhibited a strong cisternal peroxidase activity, as is usually the case with the technique used in this study.[22,24] Mitochondria were equally reactive in all cells. The two controls, performed by omitting either the OKT6 monoclonal antibody or the HRP-conjugated antibody, exhibited only endogenous peroxidase activity and the mitochondrial membrane reaction.

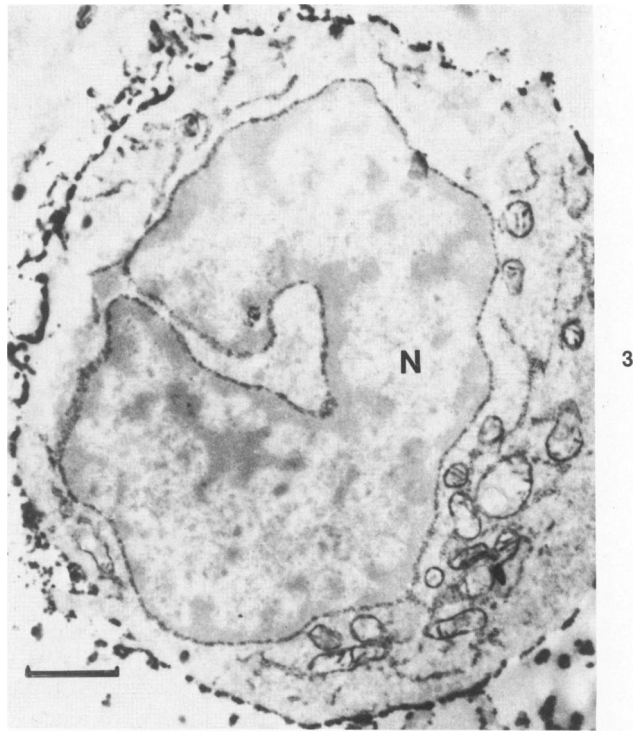
OKT4 Monoclonal Antibody

All the cells labeled with OKT4 monoclonal antibody had morphologic characteristics of lymphocytes, and their surface labeling was discontinuous. In two patients with pulmonary histiocytosis X, none of the Langerhans cells observed were labeled with OKT4 antibody. Other monoclonal antibodies, including OKT3 and OKT8, were used in a few samples. They did not label Langerhans cells, but only cells with the morphologic characteristics of lymphocytes.

Discussion

This study was performed in two steps, which resulted in complementary conclusions. The first step, using immunofluorescence techniques, showed that cells reactive with OKT6 monoclonal antibody were consistently present in BAL fluid from patients with confirmed pulmonary histiocytosis X. However, the number of these cells was very variable from case to case, and small numbers of reactive cells were also found in a few patients with conditions unrelated to histiocytosis X. The second step, using electron microscopic immunohistochemical techniques, provided evidence that all cells reactive with OKT6 antibody and revealed by peroxidase-bound immunoglobulin exhibited the general characteristics of Langerhans cells or histiocytosis X cells. In particular, most of these cells contained typical LC granules. In addition, they showed endogenous peroxidase activity of the cisternal type, a feature which is common to Langerhans cells and histiocytosis X cells.[22,25,26] Cells reactive with OKT6 were found in smaller numbers with the use of immunoelectron microscopy than with the use of immunofluorescence. Several factors may account for this difference.

First, the numbers of cells counted by immuno-



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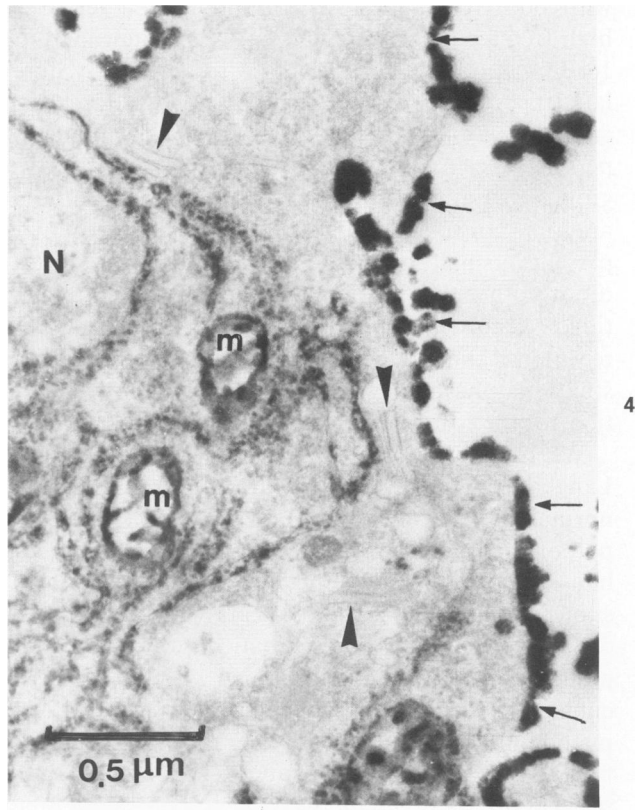

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Figure 3 – OKT6-positive cell showing DAB reaction product along the outer surface of the plasma membrane. *N*, nucleus. **Figure 4** – Detail of an OKT6-reactive cell with positive deposits on the plasma membrane (→). Notice the LC granules (➤) and the peroxidase activity in the RER and perinuclear cisterns. Mitochondrial membranes containing cytochrome oxidase are also positive. *N*, nucleus; *m*, mitochondria.

fluorescence and by immunoelectron microscopy were different: at least 1000 cells in the former and from 300 to 1000 in the latter, due to technical difficulties (many cells are lost during the different steps of immunoelectron microscopy).

Secondly, cells observed by light microscopy are entire cells, while the thin sections used for electron microscopy contain only slices of cells.

Thirdly, larger cells produce larger numbers of cell profiles in ultrathin sections than do smaller cells, and Langerhans cells are smaller than alveolar macrophages. Therefore, the percentage of Langerhans cells is usually underestimated when electron-microscopic techniques are used for cell counting[10]; however, ultrastructural study is indispensable in identifying specific cellular details such as LC granules.

From the two series of experiments, it seems reasonable to conclude that the cells that showed a reaction with OKT6 by the immunofluorescence technique are Langerhans cells (or histiocytosis X cells), as are those cells labeled with the same antibody with the use of the immunoperoxidase reaction. In patients with histiocytosis X, cells which were reactive with both techniques were found with a relatively high frequency (>3% of the total cell count in 10 of 16 BALs); however, 6 patients with apparently unrelated disorders also had a small percentage of reactive cells in BAL fluid. Thus, the degree of overlap did not permit a clear-cut separation between these two groups of patients. In this respect, three hypotheses are to be considered: 1) that Langerhans cells may be present in small numbers in normal lung and in conditions other than histiocytosis X; 2) that yet unidentified cells other than Langerhans cells possess OKT6 receptors; and 3) that some of the patients in our study may have had an unidentified pulmonary histiocytosis X (or bullous sequelae of the disease).

We have previously investigated the presence of Langerhans cells in lung tissue and BAL fluid from normal subjects and from patients with a variety of pathologic conditions.[27-29] In these studies, Langerhans cells were found in lung biopsies in 1 of 9 control subjects and in 20 of 160 patients with fibrotic lung disorders, including 13 of 56 patients with idiopathic pulmonary fibrosis, 2 of 9 patients with collagen vascular disease, 2 of 7 patients with hypersensitivity pneumonitis, each of 3 patients with end-stage fibrosis of uncertain cause, and 3 of 5 patients with bronchoalveolar carcinoma. Langerhans cells were not found in any of the 41 patients with sarcoidosis, the 35 patients with interstitial lung diseases associated with inhalation of inorganic dusts, the 7 patients with pulmonary lymphangioleiomyomatosis, or the 2 patients with chronic eosinophilic pneumonia. In the control patient, Langerhans cells were found, albeit very infrequently, between bronchiolar epithelial cells in a histologically normal area of tissue removed because of a fungal granuloma.

In patients with fibrotic lung disorders, Langerhans cells were found in the epithelial layer of bronchioles and alveoli containing proliferating epithelial cells, ie, either cuboidal epithelial cells of bronchiolar origin or type II alveolar epithelial cells. The motility of Langerhans cells apparently was restricted, because they were not found in the air spaces in any of the biopsy specimens, and they were not recovered from bronchoalveolar lavage fluid from any of the 97 patients studied. These results do not preclude the possibility that Langerhans cells very occasionally can escape into alveolar lumens and can be recovered in BAL, where they would be much more readily demonstrable by immunofluorescence or immunoperoxidase than by ordinary electron microscopy, particularly if such cells have only a small number of specific granules.

Consideration of the second possibility must take into account the fact, just mentioned above, that it may be very difficult to demonstrate LC granules in thin sections when such granules are very sparse in number. Such demonstration would require extensive serial sectioning of entire cells. Furthermore, a number of authors have suggested, on the basis of ultrastructural studies of skin[30-32] and lymph nodes,[33] that some Langerhans cells may go through a stage of development in which they truly lack LC granules.

The possibility of cross-reactions between OKT6 antibody and other cell types cannot be excluded. Nevertheless, Watanabe et al[34] have recently presented evidence suggesting that the presence of S-100 protein can be used to distinguish between two histiocytic cell lines: an S-100 protein-negative monocyte-macrophage system and an S-100 protein-positive T-zone histiocyte system, which includes Langerhans cells. At the present time, it has not been determined whether such a distinction can also be made on the basis of reactivity against OKT6 antibody.

In contrast with the labeling of Langerhans cells and HX cells that was obtained by OKT6, the results with other monoclonal antibodies, including OKT3, OKT4, and OKT8, were consistently negative. Of particular interest were the results with OKT4, because of a recent publication by Murphy et al,[18] showing that in cutaneous lesions of histiocytosis X in two children, the pathologic cells (HX cells) were weakly labeled by this monoclonal antibody, although epidermal Langerhans cells were not. With our material, we were not able to obtain the same reaction, all the cells labeled with OKT4 having the morphology of

lymphocytes. On the other hand, none of the Langerhans cells were labeled with OKT4. In patients with histiocytosis X, the percentage of OKT4-positive cells was very different from the percentage of OKT6-positive cells, suggesting that the same cells were not labeled by these two antibodies.[35] The discrepancy between our results and those of Murphy et al may have been due to the difference in material or to the technique used, which was not identical to ours; these authors used a PAP technique for immunoelectron microscopy, and they obtained a very weak reaction. We did not use the PAP method, because of some nonspecific labeling that we observed in the past with this method, while we were first studying the OKT6 reactivity of histiocytosis X cells in tissue samples and in cells obtained by BAL. Thus, we decided to use the two-step immunoperoxidase reaction on living cells, as described in the methods section of the present paper. This technique not only seemed to be more satisfactory, because it was more specific, but also preserved the cisternal peroxidase reactivity, which is helpful for the identification of Langerhans cells.

The differential cell count in BAL fluid is not changed to a great extent in pulmonary histiocytosis X, except for a small increase in the percentage of eosinophils present (from 3% to 7% usually). Histiocytosis X cells can be identified unequivocally only by transmission electron microscopy. In our experience, the most striking difference in the BAL fluid of control subjects and patients with histiocytosis X is that in the latter the total number of cells collected per milliliter of fluid is extremely high most of the time. This hypercellularity may result in part from the fact that most patients with histiocytosis X are smokers; nevertheless, these cell counts usually are higher than those in normal smokers. Therefore, the OKT6 reactivity, using both immunofluorescence and electron-microscopic immunohistochemical methods, should be tested in all hypercellular BAL fluids, especially if the clinical findings are compatible with the diagnosis of histiocytosis X. The immunofluorescence technique is performed very rapidly and could yield a diagnosis within a few hours of the lavage. However, further research is needed to establish more precisely quantitative criteria of positivity for the diagnosis of histiocytosis X, because of the small number of OKT6-positive cells detectable in BAL of patients with conditions other than histiocytosis X. Although the immunoelectron-microscopic detection requires more time and effort, it seems of great interest when used in combination with the immunofluorescence technique. These combined techniques eventually could substitute for open lung biopsy, at least when they give unequivocal results.

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