A Monoclonal Antibody Specifically Reactive to Human Langerhans Cells

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We prepared a mouse monoclonal antibody that reacts specifically to human Langerhans cells (LC). The protein recognized by this antibody was mainly in the membranes of Birbeck granules and related structures. Using this antibody, we could identify LC in various tissues; these cells were in the skin, stratified squamous mucosal epithelia, lymph nodes, and the thymus. The antibody did not react with monocytes, tissue macrophages, lymphoid dendritic cells, follicular dendritic cells, or interdigitating cells. The antigen purified with this antibody was a heterogenously glycosylated protein of M_r ~40,000 without interchain disulfide bonds. This antibody may be useful for identifying LC in various human tissues with or without abnormalities, and for studying the origin and fate of Birbeck granules of LC. *J Invest Dermatol 87:602–607, 1986*

Langerhans cells (LC) were reported to function as the antigen-presenting cells in the skin. They express immune response gene-associated antigens on their cell membranes [1,2]. These cells seem to mediate contact sensitivity in vivo [3–6] and the antigen-specific response of T lymphocytes in vitro [2]. Langerhans cells also have surface receptors for the Fc portion of immunoglobulin G (IgG) and for the third component of complement, C3 [7]. They originate from the bone marrow [8,9], but details about the precursors of LC are not known.

Langerhans cells in the epidermis were first identified by their dendritic features [10] and later by the presence of Birbeck granules in their cytoplasm [11]. Epidermal LC are dendritic in shape [12]. Dendritic cells exist in many tissues, including those of the skin [13], spleen, lymph node [14,15], thymus [16], kidney [17], and heart [13]. However, LC are different from other dendritic cells, such as the lymphoid dendritic cells described by Steinman et al [14,15]. The only reliable morphologic way to identify LC is by the detection of their Birbeck granules, which can be done only under the electron microscope. Membrane-bound ATPase is often used as a marker for these cells [18]. S-100 antigen has been shown to exist in LC [19]. Langerhans cells can also be detected in skin using monoclonal antibodies such as OKT6 [20,21], NA1-43 [22], and V1-CY1 [23]. However, these antibodies are not specific; OKT6 also reacts with thymocytes, as does NA1-43, and V1-CY1 also reacts with human B cells as well as monocytes.

Here, we report a mouse monoclonal antibody that reacts specifically with human LC. In an immunoelectron microscopic study we showed that this antibody bound to Birbeck granules in LC. Using this antibody, we found LC in various organs. Then, we isolated and characterized the specific antigen reactive to this monoclonal antibody.

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Abbreviations:
LC: Langerhans cells
NP-40: Nonidet P-40
PB: phosphate buffer
PBS: phosphate-buffered saline
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TNP: trinitrophenyl

MATERIALS AND METHODS

Preparation of Monoclonal Antibodies Skin fragments were taken from specimens of resected breast. The skin was immersed for 2 h at 4°C in Dulbecco's modified Eagle's medium supplemented with penicillin (200 U/ml), streptomycin (200 μg/ml), and fungizone (5 μg/ml), cut into pieces of about 10 × 5 mm, and incubated for 20 h at 4°C with Dispase (1000 U/ml; Godoshusei, Tokyo) in the modified Eagle's medium supplemented with 20% fetal calf serum. The epidermis was stripped using forceps by the method of Kitano and Okada [24]. Single cells from the epidermal sheets were obtained by treatment first with 0.02% EDTA in phosphate-buffered saline (PBS) and then with 0.25% trypsin in PBS, for 5 min each. The cells were washed and used for the immunization. Some of the cells were stored at −80°C in fetal calf serum containing 10% DMSO for later immunization.

Seven-week-old BALB/c mice were immunized i.p. 3 times with 2×10^7 cells in 1 ml of PBS every 2 weeks. Three days after the last immunization, spleen cells from the mice were fused with mouse myeloma cells (X63Ag8.653) in a 50% solution of polyethylene glycol 1500. The fusants were incubated in flat-bottomed 96-well microculture plates (Coster, Cambridge, Massachusetts) with irradiated thymocytes of BALB/c mice, and the fused cells were selected in HAT medium. After 14 days of culture, 0.1 ml of medium obtained from each well containing colonies was assayed. Hybrid cells secreting antibodies of interest were cloned by the limiting-dilution method. To determine the immunoglobulin subclass of the antibodies, we used the Ouchterlony double-immunodiffusion method with rabbit monospecific antisera to mouse immunoglobulins (Miles, Elkhart, Indiana) [25]. To obtain a large quantity of antibody, hybrid cells were injected into the peritoneal cavity of BALB/c mice given pristane (2,6,10,14-tetramethylpentadecane, Aldrich Chemical Co., Milwaukee, Wisconsin). IgG was purified from ascites by ammonium sulfate precipitation and chromatography on protein A-Sepharose

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CL-4B columns (Pharmacia Fine Chemicals, Uppsala, Sweden) [26]. Purified immunoglobulins were stored at either 4° or −80°C in PBS containing 10 mM sodium azide.

Staining and Observations

Materials: For immunofluorescence or immunoperoxidase staining, we used normal human skin and other organs from autopsies done at Kyoto University Hospital. Bone marrow cells were obtained from a patient with skin amyloidosis without any systemic involvement. Peripheral blood cells were obtained from healthy donors.

Indirect Immunofluorescence with Monoclonal Antibodies: Normal tissues were frozen in acetone at −70°C and stored at −80°C until use. Cryostat sections 4 μm thick were prepared, overlaid with culture supernatant from hybrid cells, incubated in a moist chamber for 45 min at room temperature, and washed twice with PBS. These sections were treated for 45 min at room temperature with fluorescein-labeled rabbit antibodies against mouse immunoglobulins (Cappel Laboratories Malvern, Pennsylvania). After being washed with PBS, the sections were examined under a Nikon epifluorescence microscope (Tokyo, Japan).

Indirect Immunoperoxidase Staining: Fresh specimens of the tissue were cut into small pieces using a razor blade, immediately fixed with 5% paraformaldehyde in water for 2 h at 4°C, immersed overnight in PBS containing 10–20% sucrose and 10% glycerin at 4°C, and embedded in Tissue-Tek II O.C.T. compound (Miles Laboratories, Naperville, Illinois). Serial sections 10 μm thick were mounted on albumin-coated glass slides, and air-dried. They were stained with mouse monoclonal antibody (2 μg/ml) as the first antibody, and with peroxidase-conjugated goat antibodies against mouse immunoglobulins (25 μg/ml; Cappel Laboratories) as the second antibody. Control staining was with mouse anti-trinitrophenyl (anti-TNP) monoclonal antibody (IgG_1) [27] (20 μg/ml) as the first antibody. After being fixed again with 1% glutaraldehyde in PBS, the sections were treated with a solution of diaminobenzidine in PBS for 30 min and then with a mixture of diaminobenzidine and hydrogen peroxide for 2 min.

For immunoelectron microscopic studies, these stained sections were washed with PBS, postfixed with 1% osmium tetroxide in water for 1 h, dehydrated in increasing concentrations of ethanol, and embedded in polyethylene capsules containing epoxy resin. Ultrathin sections were stained with uranyl acetate and observed under an H-300 electron microscope (Hitachi, Japan). We chose one monoclonal antibody for the study described here. This antibody we named Lag for Langerhans cell granules, for reasons given later.

Immunoadsorbent, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), and Immunoblotting

Immunoadsorbent Isolation of Antigen Recognized by Lag: Purified Lag (IgG_1) or affinity-purified mouse anti-TNP monoclonal antibody (IgG_1) was immobilized in 2 mg/ml gel of Sepharose CL-4B (Pharmacia) activated with cyanogen bromide. The epidermal sheets that had been stored frozen in liquid nitrogen were homogenized at 0°C in the lysis buffer containing 50 mM phosphate buffer (PB), pH 7.3, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 (NP-40), and 2 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 3000 rpm for 5 min at 4°C, and the supernatant was applied to the anti-TNP antibody Sepharose CL-4B column. The fraction that passed through was incubated at 4°C for 2 h with 0.2 ml of the Lag-Sepharose CL-4B with constant shaking. The Lag-Sepharose CL-4B was washed by centrifugation with 50 mM PB (pH 7.3) containing 150 mM NaCl, 5 mM EDTA, and 0.1% NP-40, and then with 10 mM PB (pH 7.3) containing 50 mM NaCl and 0.1% NP-40. The antigen was eluted with 50 mM citric acid containing 0.1% NP-40. The eluate was neutralized with 1.5 M Tris-HCl buffer, pH 8.6, unless otherwise noted.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Immunoblotting: The prepared sample was run, after addition of 1% SDS and 0.1 M dithiothreitol, in 12% polyacrylamide gels containing 0.1% SDS (13 cm × 14.5 cm, 1 mm thick) with 3% stacking gels [28,29]. Proteins were stained after SDS-PAGE by the silver-staining method (Daiichi Chem., Osaka, Japan) [30]. In some cases, the proteins in the gel were transferred to nitrocellulose paper (Schleicher and Schuell, Inc., Keene, New Hampshire) using a Trans-Blot Cell (Bio-Rad Laboratories, Richmond, California) with 25 mM Tris, 192 mM glycine, and 20% methanol overnight at 7 V/cm [31]. These blots were incubated with PBS containing bovine serum albumin (10 mg/ml), Lag in culture medium, and then with peroxidase-conjugated goat IgG against mouse immunoglobulins diluted 1:200 in PBS containing bovine serum albumin. The reaction was visualized with 4-chloro-1-naphtol (Wako Pure Chemical Industries, Osaka, Japan).

Two-Dimensional Gel Electrophoresis: The antigen purified by affinity chromatography, in 0.15 ml of citric acid, was treated with 0.3 ml of a solution of neuraminidase (EC 3.2.1.8, 1 U/ml, Nakarai Chemicals, Ltd., Kyoto, Japan) in 0.1 M citrate buffer, pH 5.5, at 37°C for 1 h. The solution was neutralized with 1.5 M Tris-HCl, pH 8.6, and 2 ml of the buffer for lysis was added. It was applied to Lag-Sepharose CL-4B column, and the antigen was eluted with 0.5 M NH_4OH containing 0.1% NP-40. After the sample was freeze-dried, it was subjected to 2-dimensional electrophoresis with pI markers (Pharmacia) according to the method of O'Farrell et al [32]. Ampholine (pH 3.5–10, LKB, Sweden) was used as the pH carrier. The proteins in the gel after SDS-PAGE were visualized by silver staining.

RESULTS

Monoclonal Antibody Against LC Of the 283 culture supernatants assayed, 95 reacted to normal human epidermis. One specifically bound to cells with dendrites scattered in the suprabasal epidermis and hair follicles (Fig 1). Some cells were also stained in the upper dermis. Indirect immunoperoxidase assay showed that this monoclonal antibody reacted with some cytoplasmic components of suprabasal epidermal cells with dendrites (Fig 2). Neither keratinocytes nor melanocytes were stained. Tissue sections treated with anti-TNP monoclonal antibody as the first antibody were unstained.

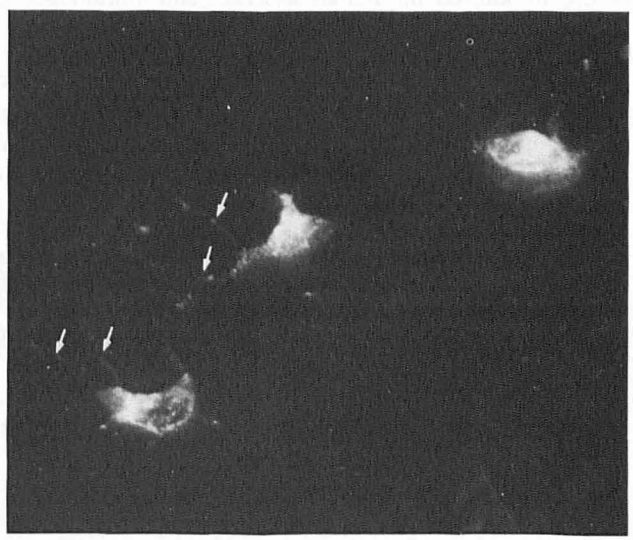

Figure 1. Indirect immunofluorescent staining of normal human skin. Frozen human skin was sectioned perpendicular to its surface. These sections were reacted with culture supernatant from hybrid cells and then with fluorescein-labeled rabbit antibodies against mouse immunoglobulins. Suprabasal cells with many dendrites (*arrows*) were stained. × 1240.

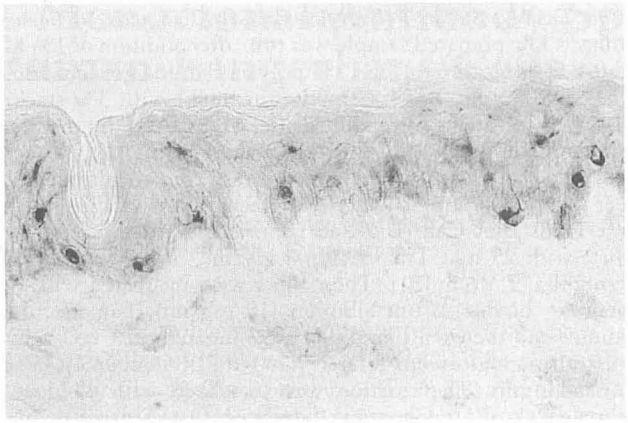

Figure 2. Indirect immunoperoxidase staining of normal human skin. Some cells with dendrites were stained with culture supernatant from the hybrid cells, followed by peroxidase-conjugated goat antibodies against mouse immunoglobulins. The peroxidase reaction was observed mainly in the inside of the cell body, not on its cell surface. × 280.

Immunoelectron microscopic observation showed that the reaction products of peroxidase were generally very close to the rod-shaped and racket-shaped structures, or to vacuoles of the nonkeratinocytic cells of the epidermis (Fig 3). Some of these rod-shaped structures had striated lamellae, and we identified them as Birbeck granules. The reaction products were clearly seen on the cytoplasmic side of the rod and the bulb portions of these granules, but not inside the granules. Some vacuoles containing reaction products were polygonal or irregularly shaped and were morphologically distinguished from the round portion of Birbeck granules, judged from their size. We also found small vacuoles and Birbeck granules with reaction products in cross-sections of dendrites of the cell. There were no reaction products on or near the surface membrane, mitochondria, or lysosomes. The reaction products were also present diffusely in the cytoplasmic space near the Birbeck granules. These results indicate that the monoclonal antibody reacted with Birbeck granules and the related structures of epidermal LC, for which reasons we have named this antibody Lag.

The reactivity of the antibody to the skin of other animals was examined by indirect immunofluorescence and immunoperoxidase staining. The pattern of staining of skin from African green monkey was similar to that from human skin. The antibody did not react at all with skin from mice, guinea pigs, rabbits, or pigs.

Detection of Lag-Positive Cells in Tissues Other Than the Skin We examined the reactivity of the monoclonal antibody (Lag) with cells in various other normal human organs. Lag-positive cells were detected in the stratified squamous epithelia of the lip, tongue, buccal mucosa, esophagus, vagina, and uterine cervix. The immunoelectron microscopic observation ascertained that those cells reacting with Lag in these organs were LC having Birbeck granules, and that most of the reaction products

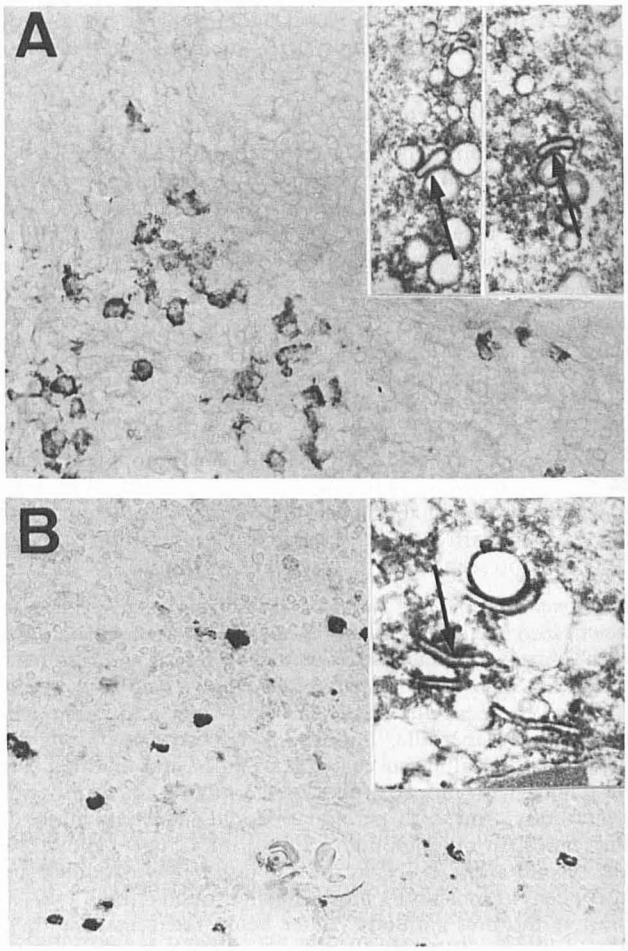

Figure 4. Indirect immunoperoxidase staining of Lag-positive cells in various human organs. *A*, In the lymph nodes, reactive cells were seen on the paracortical area and in the marginal sinuses. *B*, In the thymus, they were near the Hassal's corpuscles and within the interstitial connective tissue. These reactive cells in the lymph node and thymus are not clearly branched, but they have the Birbeck granules around which the products of the peroxidase reaction were found (*arrows*). (*A*) × 270; (*inset*), × 27,000. (*B*) × 270; (*inset*), × 27,000.

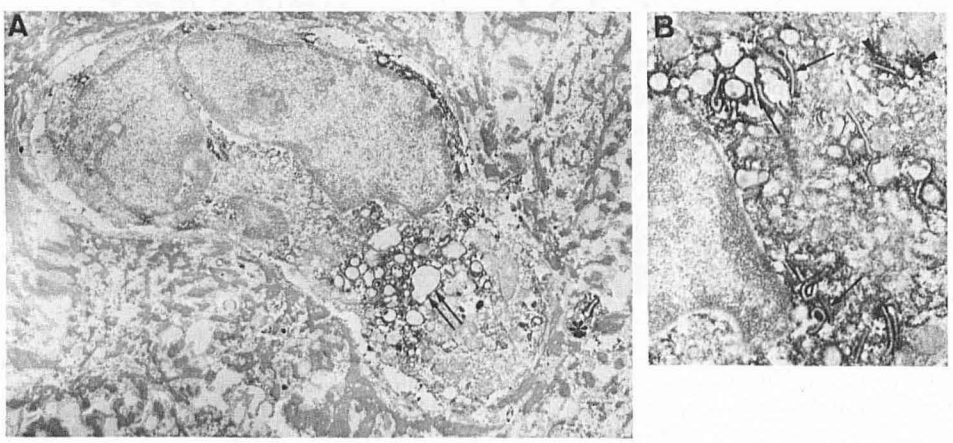

Figure 3. Immunoelectron micrograph of a cell reactive to Lag. The cell determined by Lag shows characteristic feature of LC with the cytoplasmic processes (*asterisk, A*) in normal human skin. The reaction products are mainly very close to the membrane of Birbeck granules (*arrows, B*) in the cell body, and of the irregular or polygonal vacuoles (*double arrows, A*) that are morphologically distinguishable from the round portion of the granules. The reaction products are also present diffusely near Birbeck granules (*arrowheads, B*). (*A*) × 14,800, (*B*) × 27,000.

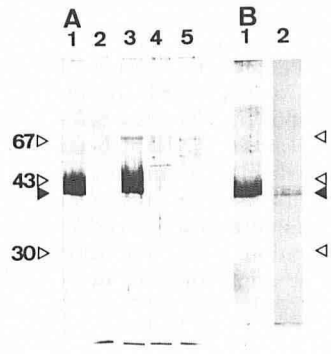

Figure 5. *A*, SDS-PAGE profile of the isolated antigen. The antigen isolated from human epidermal lysate with Lag-Sepharose has a broad band at the M_r ~40,000 and a minor band at M_r ~64,000 under reducing conditions (*lane 3*). Under nonreducing conditions, the antigen has a major band at ~40,000 (*lane 1*). Eluate of the lysate of human liver from Lag-Sepharose does not have any distinct protein band (*lane 5*); neither does the antigen-free lysis buffer eluated from Lag-Sepharose (*lane 2*), or the human epidermal lysate eluted from anti-TNP antibody Sepharose (*lane 4*). SDS-PAGE was performed in 12% acrylamide gel under reducing conditions in lanes 2–5, and nonreducing conditions in lane 1. Proteins were visualized by silver staining. *B*, Immunoblot profile of the purified antigen. The antigen isolated from a lysate of human epidermal cells was electrophoresed. The separated proteins were transferred to nitrocellulose paper, and stained with Lag as the first antibody and the peroxidase-labeled second antibody. Lag reacted to isolated macromolecules electrophoresed at M_r ~40,000 (*lane 2*). The antigen isolated in this part of the experiment has one broad band at M_r ~40,000 after SDS-PAGE (*lane 1*). It was visualized by silver staining.

of peroxidase were distributed on Birbeck granules. Reactive cells were not found in the transitional epithelia of the bladder and the columnar epithelia of the intestines.

In the lymph nodes, most cells that reacted with Lag were in the paracortical area and some were in the marginal sinuses (Fig 4*A*). In the thymus, such cells were found both in the medulla and the interstitial connective tissue (Fig 4*B*). In other areas of the lymph nodes and thymus, there were no reactive cells. The cells reactive with Lag in the lymph nodes and thymus were not so clearly branched as were LC in the epidermis. Under the electron microscope, however, these cells had Birbeck granules containing reaction products of peroxidase, and also some vacuoles containing reaction products, as in the skin. Lag did not react with some nonlymphoid cells lacking Birbeck granules but having vacuoles of irregular or polygonal shapes, which lay scattered in the lymph node or thymus.

In the spleen, kidney, pancreas, or bone marrow, we did not find any cells that reacted with Lag. In the lung, liver, or heart, in which tissue macrophages have been reported, reactive cells were absent. Lag did not react to peripheral blood cells.

Antigen Recognized by Lag The epidermal sheets were lysed, and the antigen was purified by affinity chromatography with Lag-Sepharose. The isolated antigen displayed a broad band centered at M_r ~40,000 after SDS-PAGE (Fig 5*A*). The electrophoretic patterns were similar when SDS-PAGE was carried out under the reducing and nonreducing conditions, which indicates that interchain disulfide bonds were absent. There was a minor band at M_r ~64,000 inconsistently. The eluate of liver lysate from Lag-Sepharose did not display any distinct protein band after SDS-PAGE. Similarly, no distinct band was seen with the eluate of epidermal lysate from anti-TNP antibody-Sepharose nor with the lysis buffer without antigen that was eluted from Lag-Sepharose.

Various amounts of lysate of the epidermal sheets were electrophoresed, and the proteins were transferred to nitrocellulose paper. Staining on this paper by Lag and the peroxidase-labeled second antibody was negligible (data not shown). When the antigen isolated as above was transferred after SDS-PAGE, an indirect immunoperoxidase assay was performed (Fig 5*B*). Lag was found to react with the isolated molecule (M_r ~40,000). This result confirms that the macromolecule isolated was the antigen recognized by Lag. It also indicates that the antigenic determinant is intact after SDS-PAGE, and that the seeming lack of antigen in the blotted proteins of the whole lysate was caused by the ratio of antigen to that of proteins from whole epidermal sheets being too small.

The broadness of the band in the SDS-PAGE profile in Fig 5 suggested that the antigen recognized by Lag was glycosylated. To test this hypothesis, the antigen was further characterized by 2-dimensional PAGE, in which the first dimension was performed under nonequilibrium conditions to retain basic as well as acidic proteins. The purified antigen had several components similar in molecular weight but different in isoelectric point from pI 4.7–6.5 (Fig 6*A*). Digestion of the antigen with neuraminidase

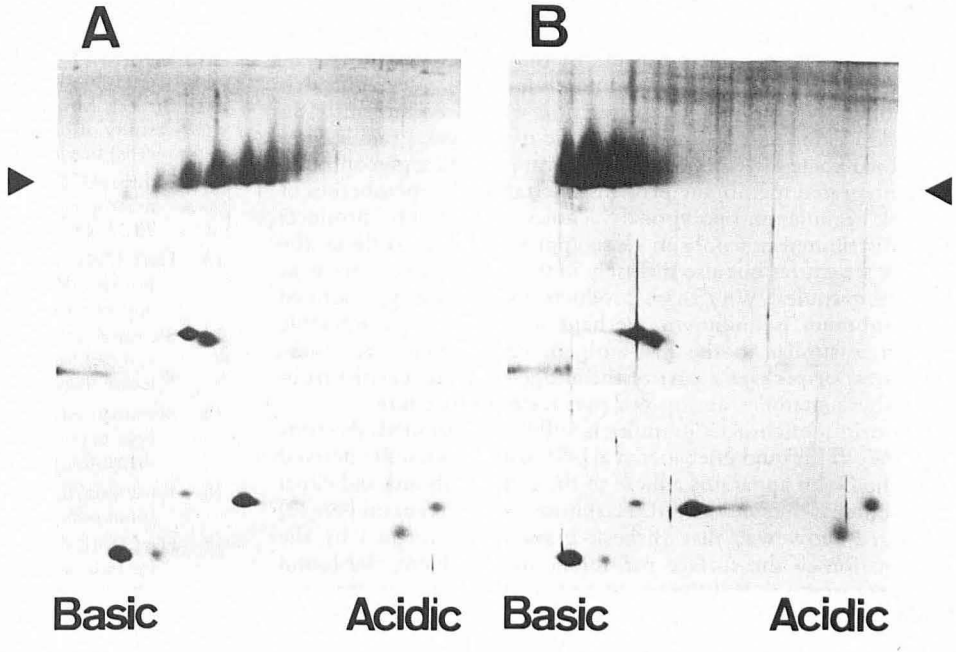

Figure 6. Analysis of the antigen by 2-dimensional PAGE. The antigen, purified from a lysate of human epidermal sheets with Lag-Sepharose, was subjected to 2-dimensional electrophoresis and visualized by silver staining. *A*, The isolated antigen without further treatment. *B*, Materials digested with neuraminase (0.7 U/ml at 37°C for 1 h). Note that the neuraminidase treatment results in a shift in the protein spots to the basic side of the gel and a decrease in their number. The spots at lower molecular weights are pI marker proteins.

resulted in a shift of the components to more basic points (pI 5.9–6.7) and in the decrease of the number of components (Fig 6*B*). These results indicate that the antigen purified with Lag was heterogeneously glycosylated, and that the heterogeneity of the isoelectric point of the antigen was mainly due to the sialic acid in its sugar chains.

DISCUSSION

Langerhans cells are considered to play an important role in the immunologic reactions in the skin; they are present in the epidermis, skin appendages, and certain stratified squamous epithelia [12]. Silberberg et al [33] reported that such cells from the epidermis seemed to migrate to the dermis, and then to the paracortical area of regional lymph nodes, after contact sensitization. Langerhans cells have been observed in the medulla of the thymus [18]. Knowledge of the distribution of these cells would help us to understand how they participate in the immunologic defenses of organisms.

Birbeck granules are the only morphologic marker for LC [11] and effort is required to identify LC by detection of Birbeck granules using electron microscopy. Here, we describe a monoclonal antibody, Lag, that reacts specifically with these granules and related structures in normal human skin. Using Lag, LC in various organs can be identified easily using light microscopy. We found these cells in several noncutaneous tissues: the stratified squamous mucosal epithelia, lymph nodes (paracortical areas and marginal sinus), and thymus (medulla and interstitial connective tissue). Lag did not react with cells in the liver, heart, lung, spleen red pulp, or peripheral blood in which tissue macrophages or monocytes are thought to be present. No Lag-positive cells were found in the white pulp of the spleen or in peripheral blood where there were lymphoid dendritic cells. Lag did not react with the cells in lymphoid follicles in which follicular dendritic cells were present. These results show that Lag does not react with monocytes, tissue macrophages, lymphoid dendritic cells [14,15], or follicular dendritic cells [34]. Interdigitating cells were reported in the medulla of the thymus and in the thymus-dependent paracortical area of the spleen and lymph nodes. Typical interdigitating cells are similar in morphology and distribution to LC, but they lack Birbeck granules [35]. We conclude that Lag does not react to typical interdigitating cells. Birbeck granules are observed frequently in cells that resemble interdigitating cells in the thymus [36]. It is not clear whether LC and interdigitating cells represent different cell populations. It is probable that LC can be classified precisely and specifically with the monoclonal antibody, Lag.

The antigen recognized by Lag is a glycoprotein with M_r ~40,000, without interchain disulfide bonds. The sugar chain of membrane glycoproteins is generally outside of the surface membrane or inside the intracellular organelle membrane [37]. We found that the products of the peroxidase reaction were predominantly on the cytoplasmic side of the membrane of Birbeck granules and polygonal vacuoles. These results indicate that this glycoprotein is an integrated membrane protein penetrating the membranes of Birbeck granules and polygonal vacuoles. The reaction products were distributed not only in close approximations to these distinctive structures but also diffusely in the cytoplasmic space near Birbeck granules. Why these products are not always confined to membranes is unknown. Perhaps molecules with antigenic properties similar to the Lag-antigen were present near these structures, or perhaps a part of the antigen was dissociated from the Birbeck granules during our preparation procedure.

The origin of Birbeck granules is still being disputed. According to Wolff [38] and Niebauer et al [39], Birbeck granules derived from the Golgi apparatus adhere to the cell membrane and expel their contents outside the cell. Hashimoto and Takahashi [40–43] suggested, however, that Birbeck granules are formed by the invagination of the surface membrane of LC. Here, we found that these granules had the specific membrane glycoprotein, the Lag-antigen, which was not found in the surface membranes or lysosomes. As the sialic acid residue was supplemented to the sugar chains of glycoproteins in the Golgi apparatus [44], it is obvious that the marker protein of Birbeck granules, Lag-antigen, originates from the Golgi apparatus. Irregular or polygonal vacuoles did have this antigen. These vacuoles, which can be morphologically distinguished from the bulb part of Birbeck granules, may be the related structures of these granules during formation or degradation. It remains to be investigated in which stage the Lag-antigen is integrated into the Birbeck granules during their formation or maturation. This monoclonal antibody may be useful in studying the origin and the fate of the Birbeck granules in LC.

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