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Monoclonal antibody against histiocytosis X cells

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Summary. Monoclonal antibody against histiocytosis X cells (HXCs) was established. The antigen was the cell membrane of HXCs from the submandibular lesion of a 63-year-old man who had been dignosed as an adult type of histiocytosis X (HX) and whose HXCs had numerous Birbeck granules (BGs). The obtained monoclonal antibody, named MI1, reacted with the antigenic cell membrane of HXC. Immunoblotting showed that MI1 bound to the cell membrane of 28500 mw. MI1 also reacted with interdigitating reticulum cells (IDCs) in the tonsil and Langerhans cells (LCs) in the epidermis. MI1 reacted with the BGs which connected to the cell membrane, but not with those located near the nucleus.

Key words: Histiocytosis X, Birbeck granule, Monoclonal antibody

Introduction

HX was first described by Lichtenstein (1953), who said that Letterer-Siwe, Hand-Schüller-Christian disease and eosinophilic granuloma of bones were understood to be a single disease entity, of which the clinical features vary from each other according to the organs involved. In the affected lesion, there are many HXCs with a variable number of membranous granules which were found by Basset et al. (1972) in the bone of eosinophilic granuloma, and which are morphologically identical to the BGs in the epidermal LCs (Birbeck et al., 1961). It is said to be virtually the sole marker distinguished the HXC from other histiocytes. BGs are, also found in the cells taken from the lesions of dermatophathic lymphadenitis (Raush et al., 1977), mycosis fungoides (Lowen and Lewis, 1976), adult T-cell leukemia (Shamoto, 1983), and malignant histiocytosis (Imamura et al., 1971).

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Many workers have documented the relationship between the LCs and the HXCs. The existence of BGs, as well as other light and electron microscopical similarities, suggests that both cells are identical, or that the HXCs are suspected to be a pathological condition of the LCs. There are, indeed, some investigators who propose that eosinophilic granuloma should be renamed «Langerhans cell proliferative disorder» (Telpitz and Gross, 1975) or «Langerhans cell granuloma» (Wolff, 1972) aside from any aspect of its neoplastic nature.

HXCs possess Ia-like antigens (Thomas et al., 1982; Favara et al., 1983) which are the markers for monocyte/macrophage. This is true of LCs (Stingle et al., 1977; Rowden et al., 1977). HXCs demonstrate a common S-100 protein (Nakajima et al., 1982) on paraffin sections, but they show no activity for lysozyme (Burgdorf et al., 1981) or α_1-antichymotrypsin (α_1-ACT), as do LCs (Beckstead et al., 1984). A recent study revealed that HXCs reacted with anti-T4 (helper/inducer T-cell) (Wood et al., 1983), and T6 (thymocyte/LC) (Murphy et al., 1983).

An immunohistochemical phenotype of the LCs is an analogue to the HXCs, expect for T4 which is less possitive in the LCs (Favara et al., 1983; Harrist et al., 1983a). This is almost true of IDCs.

Materials and methods

Two cases of HX were examined. One was from a 63-year-old man whose palpeblar lesion had been diagnosed as an adult type of HX. (Feuerman and Sandbank, 1976). The other was from 6-year-old female who was diagnosed as having eosinophilic granuloma.

Biopsy specimens of HX were obtained from submandibular lesion involved in the former and gingival lesion involved in the latter. They were prepared for fresh-frozen and periodic acid-lysine-paraform (PLP)-fixed sections.

The tissue from an adult type was also used as a source of the antigen for the establishment of

monoclonal antibodies against the HXCs.

1. Establishment of monoclonal antibody against HXC (MI1)

Minced under sterile conditions, the suspended cells from the submandibular tissue of the adult type were thrown into distilled water (hypotonic shock method) and centrifuged. Sixty μg of the antigenic membrane fraction were dissolved in 1 ml of saline, emulsified with an equal volume of Freund's complete adjuvant and injected intraperitoneally into three BALB/c female mice. Two weeks later, this immunization was followed by a boosting of 500 μg of antigen into the same site.

Three days later, 10^8 spleen cells of these immunized mice were fused with 10^7 P3UIG myeloma cells using 50% polyethylene glycol as a fusing agent. The cell mixture was seeded to the 96 well plates and after being cultured for 24 hours, the medium was replaced by hypoxanthine, aminopterin, thymidine (HAT) selective medium and the cell mixture was further cultured for 1 week. The medium was then replaced by hypoxanthine-thymidine (HT) medium. About 2 weeks later, the HT medium was replaced by RPMI 1640 medium (GIBCO) containing 20% fetal calf albumin (FCS), and the supernatant from wells containing colonies of hybrid cells were tested for antibody production by radioimmunoassay (RIA) (Stocker and Heusser, 1979).

The hybridomas, showing clear activities, were cloned by limiting dilution on BALB/c spleen feeder cells in a microtiter plate. The established hybridoma cell lines were maintained *in vitro* culture.

2. Electrophoresis and immunoblotting

The molecular weight of antigenic protein for the MI1 was determined by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 12% gel containing 8 M urea according to the method of Weber and Osborn (1969).

Calcium-binding protein, such as calmodulin, parvalbumin and oncomodulin were removed by boiling for 5 minutes in Tris-HCl buffer containing 2% SDS and 5% 2-mercaptoethanol (2ME).

Antigenic proteins were applied at 1 μg per well on the gel. The gel was stained with 0.1% Coomasie brilliant blue solution containing 45% ethanol and 10% acetic acid, and kept overnight, then bleached with destaining solution containing 25% ethanol and 10% acetic acid.

The proteins were transferred to a nitrocellulose membrane in the electrophoretic substrate composed of 0.3% Tris, 1.44% glycine and 20% ethanol. The localization of the protein was detected by the indirect immunoperoxidase method. Transferred membrane was soaked in PBS containing 3% Bovine serum albumin (BSA) for 3 hours. After washing in PBS which contained 0.005% Tween 20, the membrane was overlayed by MI1 for 1.5 hours. The brief wash followed by incubation with peroxidase-conjugated anti-mouse IgG (DAKO). The enzyme reaction of the washed membrane was developed with DAB-H_2O_2 containing 0.03% DAB. Cytochrome complex was used for the marker protein. The omission of MI1 was performed as a negative control.

3. Determination of immunoglobulin class of MI1

This was carried out according to Ouchtalony's method. Monoclonal Igs were anti-mouse IgM, IgG1, Ig2a, IgG2b and IgG3. PBS was used as a negative control.

4. Light and electron microscopical preparation

Normal skin, tonsil, gingiva and lymph node as well as HXC lesion were prepared.

Each biopsy specimen was fixed with 10% formalin and embedded in paraffin. The paraffin sections were dewaxed and stained with Haematoxylin-Eosin (H-E), Masson trichrome, periodic acid Schiff (PAS), silver and phosphotungstic acid Schiff (PTAH). The sections were immunohistochemically reacted with antibodies to lysozyme, α_1 -antitrypsin (α_1-AT) α_1 ACT, S-100 protein, ferritin, factor VIII and immunoglobulin (Ig) series following Taylor's method (1975). Enzyme activities of ATPase and ACPase were performed with azo dye solution.

The section were stained with ANS (Imai et al., 1985) which is a polyclonal antibody (IgG) bound to the membrane protein of 17500 mw. of HXCs.

Some of the remaining tissue was fixed with a PLP solution containing 4% formaldehyde for 6 hours and soaked in phosphate buffer saline (PBS), pH 7.4, with grading sugar every 6 hours.

Part of the tissue was frozen in an OCT compound (Miles) and stored at -70º C until sectioning. The remnants was chopped into 1 mm cubes and fixed with 1.25% glutaraldehyde for 4 hours for ultrastructural examination.

The PLP-fixed tissue was cut into a thickness of 7 μm with a Cryostat, and air-dried. The sections were washed in PBS, and endogenous peroxidase was blocked with 0.1 M $NaIO_4$ for 15 minutes. After adequately rinsing in PBS, the residual protein-binding sites were treated with 10% normal swine serum in PBS. Overnight incubation with 1st antibodies followed by rinsings and overlaying with swine anti-rabbit IgG (DAKO) for 3 hours. Following this, additional washing in PBS and labelling with peroxidase anti-peroxidase (PAP) for 3 hours were carried out. After washing in hydroxymethol amino-methane (Tris)-HCl buffer, solution which contained 0.03% DAB and 0.005% H_2O_2 in 0.05 M Tris-HCl buffer. Methylgreen and occasional haematoxylin were used for counter staining.

In the case of the monoclonal antibodies, after blocking endogenous peroxidase, succesive incubations were done with normal sheep serum for 30 minutes, and

followed by the antibodies at 4º C overnight, and then by peroxidase-conjugated sheep anti-mouse Ig (x 30, Amersham). Anti-T3, T4, T6, T8, Ia1 and M1 antibodies were used for the first antiserum.

For the immunohistochemical study of MI1, Cryostat sections were obtained from fresh-frozen and PLP-fixed tissues. Hybridoma supernatant was used for the first antibody.

In case of immunoelectron study, the sections which reacted in the DAB-H_2O_2 solution were succesively washed and fixed in 1% glutaraldehyde for 15 minutes, and post-fixed in 2% OsO_4 for 30 minutes. This was followed by alcohol dehydration and embedding by the inverted gelatin capsule method in an Epon-methyl nadic anhydrite (MNA) mixture.

5. Electron microscopical preparation

After rinsing with 0.1 M cacodylate buffer, the remaining blocks of biopsied tissue were post-fixed with 2% OsO_4 containing 0.1 M cacodylate buffer. Dehydration in graded alcohol was followed by Epon-Araldite mixture embedding.

Semithin sections sliced by an LKB ultramicrotome were stained with toluidine blue for light microscopical observation. Ultrathin sections were stained with uranyl acetate and lead citrate and were observed with a Zeiss EM109 electron microscopy.

Results

1. Light microscopical findings of HXCs

The lesions of the two patients showed the typical features of the HX (Fig. 1). The majority of the infiltrating cells were HXCs, which averaged 20 μm in diameter and irregular eccentric nuclei. HXCs were mostly accompanied by a variable number of eosinophils, plasma cells, lymphocytes and foam cells.

The nuclei of the HXCs demonstrated indented or lobulated appearance. There were sporadic mitotic figures. None of the HXCs showed phagocytosis. They were negative for PAS and PTAH. The lesions showed no fibrosis, and few proliferation of reticulin fibres.

HXCs showed activity for ATPase, but not for AcPase.

2. Electron microscopical findings of HXCs

Each lesion accumulated numerous HXCs, and showed similar features except for the variability of collagen fibres and infiltrating eosinophils.

HXCs showed a rough surface with a lot of digitating processes, relatively electrolucent cytoplasm and a large indented nuclei with finely dispersed chromatin and without thickening of the nuclear membrane (Fig.2). There were a great number of swollen mitrochondria and dilated endoplasmic reticulum.

Intracytoplasmic vacuoles varied with the cases, and no phagosome was found. There were many free ribosomes. There were plenty of electrodense myelinosomes in some HXCs (Fig. 2).

Some of the HXCs displayed a small number of centromeres in their cytoplasm. There were few microfilaments and microtubules.

There were variable numbers of BGs (Fig. 3) in the HXCs of each case. BGs appeared to be composed of 5 laminar structure, about 430 Å in width, ranging up to 1 μm in length, with two limiting membranes, 50 ~ 60 Å in width, and the central dot line of small particles, which were approximately 50 Å in diameter, were spaced regularly at 90 Å distances.

BGs were found not only in the Golgi area but also in the weighborhood of the cell membrane. Some BGs connected to the cell membrane and some opened in both ends. Racket-shaped ends of BGs were often crowded in the Golgi area (Fig. 3).

3. Immunohistochemical study of the HXCs, LCs, and LDCs (Table 1)

The HXCs from an adult type showed positivity for S-100 protein and ANS. S-100 protein reacted with the cytoplasm and the nucleus as well as with the cell membrane. On the other hand, ANS reacted with the cytoplasm and the cell membrane with the T-zone histiocyte in the tonsil and the lymph node. HXCs were negative for lysozyme, α_1-AT and α_1-ACT, for which phagocytic mononuclear cells and eosinophils were positive. There were variable numbers of Ig-positive plasma cells, whereas HXCs indicated negative for Igs. HXCs reacted with anti-Ia1 and T6, but not with T3, T8 and M1. The activity with T4 was partial and weaker than that for T6. The LCs in the epidermis and the IDCs in the lymph node reacted for these monoclonal antibodies like the HXCs, except for T4. T4 showed less positive in the LCs and LDCs.

4. Establishment of monoclonal antibody (MI1)

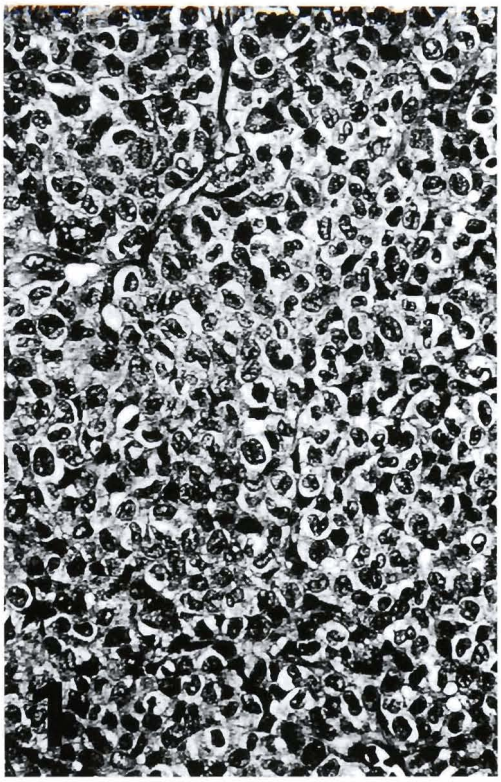
The supernatant from the first fusion reacted with collagen fibres, occasionally with endothelial cells, polymorphonuclear cells and keratinocytes in addition to HXCs and epidermal LCs. The supernatant of the well showing high Ig titer by RIA sometimes stained the tissue nonspecifically. Cloning twice by the limiting dilution method brought about available clone which produced MI1.

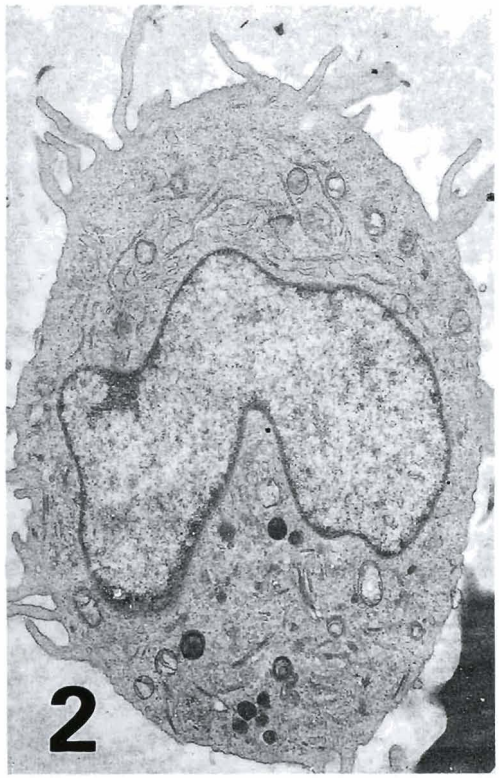
5. SDS-PAGE and immunoblotting of MI1

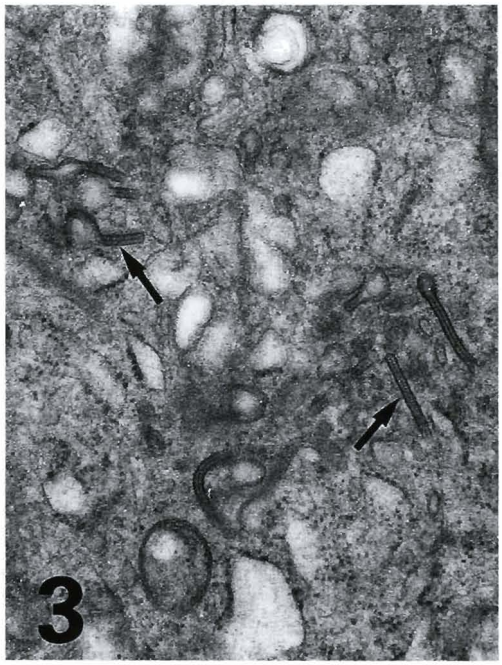
Immunoblotting revealed that MI1 bound with a band of about 28500 mw. of antigenic membrane protein. The band was faint but specific (Fig. 4).

6. Determination of immunoglobulin class of MI1

There was a clear dense line between MI1 and







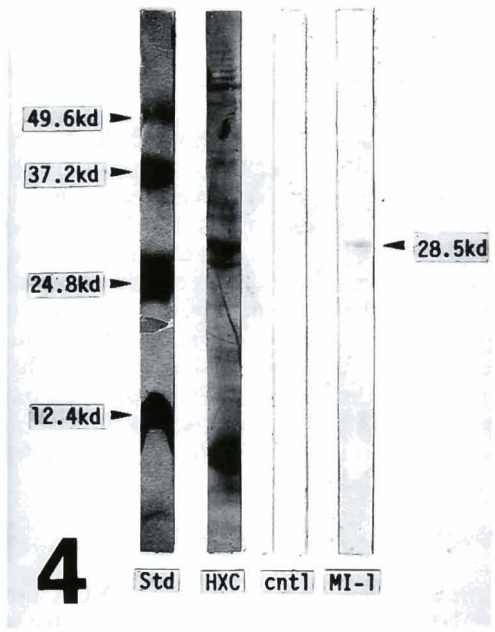


Fig. 1. In the affected lesion of an adult type of HX, proliferating HXCs are seen diffusely. HXCs have abundant pale cytoplasm. Mitotic figure is seen occasionally. HE x 200

Fig. 2. HXC has indented nucleus with sparse chromatin and irregular cytoplasmic processes. In the cytoplasm there are many mitochondria, polyribosomes and myelinosomes and BGs. x 5,300

Fig. 3. HXC has many rod or racket-shaped BGs (➞) in the Golgi area. x 26,500

Fig. 4. SDS-PAGE shows that MI1 binds to HXC membrane fraction of 28,500 mw. Std and cntl mean standard protein and control, respectively.

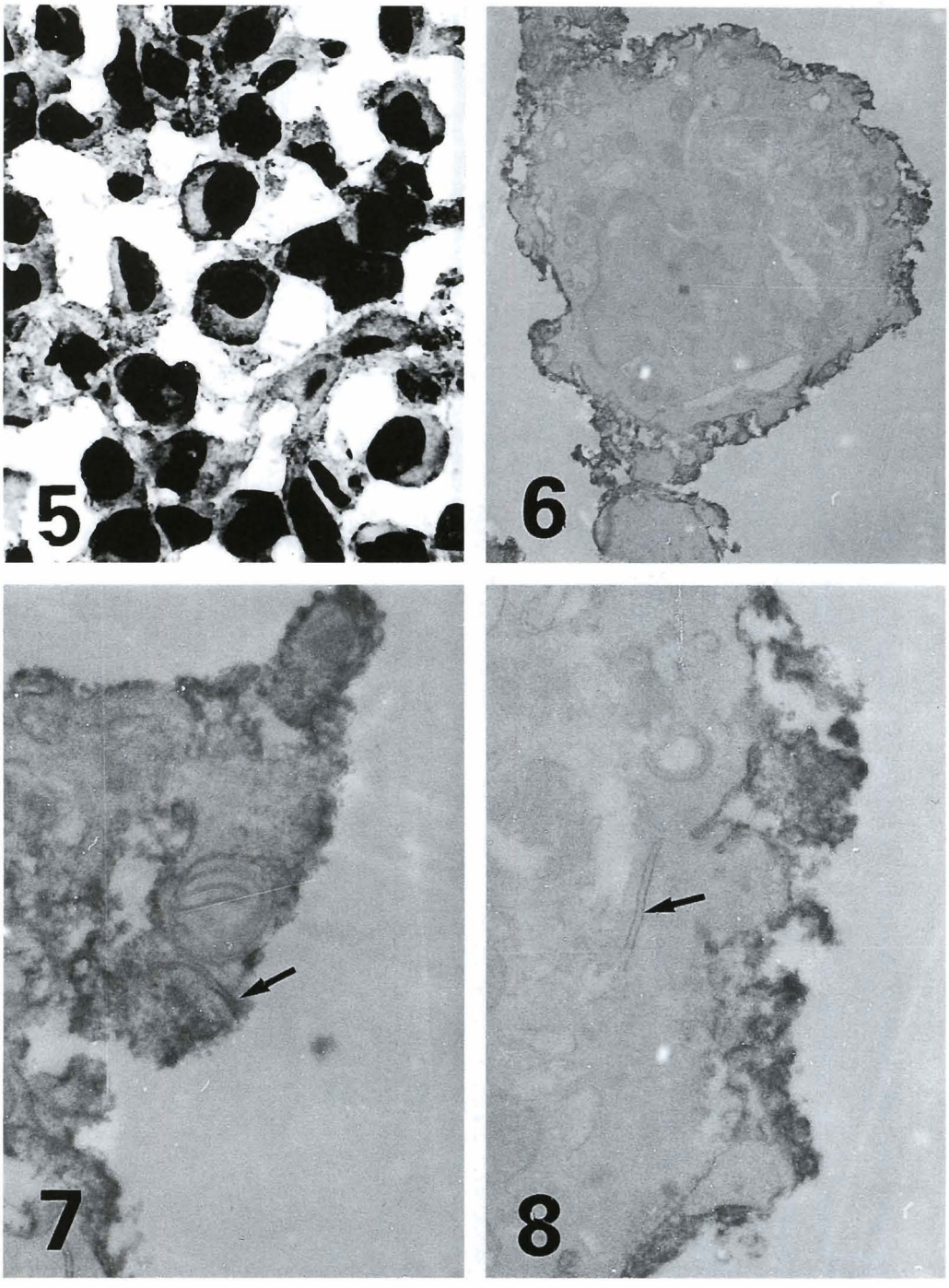


Fig. 5. MI1 reacts with cell membrane of HXCs from an adult type. Indirect method. x 400

Fig. 6. Electron microscopy shows MI1 is positive for the cell surface of HXC from adult type. Indirect method. x 4,700

Fig. 7. BG (➔) connecting to the cell surface is positive for MI1. x 15,000

Fig. 8. BG (➔) in inner cytoplasm is negative for MI1. x 15,000

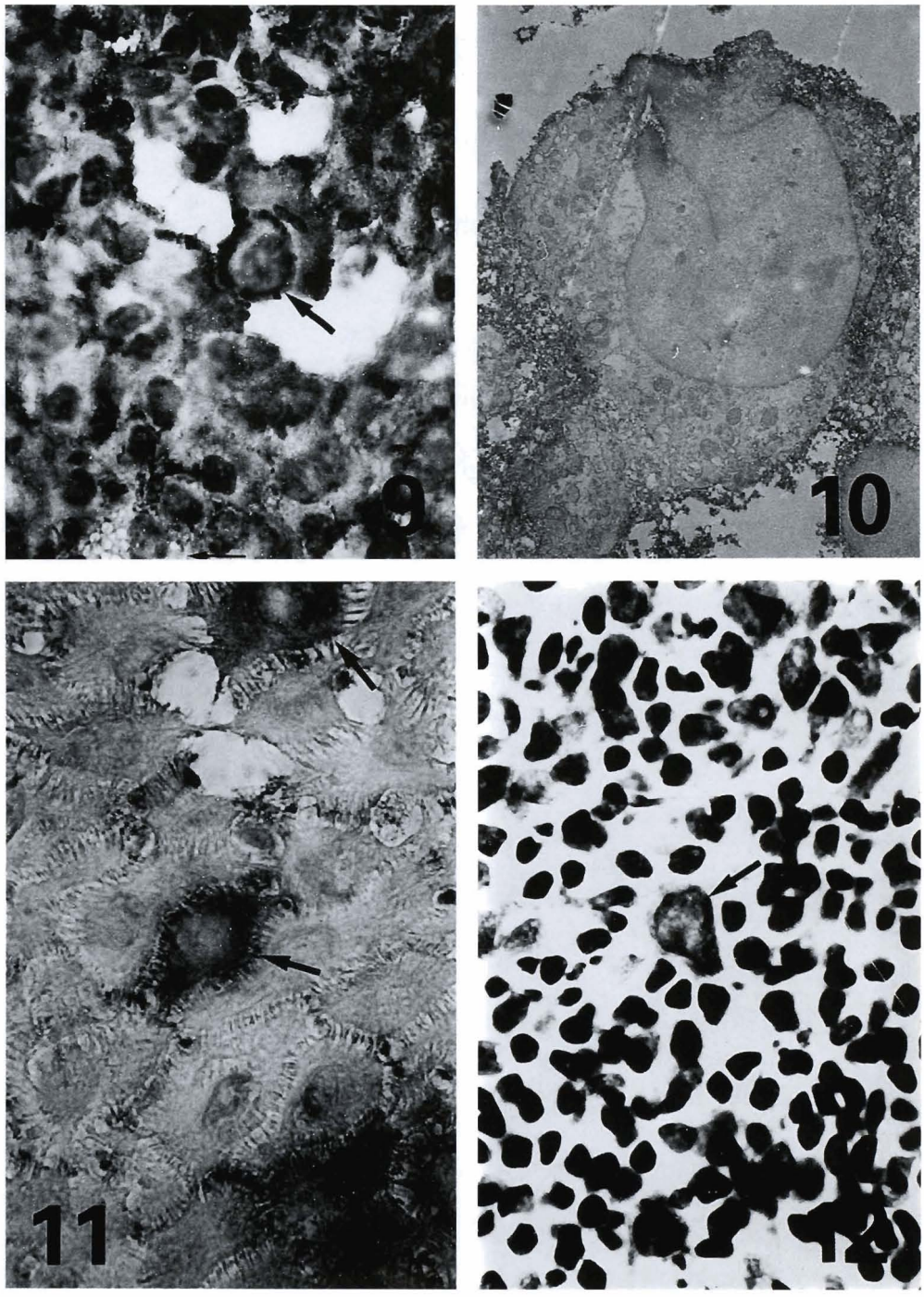


Fig. 9. MI1 also shows positivity for the HXC (➔) from eosinophilic granuloma. But phagocytic macrophage (➔) do not react with MI1. Indirect method, x 400

Fig. 10. Immunoelectron microscopy reveals MI1 react with HXC from eosinophilic granuloma. Indirect method. x 5,200

Fig. 11. LCs (➔) in the squamous cell layer are diffusely positive for MI1. Indirect method, x 600

Fig. 12. Some IDC (➔) in tonsil showed partial positivity for MI1. Indirect method, x 400

Table 1. Histochemical, immunohistochemical and morphological feature of Langerhans cell (LC), histiocytosis X cell (HXC), interdigitating reticulum cell (IDC) and phagocytic macrophage (Mϕ).

	HXC	LC	IDC	Mϕ
ATPase	+	+	+	–
Acid phosphatase	–	–	–	++
Ia-like Ag	++	++	+	++
lysozyme	–	–	–	+
-ACT	–	–	–	+
S-100α	–	–	–	+
S-100β	+	+	+	–
ANS	+	+	+	–
T3	–	–	–	–
T4	+	–/+	–/+	–
T6	+	+	+	–
T8	–	–	–	–
Ia1	+	+	+	+
M1	–	–	–	+
Birbeck granule	+	+	–	–
phagocytosis	–	–	–	+

Table 2. Reactivity of MI1

	MI1
HXC	++
LC	+
IDC	+/–
Phagocytic macrophage	–
plasma cell	–
lymphocytes	–
neutrophil	–
endothelial cell	–
squamous cell	–
fibroblast	–
collagen fibre	–/+

monoclonal rabbit anti-mouse IgM, but MI1 did not show any line with other Igs and PBS.

7. Immunohistochemical and immunoelectron microscopical study using MI1

MI1 reacted with HXCs of adult type (Fig. 5), and also with HXCs of eosinophilic granuloma (Fig. 9). MI1 reacted with LCs in the epidermis and squamous epithelium of gingiva (Fig. 11) and tonsil.

The electron microscopical immunoperoxidase of the MI1 led to the obvious reactivity to the antigenic HXCs (Fig. 6). The activity was strong in the cell membrane and the outer cytoplasm, while the nucleus and inner cytoplasm showed no electrodense deposit. Electrodense deposits were found in some of the BGs which were located close to the cell membrane (Fig. 7), while they were not found in the BGs in inner cytoplasm (Fig. 8). Other intracytoplasmic organelles did not represent clear reaction for MI1.

The tissue from gingival eosinophilic granuloma showed that MI1 reacted somewhat in the inner cytoplasm of the HXCs (Fig. 10), while, hagocytic macrophage showed no electrodense deposit.

LCs in the squamous epithelium showed diffuse positivity for MI1 (Fig. 11). IDCs in T-zone of the tonsillar tissue proved to have variable staining (Fig. 11). Some of them showed partial positivity on the cell membranes of the HXCs (Fig. 12).

The infiltrating inflammatory cells, endothelial cells, and fibroblasts did not express obvious dense deposits.

Collagen fibres were less positive in the electron microscopical study than in the light microscopical study.

Discussion

A male patient of the two studied cases was diagnosed as adult type (Feuerman and Sandbank, 1976) of HX. It is said that adult type is included in the chronic disseminated histiocytosis (Lichtenstein, 1953). In this case, although he had been suffering from gastric lymphoma and been operated on 4 years before, there was no clinical manifestation of HX other than skin lesions. The HXCs in this submandibular lesion had a large number of BGs, while, the HXCs in gingival lesion from the other case had a lesser number of BGs. Therefore, the tissue from an adult type was used for a source antigen, becuase it was possible that BGs came from cell membrane.

HXCs show a weak positivity for AcPase, non-specific esterase and strong positivity for ATPase. HXCs possess Ia-like antigens (Thomas et al., 1982; Favara et al., 1983) as do LCs. (Stingle et al., 1977; Rowden et al., 1977). Moreover, they are negative for lysozyme, α_1-ACT and α_1-AT. Therefore, it has been generally accepted that the HXC as well as the LC and the IDC comprise an element of the mononuclear phagocyte system (MPS) (van Fourth et al., 1972). The subunit localization of the S-100 protein, however, was different from phagocytic macrophage. LC, IDC and HXC react exclusively with ß subunit, while phagocytic macrophage only reacts with α subunit (Isobe and Okuyama, 1981).

The examination of a foetus with S-100 protein indicated that the LC and the IDC were not components of the MPS, but rather that they constituted a different cell type, namely S-100$^+$-zone histiocyte (Watanabe et al., 1983). This was followed by Ralfkier et al. (1984).

Further monoclonal immunohistochemistry revealed that LCs and IDCs had a similar phenotype, which were Ia1$^+$, OKT6$^+$, Ig-complex$^-$ and Mo2$^-$ (Ralfkier et al., 1984), and LCs can be differentiated only by the presence of BGs from IDCs. Other investigators (Harrist et al., 1983b) added OKT3$^-$, OKT4$^{-/+}$, OKT8$^-$. HXCs were described not only as having the presence of OKT6 antigen but also of OKT4 antigen, which shows a focal distribution and is less common than OKT6 antigen. (Murphy et al., 1983) Wood et al. (1983) similarly reported the activity for OKT4 in the HXCs.

Lag (Kashihara et al., 1986), which was specifically reactive to human LC and of which isolated antigen showed a broad band about 4300 mw, did not react with IDC. Therefore, Kashiwara et al. said LC and IDC might not be of the same origin.

Newly-established monoclonal antibody MI1 associated the membrane antigen of 28500 mw, while OKT4 bound that of 60000 mw. and OKT6 that of 12000 or 49000 mw. MI1 bound LCs and IDCs as well as HXCs. MI1 stained predominantly on the cell membrane of the HXCs. On the other hand, the LCs of the epidermis showed positivity in inner cytoplasm as well as cell membrane, and IDCs from normal tonsillar tissue displayed less positivity in the plasma membrane. These data seem to suggest that the HXCs could be differentiated from the LCs and IDCs by MI1.

There are many electron microscopical studies on the HXC and BG, but the origin of the BG is unsettled. Tarnowsky et al. (1967) insisted that BGs came from an indentation of the cell membrane and thought that they were the specialized forms of the phagocytosis which no longer associated with the classical phagocytosis or pinocytosis. Wolff et al. (1967) said that BGs came from secretory granules of the Golgi apparatus, becuase peroxidase had not been taken into the BGs located in the Golgi area. A similar study was made by Kurozumi et al. (1973). There are still others who think BGs might be formed in both ways (Cancilla et al., 1968).

S-100 protein showed no dense deposit on the BGs. Monoclonal immunohistochemical studies represented the cell membrane antigen in the BGs. BGs reacted with anti-OKT6 antibody, but not with the other intracytoplasmic organelles (Murphy et al., 1991, 1983). This seems to lead to the probable conclusion that the BGs derive from an invagination of the cell membrane.

MI1 reacted with some BGs that connected to the cell membrane, and did not react with the BGs which were located near the nucleus. This finding possible coincides with the OKT6 positivity to the BGs. Lag reacted mainly the BGs which were located in the inner cytoplasm, and did not react to cell membrane. Therefore the antigen recognized by MI1 was completely different from that recognized by Lag. Lag bound more specifically to BGs, while MI1 was less specific.

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