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Tumor Cell Line Characterization of a Malignant Histiocytosis Transplanted into Nude Mice

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Summary. The successful transplantation of a human malignant histiocytosis into nude mice allowed the examination of its atypical histiocytic cell proliferation. Histiocytic type cells were identified by positive reactions with acid phosphatase and non-specific esterase and with anti human DR or OKI1 antisera. Presence of OKT9 antigen and negative results obtained with most OKT antisera, rosettes, erythrophagocytosis and lysozyme corroborate the histiocytic immature state of the cells and preclude another type of tumor. All positive tests to prove a mature mononuclear phagocytic origin were attributable to the murine host cell reaction.

Key words: Malignant histiocytosis – Nude mouse – Histochemistry – Membrane receptors – Monoclonal antibodies

Malignant histiocytosis is an anatomo-clinical entity first described by Rappaport (1966) as a proliferation of morphologically atypical histiocytes or their precursors "throughout the haematopoietic and lymphatic tissues". In most cases diagnosis is made in relation with the clinical evolution and the examination of lymph node biopsy. Histiocytic origin of the tumoral cell line is not yet completely established in spite of ultrastructural, histochemistry (Lombardi et al. 1978; Meister and Nathrath 1980; Mendelsohn et al. 1980; Tubbs et al. 1980) and membrane receptors studies (Vilpo et al. 1980). Variability of the results may in part be related to the heterogeneity of the cell population studied.

The aim of this study is to specify the histiocytic origin of a malignant histiocytosis using the nude mouse model as a way to concentrate the tumoral cell population.

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Case Report (NEM 70432)

The disease which developed in a 12-year-old boy expressed itself as an inguinal lymph node enlargement with fever. On the first pathological report lymph nodes were considered as inflammatory. In a few weeks' time a generalized enlargement of all lymph nodes with splenomegaly appeared combined with weakness, weight loss, fever, anemia and pleural, ascitic effusions. A diagnosis of malignant histiocytosis was given on the pathological report of a second lymph node. The sinuses were full of large atypical mononucleated clear cells with an histiocytic appearance. Few of them had ingested erythrocytes. They were negative for lysosyme (PAP method). The child received chemotherapy treatment. Death ensued after 2 months.

Materials and Methods

Pleural effusion liquid of the patient was immediatly processed, one part for diagnosis and the other part for heterotransplantation into the nude mice.

Tumor Transplantation into Nude Mice

Subcutaneous (0.2 ml) or intracranial (0.03 ml) injections (12×10^6 cells/ml) were administered to approximately 5 week-old-nude mice with a Swiss background. The animals were maintained in plastic cages according to conventional methods. Food, bedding cages and tops were autoclaved prior to use. Growing tumors (1 cm^3) were re-inoculated subcutaneously into 4–5-week-old nude mice.

Cell Suspension

Transplanted tumors when 1 cm^3 were minced in Hanks' balanced salt solution. The cell suspension was filtered through 4 layers of sterile gauze and then centrifuged. Cell viability was evaluated by trypan blue dye exclusion test. Some experiments were performed on cells which had passed through lymphocyte separating medium (MSL, Eurobio, Paris, France) which eliminated erythrocytes and dead cells (Thorsby and Brotlie 1970).

Culture Technique

The transplanted tumors were excised, minced into small fragments which were placed into plastic culture dishes (Nunc, Denmark) with two drops of MEM medium (Minimum Essential Medium, Pasteur Institute, Paris, France) supplemented with 10% decomplemented foetal calf serum, 2×10^5 U/l penicillin and 0.1 g/l streptomycin. MEM medium was added few hours later and changed once a week.

Human Species Characterization

Immunofluorescent Studies. An anti-mouse serum was prepared by immunizing a rabbit 5 times at 10–15 days' intervals, with 1 cm^3 of homogenized mouse tissue (heart, liver, kidney and lung). The antiserum was absorbed, 5 times for 2 h at 4° C with human tissue homogenates (tonsils). The obtained anti-mouse serum was unreactive with human spleen and tonsil cell suspensions.

Glucose Phosphate Isomerase and Glucose 6 Phosphate Deshydrogenase Isozymes. Tumor extracts were tested by electrophoresis for glucose phosphate isomerase, GPI, (Kahn et al. 1977) and for glucose 6 phosphate deshydrogenase, G6PD, (Kahn et al. 1976). The ratio of human to murine isoenzymes was performed by immunoneutralization with specific antisera directed against human GPI (Bertrand et al. 1976) and G6PD (Kahn and Dreyfus 1974).

Besides, results with anti-human DR, lysozyme and monoclonal antibodies concern only human tumor cells and consequently help in the species characterization.

Tumor Cell Line Identification Criteria

Light microscopy. Either the initial pleural effusion or the cell suspensions were cytocentrifuged onto glass slides and stained with H.E. or May Grunwald Giemsa. Transplanted tumor tissues were fixed in 15% formol and embedded in paraffin, 5 μm thick sections were stained with H.E.

Electron Microscopy. Fixation was done at 4° C in 4% glutaraldehyde in 0.57 M cacodylate buffer (pH 7.35) and followed, after a rinsing, by 2 h of post fixation in 2% osmium tetroxyde in cacodylate buffer. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips EM. 300, 60 KV.

Histochemistry. 6–8 μ thick frozen sections were stained for acid phosphatase (Pearse 1968 a) and non specific esterase (pH 7.5), using the naphtyl acetate method (Pearse 1968 b).

Lysozyme. The lysozyme dosage was done on MEM media incubated with transplanted tumor cells for 2 h, using M Lysodeichtiens as micro-organisms test and egg white lysozyme as the standard. Antihuman lysozyme antiserum was used with the rocket electrophoresis in the species identification.

Surface Receptors. Their detection was done on cell suspension.
a) Rosettes Method. E receptors were detected by sheep red blood cells according to the method of Fröland (1970), EA rosettes were performed with sheep red blood cells labelled with rabbit IgG (Johnsen and Madsen 1978) EAC rosettes were made according to the method of Bianco et al. (1970). Rosettes forming cells were immediatly numbered.
b) Cell surface immunoglobulins. Cell surface immunoglobulins were tested with fluorescein conjugated goat antiserum against cell surface IgM, IgG, IgA of human immunoglobulins (Behring).

Tumoral cell suspensions, before or after MSL, were centrifuged and incubated with antisera for 30 min at room temperature, washed three times with phosphate buffered saline (pH 7.4), suspended in buffered glycerine and examined under fluorescence microscope.

Phagocytosis. Red cell phagocytosis was observed 3 h after EAC rosettes method either on the cell suspension or on in vitro sedimented cells adherent to cover slips in Leighton tubes. Latex phagocytosis was similarly performed (Difco, Detroit, MI, USA).

Anti-Human DR Serum. Rabbit anti-human DR serum was produced by immunization with immune complexes as reported by Welsh and Turner (1977). The serum was absorbed twice on mouse spleen cells.

Indirect immunofluorescence tests were performed with purified antibodies absorbed on a mouse IgG sepharose 4 B immuno absorbant and labelled with fluorescein isothiocyanate.

Cell Typing with Monoclonal Antibodies. Monoclonal antibodies directed at human T cell and other mononuclear cell subsets were kindly provided by P.C. Kung and G. Goldstein, Ortho Pharmaceutical Corporation, Raritan, NJ, USA. The antibodies were produced by mouse hybridomas as already described (Kung et al. 1979).

An indirect immunofluorescence assay was used to characterize tumor cells as already described (Bach et al. 1981). In brief, cells (0.5 to 1×10^6) were mixed with optimal concentrations of monoclonal antibodies (OKT3: 20 μg/ml, OKT4: 5 μg/ml, OKT5: 5 μg/ml, OKT6: 2 μg/ml, OKT8: 4 μg/ml, OKI1: 3 μg/ml, OKM1: 3 μg/ml) in 50 μl of Hanks' balanced salt solution (BSS). Cells were placed in an ice bath for 30 min. then washed twice with BSS containing 5% foetal calf serum (FCS) plus 0.2% sodium azide. Fifty microliters of fluorescein isothiocyanate conjugated goat antimouse IgG antibodies (FITC-GAM) (0.2 mg/ml in FCS-Azide-BSS) previously absorbed on human IgG, were added to the pellet. After a further 30 min

time at 4° C followed by two washings, cells were resuspended in 50 µl FCS-azide-BSS and percentages of fluorescein labelled cells were scored immediatly under a fluorescence microscope.

Results

Original Pleural Effusion

The initial cell suspension included malignant cells, as well as cells from other lineages, such as lymphocytes, polymorphonuclears, macrophages and mesothelial cells. Tumor cells, 12–15 µ in diameter, were easily detected morphologically according to their slightly basophilic cytoplasm and large irregular nucleus (Fig. 1). Electron microscopic studies revealed that the tumor cells contained single voluminous nucleus with a large nucleolus. The cytoplasm contained few organelles and the plasma membranes had rare microvilli. Some of them had engulfed polymorphonuclear cells. Tumor cells reacted positively with acid phosphatase and non specific esterase although the strongest reaction was found with the macrophages and mesothelial cells.

Transplanted Tumors

Tumor growth was obtained two weeks after the subcutaneous injection of pleural effusion. However there was no successful intracranial inoculation. The tumor has been serially passed from one mouse to another 35 times to date, with a mean latent period between passages of 2–4 weeks.

A. Species Determinations

Immunofluorescent study of the cell suspension revealed 15–20% murine cells. The murine population was also evidenced by GPI or G6PD electrophoresis of tumor extracts and was quantitatively estimated to be 20–30% by immunoneutralization of isozymes. The ratio was of the same range independently of the tumor passage number. Both immunofluorescent and isozymic studies proved the exclusively murine nature of in vitro transplanted tumor cells.

B. Tumor Cell Line Identification of the Transplanted Tumor

Cell characterization was conducted either on a suspension or on the solid tumor. The viability index of the cell suspension was 40–50% before MSL separation and always >95% afterwards. Morphological controls performed before and after MSL separation prove that this procedure eliminates only erythrocytes and dead cells.

Morphology. Sequential passage of the transplanted tumors in nude mice was performed 35 times without any modifications. The major cell population was similar to the large mononucleated tumor cells found in the pleural effusion (Fig. 2). A thin capsule surrounded most of the tumor with areas

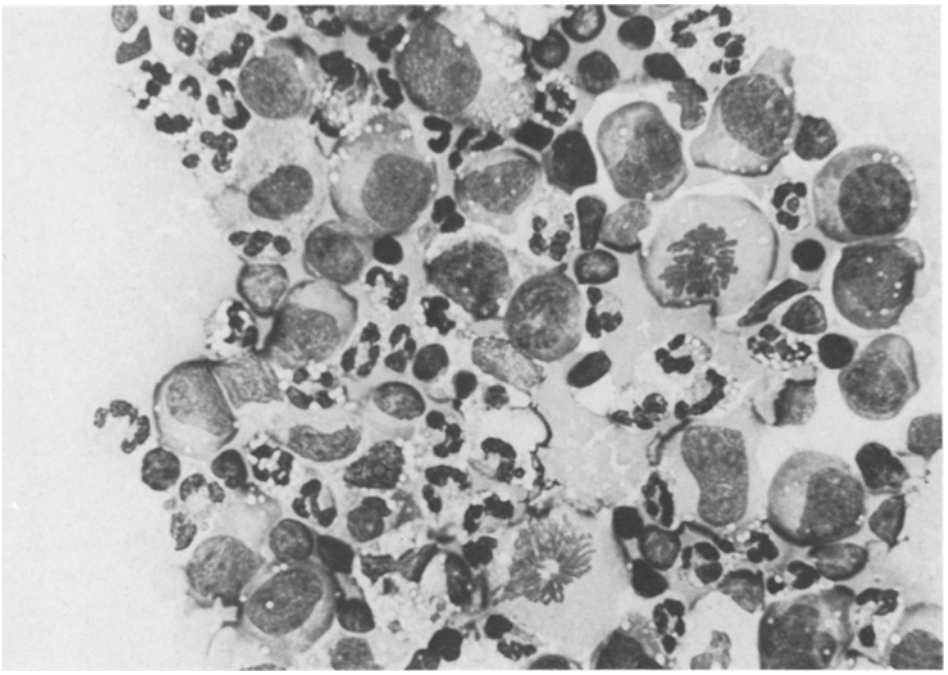

Fig. 1. Initial pleural exsudate. Tumor cells with large nucleus and frequent mitosis are intermingled with cells from other lineages (Giemsa ×400)

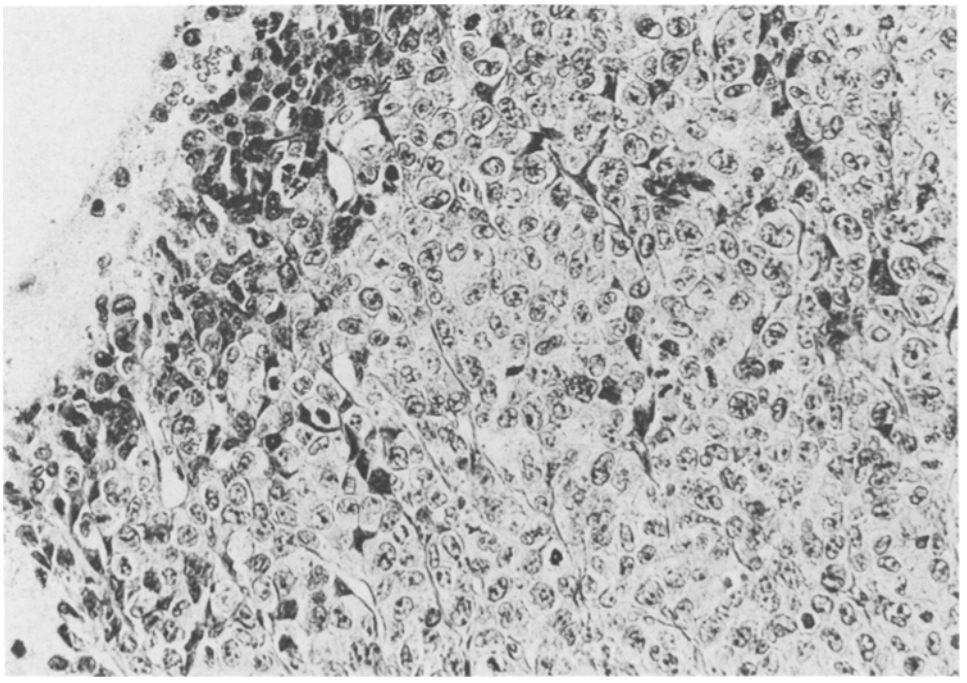

Fig. 2. Transplanted tumor circumscribed by a thin membrane. The histological aspect is monomorphic (HE ×250)

of mouse muscle invasion. The electron microscopic study of the transplanted tumor revealed characteristics similar to those found for the initial pleural effusion. Transplanted tumor cells contained a single voluminous nucleus with a well-developed nucleolus (Fig. 3). The cytoplasm had few organelles. No viral particles were observed. In some cases, the tumor cells were intermingled with other well differentiated cells as skeletal muscle cells, endothelial cells, fibroblasts and macrophages. The latter had a darkly stained cytoplasm with some heterogeneous inclusions and rare myelinic figures.

Histochemistry. The large cells from the transplanted tumors had a low positivity for acid phosphatase and non specific esterase. However, some isolated cells exhibited an intense positivity.

Membrane Receptors and Phagocytosis. On the transplanted tumor cell suspension, E, EA and EAC rosette formations and phagocytosis were negative. On the contrary, if the cell suspension was allowed to sediment for several hours onto coverslips in Leighton tubes, EA and EAC rosette formation and active phagocytosis of latex and EAC erythrocytes could be detected on rare attached cells. The latex phagocytising cells were found as being of murine origin by indirect immunofluorescent studies using rabbit antimouse serum (Fig. 4); controls on human alveolar macrophages were negative with the same antimouse serum.

No synthesis of immunoglobulins (IgM, IgG, IgA) was revealed by fluorescence technique on 3 different passages.

Lysozyme. Only murine lysozyme could be detected in the culture medium (0.85 γ/ml) after 2 h of incubation.

Human DR Antigen. Fifty percent of the tumor cells were positive for antihuman DR serum using an indirect labelling procedure. The same results were obtained with human alveolar macrophages, but mouse spleen cells were negative.

Monoclonal Antibodies. Four tests were performed on transplanted tumor cell suspension after MSL separation. 85%–95% cells react with OKT9 and OKI1 antibodies. All the other antisera tested gave negative results.

C. Identification of in vitro Cells Obtained from Transplanted Tumors

After 7–14 days of culture, two cell populations could be observed. Isolated fibroblasts and predominant colonies of round cells. These two cells populations were maintained for a few weeks under our culture conditions, however after several passages, the fibroblastic cells overtook the culture. All the tests used (ac. phosphatase and non specific esterase, EA and EAC rosette formation, latex and erythrocytes phagocytosis) proved that the round cells are macrophages. The latex phagocytosing macrophages and the fibroblasts

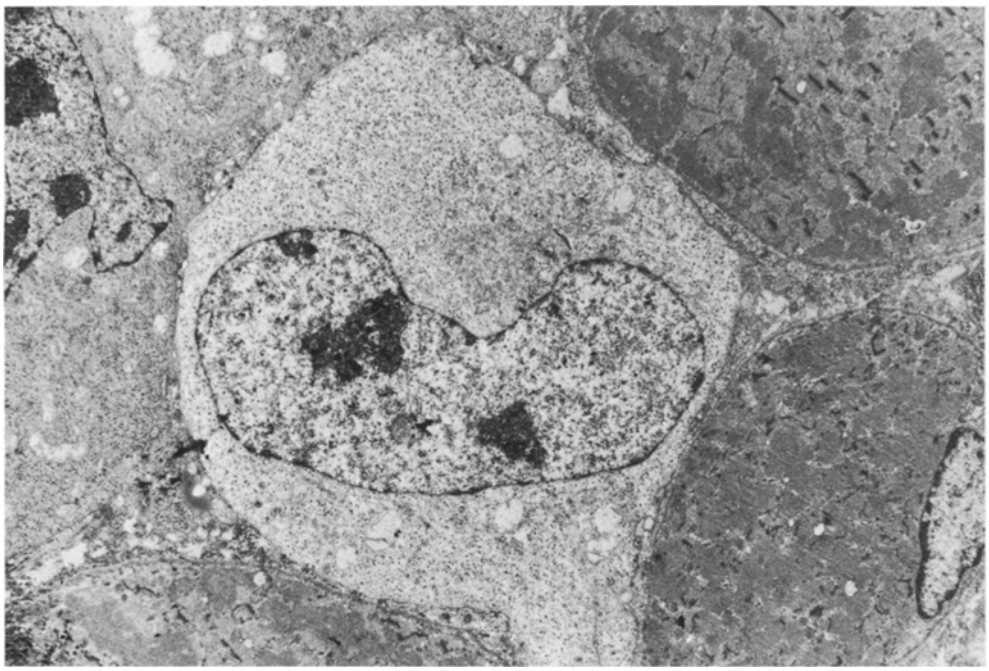

Fig. 3. Transplanted tumor cell close to muscle cells (electron microscopy $\times$ 5,600)

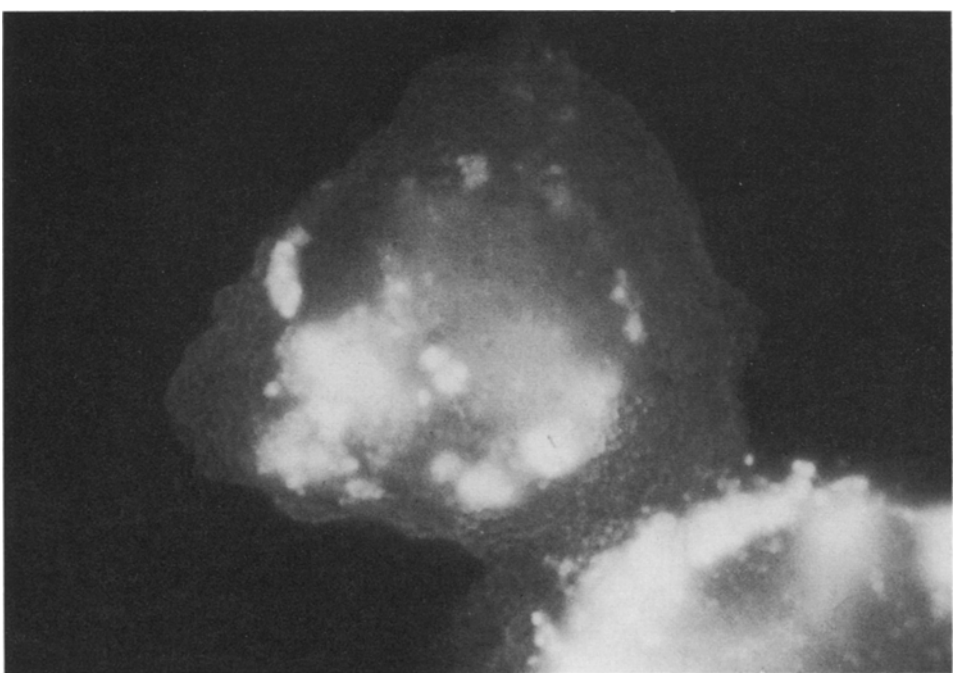

Fig. 4. Indirect immunofluorescent labelling of latex phagocytosing cells with anti-mouse serum ($\times$ 1,000)

were identified as murine by means of indirect immunofluorescence using anti-mouse serum. Controls with anti-human DR serum were negative.

Discussion

The successful heterotransplantation of a malignant histiocytosis into nude mice allowed the study of the tumoral cell proliferation and of its presumed histiocytic origin. One case of such as heterotransplantation was reported by Epstein et al. (1976) who studied factors influencing the heterotransplantation of human malignant lymphomas. These authors reported one success for a malignant histiocytosis with intracranial injection site whereas the present transplanted tumor was obtained with subcutaneous injection. In both cases, the initial transplanted tumoral cell population come from pleural effusions which may represent a good selection of particularly resistant tumor cells.

For the study of the malignant histiocytosis process, the nude mouse model puts forward two points:

1) the possibility of analyzing the host-tumor relationship by the human and murine cellular identification

2) the possibility of studying a more homogeneous tumor cell line population.

Host-Tumor Relationship

There was 70–80% of human components in the heterotransplanted tumors independently of passage number. The scattered mature macrophages observed by electron microscopy and direct histochemical assays on the transplanted tumors were detected as murine macrophage by specific lysozyme detection and by the antimouse sera labelling of phagocytizing cells. The mouse cell selectivity "in vitro", probably related to the fragility of the tumor cells (40–50% viability prior to MSL separation) allowed the complete characterization of the host macrophages. These results emphasize the participation of the host macrophages to the tumor proliferation and give an answer to a longstanding question. The presence of phagocytic cells in malignant histiocytosis has been previously discussed on morphological data as being either cells of probable stroma origin (Warnke et al. 1975; Rilke et al. 1978; Lombardi et al. 1978) or as being malignant histiocytes at various stages of maturation (Lampert et al. 1978; Mendelsohn et al. 1980; Huhn et al. 1980). The exact importance of the high stroma macrophage participation to the so-called malignant histiocytosis is not clearly understood (Nelson et al. 1981) but seems to be a constant association useful for diagnosis.

Human Tumor Cell Line Study

The isolated immature mononuclear cells from the initial pleural effusion show a cohesiveness in the transplanted tumors more distinct from that observed in the lymph node biopsy.

The histiocytic identification of this tumor was based upon the positive reactions with acid phosphatase and non-specific esterase as well as the presence of human DR or Ia antigen. In association with morphological criteria, non specific esterase is a well-known histochemical marker for histiocytic cells currently used in malignant histiocytosis (Lombardi et al. 1978; Rausch et al. 1979; Glick et al. 1980). Human DR or Ia antigen (recognized by OKI1 antibody) was used as a histio-monocyte or a B cell marker in lymphomas (Halper et al. 1980). In the present case, the tumoral cells cannot be considered as B cells since they do not synthesize IgG immunoglobulins, fail to react with EAC rosetting test and show a reactivity with acid phosphatase and non specific esterase. The negative results obtained with OKM1 rosettes, phagocytosis and lysozyme corroborate the immature histiocytic state of the cells and preclude another type of tumors. Mature cells such as fibroblasts and macrophages, were demonstrated to be of murine origin.

The fact that majority of the tumoral cells bear an OKT9 antigen was quite surprising. OKT9 is known to react with $\leqq 10\%$ thymocytes cells considered as early thymocytes (Reinherz et al. 1980). This antigen was found in association with OKT10 in three cases of human histiocytic lymphoma exhibiting clonal surface or cytoplasmic IgG but devoid of Ia like antigen (Aisenberg and Wilkes 1980). Haynes et al. (1981) showed that the antigen defined by 5 E9 and OKT9 was not thymus specific but may be related with primitive cells. These data agree with the present observation of a highly proliferative cell population not related with a T phenotype.

As a conclusion, the results obtained in the present study:

1) emphasize the association of mature benign histiocytes with the proliferation of malignant histiocytosis. If the presence of phagocytic capabilities is an aid for diagnosis (Byrne and Rappaport 1973), it must be related with the stroma histiocytes in the most undifferentiated cases.

2) confirm the immaturity of histiocytic cells in malignant histiocytosis and suggest that the human DR or Ia antigen may be a useful marker for the immature cases

3) bring a supplementary example of the relation of OKT9 with an immature tumoral cell line without T phenotype.

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