Studies relating to Langerhans cell histiocytosis

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Submitted for the degree of Doctor of Medicine to
the University of London.
Langerhans cell histiocytosis (LCH) is an uncommon disease of unknown aetiology, where characteristic histiocyte cells, designated LCH cells, accumulate in various tissues. This thesis examines the clinical, diagnostic and immunological features of LCH.

In a clinical study of 60 children with LCH, a new disease scoring system was compared with the existing Lahey system and found to be better at predicting outcome. The efficacy of the current treatment protocol used in the UK was compared with that of other International Centres where more aggressive therapeutic approaches are used.

Diagnostically, two markers for LCH cells were compared in paraffin-embedded tissue. Peanut agglutinin was found to be more specific and as easy to use as the S100 stain. Mouse monoclonal antibodies were produced against LCH cells but none proved specific.

Immunological studies defined the functional characteristics of normal Langerhans cells and compared these with the functional characteristics of LCH cells.

Epidermal cells containing 1% Langerhans cells were shown to be as potent as enriched blood dendritic cells at presenting recall antigen to autologous T-cells, and 16 times more potent than monocytes in this system. LCH cells, however, failed to act as accessory cells in mitogen driven T cell stimulation.
In order to provide a source of cells to examine LCH function an attempt was made to produce an LCH cell line. Fresh LCH cell from 10 patients with LCH were cultured under different conditions and with a variety of growth factors. Only one cell line was generated, which was shown by enzyme histochemistry to be histiocytic, but this was immature and attempts to induce maturation failed.

The nature of LCH (malignant or reactive) is controversial. Flow cytometry of fresh and paraffin-embedded LCH cells demonstrated that LCH cells are diploid supporting a reactive nature of this disease.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>LCH</td>
<td>Langerhans cell histiocytosis</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>srbc</td>
<td>sheep red blood cells</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>PHA</td>
<td>phythaemagglutinin</td>
</tr>
<tr>
<td>PPD</td>
<td>purified protein derivative of tuberculin</td>
</tr>
<tr>
<td>PBM</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>MA</td>
<td>adherent monocytes</td>
</tr>
<tr>
<td>NM</td>
<td>Nycodenz monocytes</td>
</tr>
<tr>
<td>MLR</td>
<td>mixed lymphocyte reaction</td>
</tr>
<tr>
<td>PNA</td>
<td>peanut agglutinin</td>
</tr>
<tr>
<td>MCR</td>
<td>mixed cell reaction</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>HAT</td>
<td>hypoxanthine, aminopterin and thymidine</td>
</tr>
<tr>
<td>Mlg</td>
<td>mouse Immunoglobulin</td>
</tr>
<tr>
<td>RPMI</td>
<td>RPMI 1640 buffered with sodium bicarbonate tissue culture medium</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>AFAAP</td>
<td>alkaline phosphatase and anti alkaline phosphatase staining technique</td>
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</tbody>
</table>
MT  hypoxanthine and thymidine
C'  complement
Ig  immunoglobulin
DC  dendritic cell
EC  epidermal cell
mo  macrophage
mono  monocyte

Acknowledgements

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Ethical Considerations

All studies involving the use of human tissues were carried out with approval from the Royal Postgraduate Medical School Ethical Committee.

A Home Office Animal Licence was held to carry out experiments involving animals.
INTRODUCTION

1.1 Nomenclature

Understanding of the disease now termed Langerhans cell histiocytosis has been significantly retarded by the lack of common terminology used when investigating or discussing this disease.

In 1893 Alfred Hand presented a case report of "polyuria and tuberculosis" to the Philadelphia Pathological Society. 22 years later Artur Schuller published a paper describing the various causes of skull defects in childhood which included a similar case. Henry Asbury Christian reported in 1920 a patient with "defects in membranous bones, exophthalmos and diabetes insipidus; an unusual case of dyspituitarism" but it was not until 1921 that Hand put these three cases together and the triad of diabetes insipidus, exophthalmos and lytic skull lesions became known as Hand-Schuller-Christian disease.

Erich Letterer described a fulminant disorder of the reticuloendothelial system in a 6 month old child (1924) which Sture Siwe, when he described a further similar case (1933), distinguished from "Schuller-Christian disease by the lack of lipid inclusions in the cells". This aggressive
presentation, more common in young children, became known as Letterer-Siwe disease.

In 1940 two reports of solitary bone involvement, eosinophilic granuloma, were published (Lichtenstein and Jaffe, 1940; Otani and Ehrlich, 1940). Although the overlap between these presentations had been noted it was Lichtenstein, in 1953, who grouped the three, Hand-Schuller-Christian disease, Letterer-Siwe disease and eosinophilic granuloma together under the name of Histiocytosis X.

At the inaugural meeting of the Histiocyte Society in 1986 D'Angio et al recognised that terminology had to be standardised in this disease and proposed that the term Langerhans cell histiocytosis, coined by Risdall et al (1983), be used to replace the previously used eponyms and descriptive terms for this disease. The term Langerhans cell histiocytosis highlights the pathognomonic cell in this disease— the abnormal Langerhans cell. The term has subsequently gained widespread acceptance and is now used instead of the previous terms Hand-Schuller-Christian disease, Letterer-Siwe disease, eosinophilic granuloma, Histiocytosis X, malignant, acute and chronic histiocytosis X, self-healing histiocytosis, pure cutaneous histiocytosis, Langerhans cell granulomatosis and Type II histiocytosis.
1.2 Incidence of Langerhans cell histiocytosis (LCH)

Langerhans cell histiocytosis (LCH) is an uncommon disease and the incidence in children has been estimated to be about 40 cases a year in England and Wales (Broadbent and Pritchard, 1985). The incidence in adults is unknown and many asymptomatic adults may remain undiagnosed. The disease has protean manifestations and the symptomatology in a given patient can vary substantially from time to time (Lichtenstein, 1953). It occurs in all ages but more than half the cases are in children under the age of 15 years (Oberman, 1961). LCH is more common in males than females (Greenberger et al, 1981) and certain presentations are more common in one sex than the other; for example pulmonary involvement is four times more common in males than females (Basset et al, 1978). There also seem to be racial differences with the disease being more common in those of north European origin than in blacks (Winkelmann, 1969).
1.3 Langerhans cells

1.3.1 Langerhans cells— origin.

Langerhans cells were first described by Paul Langerhans in 1868 as aureophilic dendritic cells in the epidermis. In haematoxylin and eosin stained sections of skin they appear as clear cells between keratinocytes in the suprabasal layer of the epidermis (Hoefsmit et al, 1982) at a density of 800 to 1200 per mm² in rodents (Bergstresser et al, 1979). They are normally present in the skin, mucosa (Favara, 1981), lymph node (Shamoto, 1971), and thymus (Hoshino et al, 1970).

Initially these cells were thought to represent effete melanocytes but elegant chimeric studies in mice showed that Langerhans cells were derived from the bone marrow (Katz et al, 1979). The bone marrow precursor cell of Langerhans cells has not been identified, and the trophic factors which induce migration to the skin and mucosa and maturation of the cells remain unknown. A circulating pool of precursor cells is, however, present, which can repopulate the skin if Langerhans cells are removed by tape stripping. (Streilein et al, 1982).
The indeterminate cell has been proposed as the immediate precursor of the Langerhans cell (Breathnach, 1978). This cell is dendritic and found in the epidermis and dermis. It is morphologically similar to Langerhans cells and expresses Ia (Rowden et al, 1977) and the CD1 complex (Murphy et al, 1983) but unlike Langerhans cells it lacks Birbeck granules (Breathnach, 1978).

The precursor cells of the Langerhans cell are thus controversial, but the fate of Langerhans cells after their arrival in the skin is even more so. Some groups feel that the Langerhans cell is terminally differentiated and after a period in the epidermis, dies. These cells may share a common precursor with interdigitating reticulum cells and veil cells, but mature along different pathways (Ishii and Wanatabe, 1987). Others, however, feel that the Langerhans cell migrates out of the skin where it becomes a veil cell in the efferent lymph and then becomes an interdigitating reticulum cell in the regional lymph nodes (Hoefsmit et al, 1982).

Interdigitating cells are present in peripheral lymphoid organs and thymic medulla (Silberberg-Sinakin et al, 1976). They are morphologically similar to Langerhans cells and express surface ATP-ase, S100 protein (Ishii and Wanatabe, 1987) and Ia antigens (Turner et al, 1984)
but lack Birbeck granules. Veiled cells are present in lymph and blood, are morphologically and cytochemically similar to Langerhans cells and may contain Birbeck granules (Hoefsmit et al, 1982).

1.3.2 Langerhans cells—morphology

Langerhans cells are large mononuclear cells about 12um in diameter with multiple long dendrites and an irregularly shaped nucleus (Favara et al, 1983). On electron microscopy there are many mitochondria and a well-developed Golgi system (Hoefsmit et al, 1982). There are also characteristic rod-shaped organelles known as Birbeck granules (Birbeck et al, 1961) which are 190-360nm long and 33nm wide with a central lamella and an occasional terminal dilatation giving them a tennis-racket appearance. The origin of these granules has been controversial. Zelickson (1965) suggested that they were derived from Golgi apparatus but studies on serial sections using tracers such as peroxidase (Wolff and Schreiner, 1970; Hashimoto, 1971) makes a cytoplasmic membrane origin, which seems to be from receptosomes (Hancou et al, 1986), more likely. A fuzzy coat radiates from the cytoplasmic face of the Birbeck granule (Schuler et al, 1983) which is indistinguishable from that of
clathrin coated vesicles and pits. Coated structures have been shown to be involved in the selective transport of molecules (Goldberg et al, 1979) and in locomotion (Bretscher, 1982).

Hanou et al (1987) found that when Langerhans cells were exposed to an anti-CD1 antibody conjugated to gold particles they internalized the CD1 antigen by receptor-mediated endocytosis. Following this, gold labelled Birbeck granules appeared in the cytoplasm suggesting that the granules could represent CD1 antigen intracellular transport organelles.

1.3.3 Langerhans cells- cytochemistry

The cytochemical features of Langerhans cells are summarized in Table 1.3.1
Table 1.3.1 Cytochemical features of Langerhans cells

**Enzyme histochemistry**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>++ References</th>
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<tbody>
<tr>
<td>Adenosine triphosphatase</td>
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</tr>
<tr>
<td>Adenosine diphosphatase</td>
<td>Chaker et al, 1984</td>
</tr>
<tr>
<td>a-naphthyl acetate esterase</td>
<td>Beckstead et al, 1984</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>&quot;</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>&quot;</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>&quot;</td>
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<tr>
<td>Chloroacetate esterase</td>
<td>&quot;</td>
</tr>
<tr>
<td>a-1 antitrypsin</td>
<td>Favara and Jaffe, 1987</td>
</tr>
<tr>
<td>a-mannosidase</td>
<td>Elleder et al, 1977</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Beckstead et al, 1984</td>
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**Immunological markers**

<table>
<thead>
<tr>
<th>Marker</th>
<th>++ References</th>
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<tbody>
<tr>
<td>Fc IgG</td>
<td>Stingl et al, 1977</td>
</tr>
<tr>
<td>C3</td>
<td>&quot;</td>
</tr>
<tr>
<td>Class II MHC</td>
<td>Rowden et al, 1977</td>
</tr>
<tr>
<td>CD1a (OKT6)</td>
<td>Fithian et al, 1981</td>
</tr>
<tr>
<td>CD4</td>
<td>Wood et al, 1983</td>
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**Marker proteins**

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<th>Marker</th>
<th>++ References</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100</td>
<td>Nakajima et al, 1982</td>
</tr>
</tbody>
</table>
Surface adenosine triphosphatase (ATP-ase) has been found on interdigitating cells (Müller-Hermelink et al., 1974) but is otherwise fairly specific for Langerhans cells (Wolff and Winklemann, 1967). Chaker et al (1984) have shown that adenosine diphosphatase is even more specific to Langerhans cells than ATP-ase.

\(\alpha\)-naphthyl acetate esterase is present in cells of monocyte lineage, where staining for the esterase produces a diffusely positive reaction which, like the reaction in Langerhans cells, is inhibited by the presence of sodium fluoride. Acid phosphatase is present in T cell lymphoproliferative disorders, in some mature T cells and in monocytes and macrophages. Alkaline phosphatase is present in neutrophils and peroxidase occurs in neutrophils and macrophages. Staining for chloroacetate esterase produces a positive response in mast cells and granulocytes. \(\alpha1\)-antitrypsin and lysozyme are found in macrophages (Dacie and Lewis, 1984). An intense reaction product with the stain for \(\alpha\)-mannosidase is produced only in Langerhans cells and LCH cells.

1.3.4 Langerhans cells—immunocytochemistry

Langerhans cells express the T-200 antigen which marks them as descendents of bone marrow cells (Flotte et al., 1984).
Langerhans cells are similar to monocytes and macrophages in that they express Fc-IgG and C3 receptors (Stingl et al, 1977) and Class II MHC (Ia-like) antigens (Rowden et al, 1977).

The human thymocyte antigen CD1a (T6) is present on Langerhans cells (Fithian et al, 1981) and is the most specific marker we have for Langerhans cells as it is otherwise present only on LCH cells and some interdigitating cells in the lymph node which may be Langerhans cells anyway. CD4 is weakly present on Langerhans cells (Beckstead, 1984) and is expressed more strongly when the cells are activated, but is not found on other dendritic cells.

The S100 protein is present in a variety of cells, but the b-subunit is present in Langerhans cells (Nakajima et al, 1982) but not monocytes or macrophages.

1.3.5 Langerhans cells— function

Autologous or syngeneic HLA Class-II positive accessory cells are essential in providing accessory function or factors needed by effector lymphocytes in many immune responses such as antibody production, antigen and mitogen-induced T cell proliferation and cytotoxic T lymphocyte induction (Katz et al, 1985).
The archetypal accessory cells are the dendritic cells; blood dendritic cells and interdigitating reticulum cells. Silberberg et al (1973) found that lymphocytes migrate around epidermal Langerhans cells in cutaneous hypersensitivity reactions but not in irritant reactions and suggested that Langerhans cells were important in presenting antigen to T cells. Painting of allergens onto skin in which Langerhans cells had been depleted, or in which the antigen-presenting function of Langerhans cells had been inhibited by previous ultraviolet irradiation, results in specific tolerance (Toews et al, 1980) in rodents. Stingl et al, 1978 demonstrated using cells from guinea pigs that Class II-positive epidermal cells enriched with Langerhans cells could induce an antigen-specific T-cell proliferation in guinea-pigs as strong as that induced by macrophages. Braathen and coworkers (1980, 1984) showed that human epidermal Langerhans cells presented PPD of tuberculin, used as a recall antigen, to lymphocytes in association with MHC Class II molecules. They also showed that the Langerhans cells induced an allogeneic and antigen-specific T cell response without the contribution of keratinocytes.
Langerhans cells can mediate mitogen-induced proliferation of T cells (Scheynius et al, 1983) and act as stimulatory cells in cytotoxic T lymphocyte induction (Pehamberger et al, 1982; Faure et al, 1984).

Langerhans cells, like macrophages, produce Interleukin 1 as well as other soluble immunostimulatory factors that influence T cell proliferation (Sauder et al, 1984) but the significance of this is unknown.

Although the studies cited above have demonstrated that Langerhans cells can function as accessory cells, no study has defined their functional potency and no study has compared them to other accessory cells.

1.4 PATHOLOGY OF LCH

1.4.1 Histopathology of LCH

The characteristic feature of LCH is the presence of LCH cells in involved tissue. These are large (about 12μm diameter) eosinophilic cells with lobulated nuclei which are often bean-shaped. The cytoplasm is homogenous and pink when stained with haemotoxylin and eosin. In necrotic lesions there tend to be more macrophages and eosinophils than in non-necrotic
lesions and in late lesions the histology is more xanthomatous and fibrotic. Giant cells may be present in lesions in bone, lymph node or thymus but are not seen in skin lesions. The LCH cells do not show frequent or abnormal mitoses or nuclear pleomorphism and therefore look histologically benign rather than malignant.

In the skin the infiltrate is mainly in the papillary dermis but there may be exocytosis into the epidermis. Birbeck granules are easily found within the LCH cells on electron microscopy. (Favara and Jaffe, 1987).

In the liver the infiltrate is focal being localized to the portal tracts (LeBlanc et al, 1981).

Lymph node involvement may accompany otherwise localized bone or skin disease when the draining nodes from these sites are affected and this may be an insignificant finding. The nodes may also be affected in either single system, or, more commonly multisystem disease. The infiltrate is primarily sinusoidal and lesions show sheets of LCH cells that are often syncytial in appearance. Multinucleated giant cells are common.

The histology of LCH in the spleen is similar to that in lymph node but giant cells are less common.
Hamoudi et al (1982) divided thymic changes in LCH into three main groups; severe dysplasia indistinguishable from congenital thymic dysplasia with marked depletion of cortical cells but minimal changes in the medulla; non-specific involution, and dysmorphic which was similar to the dysplastic group but in addition had destruction of Hassall's corpuscles, small atrophic lobules and histiocytic infiltration of the gland.

Lung lesions of LCH are discrete and cause destruction of the small airways with cystic change. Subpleural lesions are common and may cause pneumothoraces.

Although the opposite opinion has been expressed (Hamoudi et al, 1982) it is now generally accepted that there are no histological differences which indicate disease activity or prognosis (Risdall et al, 1983).

1.4.2 LCH cells-electron microscopy

The characteristic feature seen in LCH cells is the intracytoplasmic structure known as the Birbeck granule (see 1.3.2). There may also be trilaminar membranous loops (Basset et al, 1977). Birbeck granules are never seen in the liver and rarely in the spleen (Favara and Jaffe, 1987) but the reason
for this is unknown.

1.4.3 LCH cells-histochemistry

The features are summarized in Table 1.3.2
Table 1.3.2 Cytochemical features of LCH cells

**Enzyme histochemistry**

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<th>Reaction</th>
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<td>++</td>
<td>Beckstead et al, 1984</td>
</tr>
<tr>
<td>a-naphthyl acetate esterase</td>
<td>+</td>
<td>&quot;</td>
</tr>
<tr>
<td>acid phosphatase</td>
<td>+</td>
<td>Thomas et al, 1982</td>
</tr>
<tr>
<td>alkaline phosphatase</td>
<td>-</td>
<td>Beckstead et al, 1984</td>
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<tr>
<td>a-1 antitrypsin</td>
<td>-</td>
<td>Favara and Jaffe, 1987</td>
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<tr>
<td>a-mannosidase</td>
<td>+++</td>
<td>Elleder et al, 1977</td>
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**Immunological markers**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Reaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG-Fc</td>
<td>++</td>
<td>Elema and Poppema, 1973</td>
</tr>
<tr>
<td>C3</td>
<td>++</td>
<td>Beckstead et al, 1984</td>
</tr>
<tr>
<td>Class II MHC</td>
<td>++</td>
<td>Thomas et al, 1982</td>
</tr>
<tr>
<td>CD1</td>
<td>++</td>
<td>Chollet et al, 1982</td>
</tr>
<tr>
<td>CD4</td>
<td>++</td>
<td>Harrist et al, 1983</td>
</tr>
<tr>
<td>peanut agglutinin</td>
<td>+++</td>
<td>Jaffe, 1984</td>
</tr>
</tbody>
</table>

**Marker Proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100</td>
<td>++</td>
<td>Wanatabe et al, 1983</td>
</tr>
</tbody>
</table>
Like Langerhans cells, LCH cells produce positive results on staining for ATP-ase, α-naphthyl acetate esterase where diffuse staining is also produced by monocytes, and acid phosphatase which is present in T cells, monocytes and macrophages. LCH cells and Langerhans cells both produce negative results with stains for alkaline phosphatase (present in neutrophils) and α-1 antitrypsin (found in macrophages). Intense staining for α-mannosidase is found in LCH cells and Langerhans cells and is much greater than that produced in macrophages. (Elleder et al, 1977).

1.4.4 LCH cells—immunohistochemistry

Like Langerhans cells, LCH cells express Fc receptors, C3 and MHC Class II antigens. They also express CD1a (OKT6) (Chollet et al, 1982) and CD4 (Harrist et al, 1983) antigens.

Conflicting results have been obtained with OKM1. Harrist et al (1983) found that LCH cells were positive, Rousseau-Merck et al (1983) that they were negative, Gadner et al (1986) that strongest staining
was obtained in specimens from those with aggressive
Beckstead et al, 1984 reported that both LCH and
Langerhans cells are Leu-M3 positive.
Groh and associates (1988) found that in 12 out of
21 cases LCH cells expressed immunocytochemically
detectable amounts of CR1 (C3b) and CR3 (C3bi)
receptors and the monocyte markers CDw14 (Leu-M3).
Ki-M1 and Ki-M6. The heterogeneity of phenotype did
not correlate with the clinical disease in a given
patient.

Peanut agglutinin stain produces dense cell
surface and paranuclear staining only of LCH cells
(Jaffe, 1984), interdigitating reticulum cells and
Reed-Sternberg cells. A diffuse staining pattern is
seen in a variety of other cells including Langerhans
cells but this is easily differentiated from the more
specific staining seen in LCH cells. Staining for
S100 protein produces a positive result in LCH cells
and Langerhans cells, but not in macrophages
(Wanatabe et al, 1983). This stain is very
non-specific and positive staining occurs in
melanocytes, neural tissue and a variety of tumours
(Kahn et al, 1983) including some cases of malignant
histiocytosis (Wanatabe et al, 1983)
1.4.5 LCH cells—comparison to Langerhans cells

Since Basset and Turiaf (1965) found Birbeck granules in histiocytes in LCH, this disease has been considered to be associated with the abnormal proliferation of Langerhans cells.

Birbeck granules are not always present in LCH cells particularly those from certain sites such as liver or spleen (Favara and Jaffe, 1987) but the reason for this site variability is unknown.

On histochemical examination both LCH cells and Langerhans cells contain non-specific esterase and acid phosphatase. LCH cells and Langerhans cells are both ATP-ase positive although the staining in the former is only weakly positive.

LCH cells and Langerhans cells both express Fc and C3 receptors and CD1a and Class II MHC molecules and both express S100 protein.

CD4 is positive on LCH cells but only weakly so on Langerhans cells. LCH cells also express CR1 antigen which is usually absent on Langerhans cells. Peanut agglutinin produced dense cell surface and paranuclear staining in LCH cells but only diffuse staining with Langerhans cells.
1.4.6 LCH cells—function

LCH cells and Langerhans cells both produce Interleukin-1 (Arenzana-Seisdedos et al, 1986). Like other cells in the macrophage series, LCH cells have been shown to produce prostaglandins (Gonzales-Crussi et al, 1981). In this study LCH cells from lymph node produced mainly PGD₂, however, rather than the PGE₂ which is usually predominant in monocytes and macrophages.

1.4.7 Diagnostic criteria of LCH

Using the criteria defined by the Histiocyte Society a "presumptive diagnosis" is based on histology consistent with the diagnosis using conventionally processed material. A "diagnosis" is attained when the lesion is histologically characteristic and lesional cells have at least two of the following; positive stains for S100 protein, mannosidase, ATPase or characteristic peanut agglutinin binding. A definitive diagnosis establishes the presence of Birbeck granules in lesional cells or that these cells express the CD1a antigen (Chu et al, 1987).
1.5. **Aetiology**

1.5.1 **Early Theories**

Since Hand’s description (1893) of the disease that he attributed to tuberculosis, a variety of causes has been proposed. In the 1920’s the disease was classified as a metabolic disorder and was thought to be due to a defect in cholesterol metabolism (Rowland, 1928). This theory was discarded when serum lipids of patients were found to be normal (Thannhauser, 1947).

Juberg et al (1970) reported the occurrence of LCH in monozygous twins and reviewed the literature where they found that three out of four pairs of monozygous twins were concordant for the disease and six sibships were reported with twelve out of 23 children being affected. They concluded that at least some cases of LCH were genetically determined. However at least some of these cases would probably now be classified as one of the non-Langerhans cell histiocytoses, familial haemophagocytic lymphohistiocytosis.

When Langerhans cell granules were first discovered they were thought to be viral particles and Nezelof et al (1973) argued that the clinical course of LCH suggested an infectious aetiology.
rather than a malignant one. Glass and Miller (1968) reviewed 270 deaths between 1969 and 1964 in the United States attributed to Letterer-Siwe disease. They found a geographically random distribution and little month to month variation in incidence which was against an infectious cause.

1.5.2 Immune abnormalities

More recent theories on the cause of LCH have postulated abnormalities in the immune system of these patients. In 1974 Cederbaum and coworkers reported four patients with LCH who were found at autopsy to have abnormal thymuses. One patient, on antemortem testing, had impaired B and T lymphocyte function and the unaffected sister of a second patient had classical combined immunodeficiency. These findings suggested that LCH may be associated with impaired immunity.

B lymphocyte function With regard to abnormalities found in B cell function, a pretreatment study by Leikin and associates (1973) of 13 children with LCH found that two patients had low serum immunoglobulin levels, and there was an increase in IgM in four and IgG in two other patients. Three of the patients with hypergammaglobulinaemia had concurrent infections, and in two of the non-infected children the elevated
levels subsequently fell to normal. Lahey et al (1985) found that 74 out of 102 patients with LCH which was more extensive than a single lesion of bone had at least one elevated immunoglobulin concentration, of IgM in particular. They speculated that diminished T cell suppression may lead to polyclonal B cell activation.

T lymphocyte function  Osband and others (1981) studied thymic biopsies from seven untreated patients with multisystem LCH and found abnormal histology in five. Hamoudi and coworkers (1982) studied autopsy material from 32 patients. Four had Type 1 histiocytosis X which would probably now be classified as malignant histiocytosis (Ladisch, 1982). However of the remaining 28 patients, 23 had thymic abnormalities. These findings suggested that the T lymphoid arm of the immune system might be abnormal.

An early study by Leiken and colleagues (1973) on peripheral blood from 13 untreated children with multisystem disease showed normal lymphocyte function in most of the children. In two however their lymphocytes were hyporeactive to mitogen-induced stimulation but this abnormality disappeared after chemotherapy was given.
Thommesen and others (1978) found normal lymphocyte proliferative responses to mitogens in seven patients in remission; however Nesbit et al (1981) found at least one abnormal result of T cell function in six out of eight patients studied before treatment. Lahey et al (1985) reported a normal proliferative response of T cells exposed to the mitogen PHA in 41 out of 44 patients studied.

A study by Osband and associates published in 1981 generated considerable interest as they described a consistent defect of suppressor T cells in patients with LCH. Out of 17 patients eight had low lymphocyte H2 receptor activity (a membrane marker for T suppressor activity). Twelve patients had either circulating lymphocytes spontaneously cytotoxic to cultured human fibroblasts or an antibody to autologous erythrocytes, both suggestive of a defect of T suppressor cells. The total number of T cells as assessed using monoclonal antibodies was normal. In vitro incubation of the cells in thymic extract led to a reversal of the immunological abnormalities. In vivo treatment of the patients with thymic extract led to clinical improvement in 10 of the 17 patients and a reversal of the abnormal immune function tests. Davies et al (1983) found, in contrast, that both the total number of T cells and those expressing the T8 antigen (a marker of suppressor cells) were decreased.
in 26 children with LCH, but the degree of abnormality did not correspond to the severity of the disease. Broadbent and coworkers (1984) found a high T4:T8 ratio in two patients which reverted to normal with spontaneous regression of disease. Shannon and Newton (1986) found that eight patients with active LCH had low numbers of T-suppressor cells but that the results in 15 patients with inactive disease did not significantly differ from controls. More recent studies have found a reduction in T-helper cells (Zaizov et al, 1986) or no consistent abnormality (Ceci et al, 1986).

Kragballe and others (1981) postulated an intrinsic functional defect in the mononuclear phagocyte system in LCH as they found that monocytes from six patients with LCH, all in clinical remission, exhibited reduced antibody-mediated cytotoxicity.

A variety of different and conflicting findings has been reported after study of immune function in these patients and it seems likely that these are epiphenomena rather than causative of the disease.

1.5.3 Is it a malignancy?

For many years Letterer-Siwe disease was thought to behave in a similar manner to malignancies and to be invariably fatal (Aronson, 1951). On this basis
Letterer-Siwe disease has been treated with cytotoxic drugs (Beier et al, 1963) since the 1960's. There is, however, no evidence of a monoclonal proliferation of cells in this disease (Broadbent and Pritchard, 1985), nor is there a correlation between the degree of histological differentiation and clinical course of the disease (Risdall et al, 1983).

Several cases of LCH histology occurring in the lymph nodes of patients with lymphoma have been found. Kjeldsberg and Kim (1980) reported three patients with Hodgkin's lymphoma and three with non-Hodgkin's lymphoma who had histological LCH lesions in the same lymph nodes as the lymphoma. Burns and coworkers (1983) reported five others with Hodgkin's disease and one with non-Hodgkins lymphoma who had coincidental LCH. In a review of 659 cases of Hodgkin's disease (Colby et al, 1981) two cases also had LCH lesions in the involved lymph nodes. An additional case of coincidence of LCH with a composite lymphoma has been reported (Almanaseer et al, 1986). The histological findings could be due to a common predisposing factor or the abnormal immunity in one disease may predispose the patient to the other. These patients did not, however, have clinical LCH but these were only incidental histological findings on lymph node biopsy and the relevance of this seems doubtful.
The spontaneous resolution of most cases of single system LCH and the occasional spontaneous resolution of cases with multisystem disease (Broadbent et al, 1984) has influenced most clinicians to now regard LCH as a nonmalignant disorder.

1.6. Clinical course

Single system disease of bone is the most common form of the disease and is probably often undetected. Flat bones are more commonly affected, particularly those of the skull whilst the femur is the most common long bone to be involved (Whitehouse, 1971). The serum calcium, phosphate and alkaline phosphatase are normal (McGavren and Spaldy, 1960). Radiography reveals destructive lesions of the medulla, and usually the cortex, but bone expansion may also be found (Whitehouse, 1971). The punched-out lesions are not detected as well on radionuclide scanning as on plain radiography (Parker et al, 1980). Lesions in the vertebra can lead to vertebral collapse and may threaten the spinal cord (Berry and Becton, 1987). Bone disease can resolve spontaneously and respond well to local treatments.

Calvarial lesions may spread into the brain substance (Avery et al, 1957). The pituitary is the most common area of the brain to be affected and
diabetes insipidus nearly always precedes anterior pituitary or hypothalamic involvement. Cerebellar involvement is most common in those with longstanding disease who have diabetes insipidus (Braunstein et al, 1973). The brain may occasionally be the only site of disease but no part of the central nervous system is exempt (Kepes, 1986). Radiotherapy is the most commonly used method of treatment and response is usually good. Mild mental retardation and maladjustment disorders have been reported in long-term follow-up of patients with LCH (Sims, 1977).

Diabetes insipidus is common in those patients with multisystem disease and the incidence in children is about 30% (Sims, 1977). Radiotherapy may help reverse some cases (Greenberger et al, 1979) but the presence of diabetes insipidus does not affect prognosis (Berry and Becton, 1987). The anterior pituitary and hypothalamus may be involved, usually in association with diabetes insipidus, and thyroid infiltration has been reported (Avery et al, 1957).

Isolated lung LCH is largely a disease of young and middle-aged adults (Basset et al, 1978) and is more common in males (Basset et al, 1978) and cigarette smokers (Friedman et al, 1981). Pulmonary involvement may present with dyspnoea, cough,
tachypnoea or pneumothoraces. Radiography shows micronodular and reticular shadowing particularly in the mid-zones and bases, but often sparing the costophrenic angles. Pulmonary function tests show a restrictive pattern with impaired gas diffusion (Berry and Becton, 1987). LCH cells may be obtained by bronchial lavage (Basset et al, 1977).

Oral symptoms include premature eruption or loosening of teeth, mucosal ulceration and gingival infiltration (Berry and Becton, 1987). The mandible is commonly involved (Hartman, 1980). Involvement of the ear occurred in 21% of patients in one series, usually presenting as an otitis externa, but some cases progress to mastoiditis and deafness (Smith and Evans, 1984).

Liver involvement usually presents as hepatomegaly with mild elevation of liver function tests, or as cholestasis due to fibrotic obstruction of the biliary tree as a consequence of previous infiltration (Le blanc et al, 1981).

Cutaneous disease is usually seen as a maculopapular and often purpuric eruption on the scalp, trunk, flexures and perineal areas (Avery et al, 1957). It may also present as a single plaque.

Lymph node infiltration often occurs in draining
nodes from skin or bone lesions. Bone marrow infiltration, when it leads to haemopoetic system dysfunction, is a bad prognostic feature (Lahey, 1975).

1.7 Scoring systems

Various attempts have been made to establish a scoring system to predict prognosis and to help guide treatment decisions. Lahey (1962) proposed a scoring system in which one point was allocated for each organ system involved, as determined by clinical assessment or simple laboratory investigation. He found an almost linear relationship between mortality rate and score; the mortality was nil with a score of one, 35% with a score of 3 or 4 and 100% with a score of 8. He later (1975) defined "vital organ failure" of liver, lungs or haemopoetic systems and stressed that this conferred a poor prognosis.

Greenberger et al (1981) devised their own staging system but supported Lahey's contention that it is organ failure rather than involvement per se that is prognostically important.

A recent studies in Italy (Ceci et al,) has used its own adaptation of the Lahey system but the details of this are as yet unpublished.
1.8 Treatment

1.8.1 Local Measures

Because of the excellent prognosis and the rarity of disease-related morbidity in cases with single-system involvement (Slater and Swarm, 1980), if treatment is indicated in these patients it can often be carried out using local measures alone. In the treatment of bone lesions there has been no comparative trial comparing intralesional steroids to low-dose radiotherapy to the lesion but both methods of treatment are effective although the former carries less risk of long term side-effects.

In the treatment of multisystem disease the use of local treatment measures is particularly favoured by those who advocate a conservative rather than an aggressive approach. Topical application of mustine hydrochloride, as an aqueous solution at a concentration of 20mg/100ml can be useful for treating skin lesions of LCH. Contact sensitization seems to be less of a problem than when it is used in mycosis fungoides but there is a theoretical risk of inducing cutaneous malignancy (Atherton et al, 1986).

Ultraviolet light has been found to be helpful in treating elderly patients with skin disease (Iwatsuki
et al, 1986), PUVA (psoralen plus ultraviolet A light) being better than ultraviolet B, but both again carry a risk of inducing skin malignancies.

1.8.2 Chemotherapy

Chemotherapy is rarely indicated in single system LCH (Starling, 1980). For multisystem LCH a variety of agents has been used, either singly or in combination. In view of the known incidence of spontaneous remission and the undesirable side effects of cytotoxic drugs, some groups advocate a conservative approach to treatment (Broadbent and Pritchard, 1985). If treatment is needed at all, these groups initially use prednisolone, 2mg/kg/day in short courses of three months.

Other groups are more aggressive and always use cytotoxic drugs (Ceci et al, Gadner et al, 1987). Vinblastine (Beier et al, 1963), chlorambucil, methotrexate and 6-mercaptopurine (Starling, 1980) are all effective drugs and etoposide is very promising (Starling, 1987).

There is no proof that the response rate to combination chemotherapy is higher than to single-agent treatment (Pritchard, 1979; Komp et al, 1979) whereas toxicity is higher particularly in children under the age of one (Starling, 1981).
A North American group found that maintenance treatment improved remission duration but the numbers were small with only six patients on maintenance treatment and nine control patients (Jones et al, 1974). There has been no other study randomizing maintenance treatment versus no maintenance treatment.

1.8.3 Radiotherapy

Radiotherapy is used for bone disease for symptomatic relief of pain or when vital organs such as the spinal cord are threatened (Greenberger et al, 1979). A total dose of 450 to 600 cGy may be effective although 600 to 1000 cGy are usually given, in 200 cGy daily fractions (Cassady, 1987). Skin disease can be treated with radiotherapy and electron beam therapy restricts irradiation to the superficial layers.

Pituitary irradiation, at a dose of 600 to 1200 cGy in three to eight fractions, has been recommended (Cassady, 1987) for pituitary involvement. The evidence for its effectiveness is, however, not very good. In the Greenberger series (1979) the diagnosis was based on clinical findings rather than laboratory tests. Diabetes insipidus may be intermittent or partial in LCH (McLelland and Broadbent, 1988) so the clinical improvement in those
patients who seemed to respond could have happened spontaneously.

1.8.4 Immunotherapy

Osband and others (1981) found that a crude calf thymic extract caused reversal in vitro of the various abnormalities that they identified in 12 out of 17 patients with multisystem disease. All 17 patients were treated with daily injections of this extract and the response was reported to be at least as good as that of controls treated with chemotherapy. Clinical response correlated with an in vitro reversal of the immunologic abnormalities. A major problem in this study however, was that only historical controls were used so it is not known how the response compares to spontaneous variation in the disease or to how they would have responded to conventional treatment for that time.

Davies and associates (1983) studied 26 children and confirmed that the high blood T4:T8 ratio fell after incubation of the lymphocytes with TP5, a synthetic pentapeptide with thymic hormone activity. Three children were treated with TP5 and one with thymostimulin, an extract similar to that used by Osband et al. All had a decrease in T-cell numbers, a decrease in the T4:T8 ratio, or both, but no
patient showed clinical improvement. Treatment was withdrawn in all and conventional chemotherapy, to which all initially responded, substituted.

In a study by Ceci et al (personal communication) in Italy using thymostimulin as used by Osband et al, response rate was also very low and treatment with this was abandoned.

1.8.5 New treatments

There has been an isolated case report of two patients responding to alpha-interferon (Jakobson et al, 1987), but the significance of this is difficult to assess.

A patient with multisystem LCH who was treated by allogeneic bone marrow transplantation has been reported (Ringden at al, 1987). As this patient was 19 years old at the time of transplantation, and had already had the disease for eight years, his prognosis was, in fact, very good and he was not the best candidate for such radical treatment (Komp, 1987).

1.9 Outcome

In patients presenting with single system disease in the form of bone involvement, the disease rarely spreads to extraskeletal sites and the patients fare
extremely well. The number of bony lesions does not affect survival (Greenberger et al, 1981). In a review of 686 cases of isolated bone involvement (Slater and Swarm, 1980) a 'remission rate' of 95% was reported.

In multisystem LCH age seems to an important prognostic factor independent of the finding that older patients are more likely to have single system disease (Greenberger et al, 1981). In a series of 151 patients, 35 out of 95 (37%) under 24 months at diagnosis died compared to 9 out of 56 (16%) over 24 months (Komp et al, 1981). Survival curves flatten out about five years after diagnosis (Greenberger et al, 1981).

Nezelof et al (1979) studied 50 cases retrospectively and found that those with thrombocytopenia, anaemia, jaundice, hepatosplenomegaly, respiratory insufficiency and no osteolytic lesions had a high mortality rate. On the other hand those with skin lesions, diabetes insipidus, multiple bone lesions and the absence of functional respiratory involvement did well.

In a review by Broadbent (1986) of 70 cases of biopsy proven LCH in children presenting between 1961 and 1982 eight patients died. All were under two at diagnosis and all had vital organ failure (Lahey, 1975) of lung, liver or bone marrow. Bone disease and
absence of skin rash were favourable features. Seven of the eight died before reaching two years of age and the eighth died three years after diagnosis.

50% of survivors have residual disabilities (Komp et al, 1980; Sims, 1977) most of them in children with disease of more than five years duration. The main disabilities are diabetes insipidus, short stature, deafness and orthopaedic or lung abnormalities (Broadbent and Pritchard, 1985). Fatal outcome from disabilities is particularly seen in pulmonary disease, either from progressive fibrosis or opportunistic infection (Komp, 1980). In Greenberger's series (1981) 5% of patients had already developed malignancies directly attributable to their treatment with radiotherapy or cytotoxic drugs.
1.10 Aims of this study

1. Clinical studies.
   a) To study patients currently under the care of the Hospital for Sick Children, Great Ormond Street to see how their treatment and outcome compares to those at other centres.
   b) To see whether a newly devised scoring system is better or worse in predicting the outcome of patients than the existing most widely used system devised by Lahey.

2. Cellular immunologic studies.
   a) To produce a cell line of LCH cells which could be used for functional studies of these cells. This would help to overcome the difficulties in obtaining tissue when the disease is rare and involves mainly children so specimens are small and ethically difficult to obtain except when procedures are performed for diagnostic or treatment purposes.
   b) To apply a new technique of culturing small numbers of cells in hanging droplets to the culture of Langerhans and LCH cells.
   c) To study the functional activity of normal Langerhans cells.
   d) To look at the functional activities of LCH cells, particularly as accessory cells.
3. Immunohistochemistry.
   
a) To compare a new stain of histological specimens, peanut agglutinin, and see how it compares to the S100 stain, which is presently the most widely used stain in making the diagnosis of LCH.

    b) To raise a monoclonal antibody specific to LCH cells which could potentially be used in diagnosis and treatment.

4. Study of the nature of the disease.

   To study LCH cells using the flow cytometer to see if they show DNA aneuploidy, which would infer that LCH is a malignant or premalignant condition. If only some cells showed aneuploidy then this may be an indication of prognosis and therefore influence treatment.
METHODS

2.1 Scoring systems

2.1.1 Lahey scoring system
Devised 1962, modified 1975 (see refs)
One point is given for each of the following systems involved by history, examination or simple laboratory investigation.

1. skin
2. liver- as defined by oedema, ascites, total serum protein <55g/l, albumin <25g/l, and/or bilirubin >1.5mg/100ml
3. spleen
4. lung- tachypnoea and/or dyspnoea, cyanosis, cough, pneumothorax or pleural effusion (not radiographic abnormalities alone)
5. pituitary
6. skeleton
7. haemoglobin <10g/dl; white cell count <4 or >14x10^3/dl
8. platelets <100,000
2.1.2 Proposed new scoring system devised by V Broadbent

One point is awarded for each feature

dyspnoea
tachypnoea >40/min at rest
grunting respirations
rib recession
pallor
purpura and/or bleeding spontaneously
splenomegaly >2cm below costal margin
hepatomegaly
jaundice
ascites
fever >37.5°C not due to infection
failure to thrive
extensive skin disease (>6% of surface area)
absence of bony disease
haemoglobin <8g/dl
platelets <100,000
serum protein <50g/l
serum albumin <20g/l
abnormal clotting studies
reduced lung compliance and/or reduced vital capacity, increased pulmonary resistance, increased lung markings
2.2 Names and addresses of firms commonly used

All chemicals were obtained from Sigma Chemical Co unless otherwise stated.

All tissue culture media was obtained from Flow Laboratories unless otherwise stated.

Amersham Radiochemical Centre,
Amersham, Bucks

BDH Ltd
Broom Road, Poole
Dorset BH12 4NN

Becton Dickinson
Scientific Supplies Co Ltd
Vine Hill
London EC1R 5EB

Dakopatts
Mercia Brocades,
Brocades House, Pyrford Rd
West Byfleet,  
Weybridge, Surrey.

Flow Labs  
Victoria Park,  
Heatherstone Rd  
Irvine  
Scotland KA12 8NB

Gibco Ltd  
Trident House  
PO Box 35, Renfrew Road  
Paisley PA3 4EF

Nygaard and Co  
Diagnostics Division  
PO Box 4220 Torshov  
N-0401 Oslo 4, Norway

Sigma Chemical Co  
Fancy Road, Poole  
Dorset BH17 7NH

Sterilin Ltd  
Teddington
Middlesex

Sterling Research Labs
Onslow St, Guilford
Surrey

2.3 Solutions commonly used

**Phosphate buffered saline (PBS) pH 7.2**

sodium chloride 8.0g
potassium chloride 0.2g
disodium hydrogen phosphate 1.15g
potassium dihydrogen phosphate 0.2g
distilled water to make up to 1 Litre

**Tris buffered saline (TBS) pH 7.6**

Tris hydrochloride 6.05g
sodium chloride 8.6g
The salts were dissolved in 500ml of distilled water
then the pH was adjusted to 7.6 with 1M Hydrochloric acid. Distilled water was added to make the volume up to 1 Litre.
APAAP substrate

10mg of naphthol AS-MX phosphate was dissolved in 1ml of dimethylformamide in a glass flask. 49ml of 0.1M Tris hydrochloride buffer (adjusted to a pH of 8.2 with 1M hydrochloric acid) and 12mg of levamisole were then added. This was stored for up to one month at 4°C. Just before use 10mg of Fast Red dye was added to 10ml of solution and filtered through filter paper in a funnel directly onto the slides.

Veronal acetate buffer pH 4.0

- sodium acetate 100mg
- sodium diethylbarbitone 150mg
- 0.1M hydrochloric acid 13ml
- distilled water 13ml

Graham Karnovsky medium (Graham and Karnovsky, 1966)

2mg of diaminobenzidine was dissolved in 10 ml of TBS pH 7.6 (see above). 100ul of 28% hydrogen peroxide was added just before use.

Phosphate buffer x molar, pH y.
x molar sodium biphosphate
x molar sodium dihydrogen phosphate
The two solutions were mixed together in the proportions necessary to obtain the desired pH.

**Hexazotized pararosanilin**

2g of pararosanilin was dissolved in 50ml of 2M hydrochloric acid by heating gently. The solution was cooled to room temperature, filtered through filter paper and stored at 4°C. Equal volumes of acidified pararosanilin and freshly made sodium nitrite at a concentration of 2% in distilled water were combined and allowed to stand for two minutes.

**Scott's tap water**

sodium bicarbonate 3.5g
magnesium sulphate 20g
tap water to make solution up to 1litre

**red cell lysis fluid**

ammonium chloride 8.29g
potassium bicarbonate 1g
disodium EDTA 0.2g
The salts were dissolved in distilled water which was adjusted to pH 7.4 with acetic acid and made up to 100ml. The solution was stored at 4°C for up to six weeks and immediately before use was diluted ten-fold with distilled water.

Ethidium bromide and acridine orange

50mg ethidium bromide
15mg acridine orange
These were dissolved in 1ml ethanol and 49ml distilled water added. This was stored at -20°C and before use it was diluted 1/100 in FBS then kept in an amber bottle at room temperature for up to a month.

srbc-adsorbed FBS

FBS was combined with one-third of the volume of packed srbc and incubated at 37°C for 30 minutes then left overnight at 4°C. The cells were centrifuged at 2000 rpm and the serum removed. This srbc-adsorbed FBS was stored at -20°C until required.
Eisinger medium

Dulbeccos Modified Eagles medium with
10% FBS
10ug/ml penicillin
100ug/ml streptomycin
2.5ug/ml amphotericin B
0.8ug/ml hydrocortisone
2mmol/l l-glutamine

The pH was adjusted to 5.6-5.8 with concentrated hydrochloric acid

Esterase fixative

acetone 40ml
formalin 35% 25ml
distilled water 30ml
disodium biphosphate 20mg
potassium dihydrogen phosphate 100mg

The above were mixed together and stored at 4°C for up to a month.

2.4 Staining methods
2.4.1 Cutting paraffin embedded material

Twin frosted glass slides were coated with 0.1% poly-l-lysine in water (Sigma) and air dried. 5um sections were cut and placed on the slides and dried at 37°C overnight.

2.4.2 Cutting frozen sections

5um sections were cut and placed on glass slides which had previously been coated with 0.1% poly-l-lysine in water (Sigma) and air-dried. The sections were then air-dried at room temperature for at least one hour before use.

2.4.3 Cytospin preparations

Cells at a concentration of $5 \times 10^5$ to $10^6$ /ml RPMI tissue culture medium were used. Three drops were placed in the funnel of a Shandon cytospin and spun for 10 seconds at 10,000 rpm. The cells were centrifuged onto a glass slide which was then air dried at room temperature for at least an hour.

2.4.4 Staining for S100 protein
The sections were dewaxed in xylene for three minutes then washed in 95% ethanol for one minute followed by tap water for five minutes. The sections were then incubated for 15 minutes in 0.01% protease (Sigma) in PBS (2.3). The section was covered with peroxidase-conjugated rabbit anti-cow S100 antibody (Dakopatts) at a dilution of 1/75 in PBS with 0.5% bovine serum albumin. After 45 minutes incubation the sections were washed three times in PBS. Peroxidase activity was revealed using the Graham Karnovsky medium (2.3) which was placed on the section and then the slides were incubated in the dark for five minutes.

Sections were counterstained with haematoxylin by placing haematoxylin on the slide for one minute and then washing it off with tap water. The sections were dehydrated by placing them in 95% alcohol for 5 minutes, cleared by placing them in xylene for 5 minutes and mounted in DPX mounting medium with a cover slip over the top then allowed to dry.

Sections from a benign melanocytic naevus, where the S100 stain produces staining of melanocytes, were used as a positive control.
2.4.5 Staining for peanut agglutinin

(Leathem and Atkins, 1983b)

The sections were dewaxed in xylene for three minutes, washed in ethanol for one minute then in tap water for five minutes. Endogenous peroxidase was blocked by incubating with 1% hydrogen peroxide in methanol for 20 minutes followed by a wash in tap water then a further wash in TBS pH 7.6 (2.3). The section was then covered with peanut agglutinin 10ug/ml of TBS and incubated for 30 minutes. Following a wash in TBS the sections were incubated with rabbit anti-peanut agglutinin antibody (Dakopatts) at a dilution of 1/100 in TBS for 30 minutes and washed in TBS. The sections were incubated with peroxidase-conjugated swine anti-rabbit antibody (Dakopatts) at a dilution of 1/50 in TBS for 30 minutes then washed in TBS.

Peroxidase activity was then revealed using the Graham Karnovsky medium (section 2.2) which was placed on the sections which were then incubated in the dark for five minutes.

The sections were counterstained with haematoxylin, dehydrated in alcohol, cleared in xylene and then mounted in DPX as in 2.4.4.
Sections of kidney, where the peanut agglutinin stains the cells of the distal convoluted tubules (Leatham and Atkins, 1983a) were used as a positive control.

A specificity control was carried out as above but incubating the lectin in the presence of 0.1M galactose, to which sugar the lectin preferentially binds (Leatham and Atkins, 1983a).

2.4.6 Two-step method of peanut agglutinin stain

This was carried out as above, but incubation for 30 minutes with peroxidase-conjugated peanut agglutinin (Dakopatts) at a concentration of 1/20 in TBS was substituted for the incubations with the lectin and peroxidase-conjugated anti-peanut agglutinin.

2.4.7 Indirect peroxidase staining of frozen tissue sections

The sections were fixed in acetone for 10 seconds at room temperature, dried then washed three times in PBS (2.3). 15ul of the primary monoclonal antibody at the appropriate dilution as indicated by the instructions supplied with the antibody was placed on each section and the slides incubated for 30 minutes at room temperature. The sections were washed three
times in PBS then incubated with peroxidase-conjugated rabbit anti-mouse antibody (Dakopatts) for 30 minutes at room temperature. The slides were washed three times in azide-free PBS then Graham Karnovsky medium added as in 2.4.4. The sections were counterstained with haematoxylin, cleared in xylene, dehydrated in alcohol and mounted in DPX mounting medium as in 2.4.4. Positive and negative controls were also examined.

2.4.8 Indirect immunofluorescent staining of sections

Frozen tissue sections or cytospin preparations of cells were used. The sections were fixed in acetone for 10 seconds at room temperature, dried then washed three times in PBS (2.3). The slides were dried around each section. 15ul of the primary monoclonal antibody at the appropriate dilution in PBS as indicated by the instructions supplied with the antibody was added to each section and the slides incubated at room temperature for 30 minutes. The slides were washed three times in PBS then 15ul of FITC-conjugated rabbit anti-mouse antibody at 1: 100 dilution in PBS with 20% human serum was added. The slides were incubated for a further 30 minutes at room temperature then washed three times in PBS. The sections were then examined under a Zeiss
fluorescence microscope.
Positive fluorescence was seen as a bright green colour.
Positive and negative controls were also examined.

2.4.9 Staining by APAAP technique

(Cordell et al, 1984).
Tissue sections or cytospin preparations were fixed in acetone for 10 seconds at room temperature, dried then washed in TBS pH 7.6 (2.3) for three minutes. The primary monoclonal antibody was added at the appropriate dilution and the slides incubated in a moist chamber at 4°C overnight or at room temperature for 30 minutes. Following a wash in TBS 15ul of anti-mouse immunoglobulin (Dakopatts) at a dilution of 1/25 in TBS was added to each section. The slides were incubated in a moist chamber at room temperature for 30 minutes. The slides were washed in TBS then alkaline phosphatase-anti-alkaline phosphatase (APAAP) (Dakopatts) was added at a dilution of 1/25 in TBS for 30 minutes at room temperature. If the initial incubation had been for 30 minutes rather than overnight then the last two steps were repeated. The slides were washed in TBS then APAAP substrate (2.3) was added for 15 minutes at room temperature. The slides were washed in TBS and then tap water.
Haematoxylin was used as a counterstain and was made more blue in Scott's tap water (2.3). The slides were mounted in glycergel, an aqueous mounting medium (Dakopatts) and a cover slip placed over the sections which were allowed to dry.

Positive and negative controls were made simultaneously.

2.4.10 Non-specific esterase stain

The slides were immersed in esterase fixative (2.3) at 4°C for 90 seconds, rinsed in water and dried. 50mg of a-naphthyl acetate was dissolved in 2.5 ml of 2-methoxyethanol. 44.5ml of 0.05M phosphate buffer pH 7.4 (2.3) was added followed by 3ml of hexazotized pararosanilin (2.3). The pH was adjusted to 6.1 with 1M Hydrochloric acid and the solution filtered immediately before use. The solution was applied to the slides for 45 minutes at 37°C, washed off with tap water and the slides counterstained with 1% methyl green in veronal acetate buffer pH 4.0 (2.3) for one minute. Following a wash in tap water the sections were mounted in aqueous mounting medium.

Inhibition with sodium fluoride
75mg of sodium fluoride was added to 50ml of incubation medium simultaneously with the above reaction to test the sodium fluoride sensitivity of a cell population.

A whole blood smear was used as a positive control. Lymphocytes give a dot-like reaction which is fluoride resistant and monocytes give a more diffuse reaction product which is inhibited by the presence of fluoride (Dacie and Lewis, 1984).

2.4.11 Acid phosphatase stain

Incubating medium
32 mg sodium b-glycerophosphate
5ml 0.1M sodium acetate adjusted to pH of 5.0 with 0.1M acetic acid
5ml 0.008M lead acetate

Sections or cytospin preparations were incubated in the above medium for 30 minutes at 37°C. They were washed in tap water for 30 seconds then immersed in 200ml of 0.05% ammonium sulphide in distilled water. The slides were washed well in tap water, counterstained with haematoxylin for one minute then mounted in aqueous mounting medium and covered with
Activity is indicated by a brown reaction product and is found in T cells, monocytes and macrophages (Dacie and Lewis, 1984). A blood smear was used as a positive control.

2.4.12 a-mannosidase stain

(Method from Prof B Lake)
Optimum results are obtained using unfixed sections placed on Visking tubing 24/32 stretched over a tube into which the incubation medium is placed. Unfixed cryostat sections and cytospin preparations on glass slides were also used.

Incubation medium
2.5mg of a-naphthyl-x-mannoside (Koch) dissolved in
100ul of 2-methoxyethanol.
5ml distilled water
5ml of McIlvaine buffer (0.2M sodium phosphate, 0.1M citric acid); proportions adjusted to give a pH of 5.0
0.5ml hexazotized pararosanilin
The pH was adjusted to 5.5 using 1M hydrochloric acid then 500mg polyethylene glycol 6000 was added.

The sections were incubated in the incubation medium overnight at room temperature, then the membrane was cut out and washed in tap water. The sections were counterstained with haematoxylin, dehydrated in alcohol, cleared in xylene and mounted in DPX as in 2.4.4.

Activity is shown by a reddish-brown colour and deep staining indicating intense activity is found in Langerhans cells and LCH cells (Elleder et al, 1977). A section of skin, in which the Langerhans cells are densely stained, was used as a positive control.

2.4.13 Lysozyme stain

The test must be performed within one hour of collecting the sample. The sample was collected in a blood collection tube containing EDTA and 0.5ml of the cell suspension and 0.5ml of a fresh suspension of dried micrococcus lysodeikticus (Difco) at a concentration of 60mg/ml of normal saline were mixed in a tube and gently shaken for 10 seconds. The cells were then smeared onto glass slides and air dried. They were fixed in 1 volume of 10% neutral formalin
to 2 volumes of 96% ethanol for one minute, rinsed in
0.01mmol/l phosphate buffer pH 7.0 (2.3) then
incubated for 10 minutes in the buffer in a Coplin
jar at 37°C. The slides were dried and
counterstained with May-Grunwald-Giemsa stain using
an automated system.

Positive activity is seen as an area of bacterial
lysis in the vicinity of the cells and is found in
macrophages, monocytes and neutrophils (Dacie and
Lewis, 1984). Whole blood was used as a positive
control.

2.5 Tissue handling

2.5.1 Conditions of handling

All tissue culture work was carried out in a laminar
flow hood using a no-touch technique. Media was
either autoclaved or filtered through an 0.2um filter
(Flow) before use. Containers and glasswear were
either sterilized commercially or autoclaved before
use.
2.5.2 Media and solutions commonly used

Decontamination medium

100ml RPMI 1640 (Gibco) buffered with 3.25 ml of 7.5% sodium bicarbonate
penicillin 10mg (Gibco)
streptomycin 100mg (Gibco)
fungizone 2.5mg (Gibco)

RPMI tissue culture medium

450ml RPMI 1640 tissue culture medium buffered with
16.25 ml of 7.5% sodium bicarbonate (Gibco)
penicillin 5mg (Gibco)
streptomycin 50mg (Gibco)
5 ml of l-glutamine 200mMol (Gibco) added within 1 week of use

For RPMI with 10% foetal bovine serum (FBS), 10ml of FBS (Gibco) was added to 90ml of the above mixture.

Medium for Rheinwald Green keratinocyte culture technique
450ml RPMI 1640 tissue culture medium buffered with
  16.25 ml of 7.5% sodium bicarbonate (Gibco)
50ml foetal bovine serum (FBS) (Gibco)
5mg penicillin (Gibco)
50mg streptomycin (Gibco)
1.25mg amphotericin (Gibco)
2.5mg hydrocortisone
5ml of 200mmol/l l-glutamine (Gibco) added within 1
  week of use
2.5ug cholera toxin (Sigma)

Freezing mixture

12 ml RPMI 1640 tissue culture medium buffered with
  16.25ml of 7.5% sodium bicarbonate (Gibco)
4ml foetal bovine serum (Gibco)
4ml dimethylsulphoxide (Sigma)

Ficoll-hypaque

34% sodium hypaque (Sterling)
9% ficoll 400 (Sterling)
Approximately 500ml of hypaque and 1200ml of ficoll were combined and the proportion adjusted to produce a specific gravity of 1.078 measured using a hygrometer. This was sterilized by autoclaving.

2.5.3 Counting cells

Cells were adjusted to approximately $10^6$/ml. A 25ul aliquot was taken and combined with an equal volume of ethidium bromide and acridine orange (2.3). The cells were placed on a Neubauer haemocytometer and examined under a Zeiss fluorescent microscope using ultraviolet light. Viable nucleated cells show green fluorescence. The number of cells per ml in the original solution is equal to the number in two large boxes of the haemocytometer multiplied by $10^4$/ml.

2.5.4 Washing cells

Cells were suspended in RPMI 1640 (2.5.2) then centrifuged at 1200rpm (200g) for 5 minutes in an IEC Centra 7 centrifuge at 4°C. The supernatant was discarded and the cells resuspended in fresh RPMI by tapping the centrifuge tube gently.
2.5.5 Freezing cells

Cells were washed twice in RPMI 1640 (2.5.2). They were then counted and resuspended at $10^7-10^8$/ml in RPMI with 20% FBS (2.5.2). The cells were placed on ice then an equal volume of freezing mixture (2.5.2) added dropwise. The cells were left at 4°C for 10-15 minutes then 1ml aliquots placed in sterile tubes. The tubes were wrapped in paper tissue and frozen at -70°C. After 4-6 hours they were transferred to a liquid Nitrogen container.

2.5.6 Thawing cells

The vial of cells was removed from the liquid Nitrogen tank and thawed at 37°C. 2ml of RPMI 1640 containing 10% FBS (2.5.2) was added dropwise at room temperature, left for 5 minutes then a further 2ml added. This was repeated until the total volume was 10ml then the cells were washed three times (2.5.4) in RPMI with 10% FBS and counted.
2.5.7 Preparing cells for electron microscopy

Approximately $10^6$ cells were centrifuged at 1500rpm for 5 minutes and the supernatant discarded. The cells were then resuspended in 5ml of glutaraldehyde 2%, left for 5 minutes, respun and the supernatant discarded. Veronal acetate buffer (2.3) adjusted to a pH 7.4 was added and the cells resuspended, added to 2ml plastic capsules and respun to form a pellet. This was dehydrated in ethanol, embedded in Epon 812 and ultrathin sections stained with a saturated solution of uranyl acetate in ethanol. These were then examined using a transmission electron microscope.

2.5.8 Testing cells for Fc receptors

Sheep red blood cells (srbc) were washed three times in PBS then resuspended at a concentration of $2 \times 10^8$/ml in RPMI 1640 (2.3). 0.5ml aliquots of the srbc were incubated with dilutions of rabbit srbc antiserum (Dako) for 30 minutes at 37°C, washed three times (2.5.4) in RPMI and resuspended in 1ml of medium. An aliquot of the srbc was then examined under a microscope for agglutination. The lowest dilution of antiserum which, when combined with srbc, did not show agglutination was used as the source of
sensitised srbc.

The cells to be tested were adjusted to a concentration of $5 \times 10^6$/ml and 100 ul was combined with 200 ul of coated srbc. The cells were incubated at room temperature for 60-90 minutes then 50 ul of acridine orange was added and the number of cells surrounded by srbc rosettes was calculated. Macrophages, polymorphonuclear cells and lymphocytes all have Fc receptors and PBMs were used as a positive control.
2.5.9 Growing epidermal cells by Rheinwald Green technique

(As described by Rheinwald and Green, 1975)

3T3 fibroblasts were used as feeder cells. The 3T3 cells were split to one-third of their original concentration by adding fresh medium approximately every 10 days. To use as feeder cells the cells were adjusted to a concentration of $10^6$/ml and were irradiated with 6.5 Gy using a blood irradiation machine, to prevent cell division.

These cells were plated out at $5 \times 10^5$ cells/25cm$^2$ petri dish. After 24 hours freshly separated epidermal cells were added at a concentration of $5 \times 10^5$/ml in Rheinwald Green medium (2.5.2).

2.5.10 Growing epidermal cells by Eisinger technique

(As described by Eisinger et al, 1979)

Freshly separated epidermal cells at $1 \times 10^6$/cm$^2$ were grown in Eisinger medium pH 5.6-5.8 (2.5.2). The cells were incubated at 37°C in 5% CO2 and fed twice weekly. Confluent monolayers were generated after 10 days. The cells were incubated at 37°C in 5% CO2.

2.5.11 Separation of bone marrow cells
Aspirated bone marrow was placed in a sterile container with 200 U/ml of preservative-free heparin. The tissue was then placed in decontamination medium (2.5.2) for 30 minutes then centrifuged at 1500 rpm for 5 minutes and the cells resuspended in RPMI (2.5.2). The cell suspension was then layered onto a similar volume of ficoll-hypaque density gradient centrifugation medium (2.5.2) in a centrifuge tube and spun at 1500rpm (250g) for 30 minutes at room temperature. Cells were removed from the interface and washed three times (2.5.4) in RPMI tissue culture medium.
2.5.12 Separation of spleen cells

The tissue was cut into pieces about 3 mm³ and placed in decontamination medium (2.5.2) for 30 minutes. The tissue was washed three times (2.5.4) in RPMI 1640 (2.5.2) then passed through a metal sieve. The resulting cell suspension was passed through a gauze swab (to remove particulate matter) into a centrifuge tube. This was washed through with RPMI 1640. The cells were then layered onto a similar volume of ficoll-hypaque density gradient centrifugation medium (2.5.2) and centrifuged at 1500 rpm for 30 minutes at room temperature. Cells were removed from the interface and washed three times (2.5.4) in RPMI.

2.5.13 Separation of LCH cells from skin

The skin was cut into 3 mm wide strips and placed in decontamination medium (2.5.2) for 30 minutes. The skin was then washed three times (2.5.4) in calcium and magnesium-free PBS (Flow) then placed in 0.25% trypsin in calcium and magnesium-free PBS at 4°C overnight. The epidermis and dermis were separated and the dermis placed in 0.05% trypsin/EDTA (Flow) and passed through a metal sieve. This suspension was filtered through sterile gauze to remove particulate
matter. The cells were washed three times (2.5.4) with RPMI 1640.

2.5.14 Separation of epidermal cells by suction blister

A suction blister 8mm in diameter was raised on forearm skin using a vacuum pump attached to a manufactured perspex chamber with an 8mm aperture. The blister roof was removed with scissors and placed in decontamination medium (2.5.2) for 30 minutes. The roof was washed three times (2.5.4) in calcium and magnesium free PBS (Flow) and separated into a single cell suspension by overnight trypsinization using 0.25% trypsin in calcium and magnesium free PBS (Flow) at 4°C as described by Eisinger et al, 1979. The epidermis was then placed in 0.05% trypsin/EDTA (Flow) and passed through a metal sieve. The cells were washed three times in RPMI 1640 (2.5.2).

2.5.15 Separation of peripheral blood mononuclear cells (PBMC)

15ml of ficoll-hypaque (2.5.2) was placed in a 50ml centrifuge tube and 30ml of fresh heparinized blood (10 U heparin/ml blood) were layered on top. The tube was then centrifuged at 1500 rpm for 30 minutes. The
mononuclear cells which were present at the interface between plasma and ficoll-hypaque were removed with a pipette, washed three times (2.5.4) with RPMI 1640 (2.5.2) and made up to the required concentration with RPMI 1640.

2.5.16 Separation of T cells from blood by E-rosetting

As described by Kaplan and Clark, 1974.

Fresh sheep red blood cells (srbc) (less than a week old) were washed three times (2.5.4) in PBS then resuspended to 1ml packed cells/100ml serum-free RPMI 1640 (2.5.2).

PBMs were separated as 2.5.15 and suspended at a concentration of 2x10^6/ml in RPMI 1640 with 20% srbc-adsorbed FBS (2.5.2). Equal volumes of PBMs and 1% srbc were mixed and incubated at 37°C for 5 minutes. This was centrifuged at 200g for 10 minutes and incubated at 4°C for at least an hour, but preferably overnight.

The supernatant was decanted and the cells gently resuspended and layered onto ficoll-hypaque. The tubes were centrifuged at 200g for 40 minutes following which the srbc-rosetted cells are in the pellet. 1ml of red cell lysis fluid (2.3) was added to the cells for 5 minutes and the T cells washed
three times in RPMI 1640 (2.5.4).

2.5.17 Separation of monocytes using Nycodenz—monocyte

As described by Boyum, 1983
Venous blood was collected into bottles containing sodium-EDTA. 3ml of blood was layered onto 3ml Nycodenz (Nyegaard) in a 15ml centrifuge tube and spun in a centrifuge at 600g for 15 minutes at room temperature. The cells from the interface were removed with a pipette and diluted to a volume of 7ml with 0.9% saline and spun at 600g for 7 minutes. The cells were resuspended in 1ml calcium magnesium free PBS (Flow) and layered over 3ml of autologous plasma in EDTA. The tube was centrifuged at 50g for 10 minutes and the supernatant removed. The cells were then washed three times (2.5.4) in RPMI 1640 (2.5.2).

2.5.18 Separation of adherent monocytes

Bacteriology grade petri dishes were coated overnight at 37°C with FBS and washed once with PBS (2.3). PBM’s (2.5.15) at a concentration of approximately 10^7/ml in RPMI with 10% FBS (2.5.2) were placed on the dishes and incubated overnight at 37°C. The non-adherent cells were gently removed by
suction with a pipette. The adherent cells were removed with 0.05% trypsin/EDTA (Flow) at 37°C for 5 minutes. This population comprised the adherent monocyte population. Cells were collected in 5ml FBS and washed three times in RPMI 1640 (2.5.4).

2.5.19 Separation of blood dendritic cells

As described by Gaudernack and Bjercke, 1985. Falcon (Becton Dickinson) bacteriology grade petri dishes were coated with gelatin 2% in water and incubated for two hours at 37°C. The gelatin was removed by suction and the plates allowed to dry. These were then stored at 37°C for up to a month.

10ml of autologous or pooled serum was placed on the gelatin coated plates for 40 minutes at 37°C. The plasma was aspirated and the plates washed once with calcium and magnesium-free PBS (Flow).

PBMs (2.5.15) at a concentration of 1-2x10⁷/ml RPMI 1640 (2.5.2) with 20% pooled human serum were placed on the plates and incubated at 37°C for 2 hours. The adherent cells were removed with EDTA 10mM in RPMI (Flow) at 37°C for 10 minutes.

Bacteriology grade petri dishes were coated with human Immunoglobulin G (at a concentration of 1mg/ml of 0.05M Tris hydrochloride in water adjusted to a pH of 9.5 with 1M hydrochloric acid) at 4°C overnight.
The plates were washed once with PBS and the eluted cells placed on the dishes for 30 minutes at 4°C. The non-adherent cells formed the dendritic cell population.

2.5.20 Separation of cells by panning

Petri dishes (Falcon) were coated with rabbit anti-mouse immunoglobulin (Dako) diluted 1/100 in 0.05M Tris hydrochloride in water, adjusted to a pH of 9.0 with 1M hydrochloric acid. The plates were left overnight at 4°C then washed three times in PBS (2.3) with 0.1% bovine serum albumin (BSA). The cell suspension containing the cell of interest was suspended in PBS and the monoclonal antibody was added at the appropriate dilution (for OKT6 (Ortho) this was 1ul/10⁶ cells). The cells were incubated for 30 minutes at 4°C then washed three times (2.5.4) in PBS with 0.1% BSA and made up to 10ml with PBS containing 0.1% BSA. The cells were then incubated in the anti-mouse antibody coated plate for 70 minutes at 4°C swirling occasionally. The non-adherent cells were aspirated and the plate washed three times with PBS. Adherent cells were harvested using trypsin 0.25% in calcium, magnesium-free PBS (Flow) for 5-10 minutes at 37°C. The cells were removed, suspended in 5ml of FBS then washed three times in RPMI 1640
2.5.21 Conditional growth media for cells

Cells generated in these studies were grown in a variety of conditions to attempt to favour the development of a cell line. Cells were grown in both tissue culture treated plastic petri dishes (Becton-Dickinson) and in glass petri dishes. The cells were fed weekly and split if they became overgrown. The following growth media were used:

A RPMI tissue culture medium (2.5.2) with 20% FBS

B RPMI with 20% FBS
   + cholera toxin 50ng/ml (Sigma)

C RPMI with 20% FBS
   + TP5 (synthetic thymopoetin, Sigma) 100ng/ml

D RPMI with 20% FBS
   + 50% spent media from epidermal cell cultures using Rheinwald Green technique (2.5.9)

E RPMI with 20% FBS
   + 50% spent media from 3T3 cells in culture (2.5.9)
F RPMI with 20% FBS
+ Phythaemagglutinin 3.125ug/ml

G RPMI with 20% FBS
+ Concanavalin A 12.5ug/ml

H RPMI with 20% FBS
+ 50% spent media from epidermal cell culture
  using Eisinger technique (2.5.10)

2.5.22 Conditions used to induce differentiation
in immature cells.

Various culture conditions were used to attempt to
induce differentiation of a generated cell line. The
cells were grown under these conditions and aliquots
removed daily for 10 days. Cytospin preparations were
made of the cells removed and they were stained by
various immunocytochemical and histochemical
techniques (2.4). Some of the cells were removed and
for electron microscopic study (2.5.7).

A Co-cultivation with epidermal cells

Cells were placed at a concentration of 5x10^⁶/ml in
a petri dish containing epidermal cells cultured
using either the Rheinwald Green technique (2.5.9)
or the Eisinger technique (2.5.10).

**B Cultivation with TP5**

TP5 (synthetic thymopoietin) was added to the culture medium at final concentrations of 1000, 500, 100, 50, 25ng/ml. The culture medium was replaced every three days.

**C Cultivation with phorbol ester**

(Ralph et al, 1982)

Phorbol 12-myristate 13 acetate (Sigma) was dissolved in a minimal amount of dimethylsulphoxide, diluted with RPMI and autoclaved. This was added to the culture medium at final concentrations of 500, 200, 50, 10, 1ng/ml. The culture medium was replaced every three days.

**D Cultivation with calcitriol**

(Rigby et al, 1984)

Calcitriol (a gift from Roche) was added to the culture medium at final concentrations of $10^{-5}$, $10^{-6}$, $10^{-7}$, $10^{-8}$, $10^{-9}$, $10^{-10}$ M. The culture medium was replaced every three days.
**F Cultivation with retinoic acid**

(Olsson and Breitman, 1982a)

50mg of retinoic acid was dissolved in 1ml of 70% ethanol, diluted in RPMI 1640 (2.5.2) and filter-sterilized. It was added to the culture medium at final concentrations of

0.1, 1, 10uM

The culture medium was replaced every three days.

**F Retinoic acid and cholera toxin**

(Olsson et al, 1982b)

Cells were cultured as in E with the addition of 1nM cholera toxin to the culture medium.

**G Retinoic acid and Prostaglandin E2**

(Olsson et al, 1982b)

Cells were incubated in retinoic acid as in E for 24 hours, Prostaglandin E2 was dissolved in ethanol then diluted to 10nM in culture medium. This was added to the cells and the cells were harvested the following day.

**H gamma-interferon**
gamma-interferon was added to the culture medium at final concentrations of 1, 10, 100, 1000U/ml. The medium was replaced daily and cells were harvested daily for five days.

2.5.23 Mixed lymphocyte reaction—96 well plate

Peripheral blood mononuclear cells (PBM) were prepared as in 2.5.15 and resuspended at a concentration of 3 x 10^6/ml in RPMI with 5% FBS. For each patient 100ul of cells was added to 9 wells in a sterile 96 well flat bottomed Costar plate. To the first 3 wells a further 100ul of the patient's PBM were added.

PBMs from 2 normal volunteers at a concentration of 3 x 10^6/ml in RPMI with 5% FBS were irradiated at 17.5cGy/min for 2 minutes in a blood irradiation machine to prevent any further cell division. 100ul of the irradiated normal PBMs were added to the second and third triplicates of wells. PBMs from each normal subject were set up against irradiated cells from the other to establish the normal response.

The plates were then incubated at 37°C in 5%CO2 for 4 days, pulsed with ^3H Thymidine (50ul/well of Thymidine 5mCi/ml (Amersham) diluted 1/50), incubated
for 18 hours at 37°C and harvested onto filter paper using a Titertek cell harvester. The paper was then placed in plastic vials with 1ml scintillation fluid and counted on a beta-counter.

Stimulation Index = \frac{\text{mean sample dpm} - \text{mean background dpm}}{\text{mean control} - \text{mean background}}

If the SI of the patient was less than half of that of the control then the response was considered abnormal.

2.5.24 Mitogenic T cell stimulation-96 well plate

PBMs were prepared as in 2.5.15 and adjusted to a concentration of $3 \times 10^6$/ml in RPMI 1640 with 5% FBS (2.5.2). 100ul of cells were added to 12 wells of a sterile 96 well flat bottomed Costar plate. To three wells of each sample 100ul of mitogens were added and to the other three wells 100ul of RPMI as a negative control. Mitogens used (100ul/well) were

- Phytohemagglutinin (PHA) 0.065ug/ml, a T cell mitogen
- Concanavelin A (Con A) 0.25ug/ml, a T cell mitogen
- Pokeweed mitogen (1/600), a T cell dependent B cell mitogen

Optimal concentrations of the mitogens had been obtained by plotting dose response curves using PBMs from three volunteers.

The cells were incubated at 37°C in 5% CO2 for 3 days, then pulsed with tritiated Thymidine and
2.5.25 Recall antigen (PPD) stimulation using the Terasaki technique

Cells were cultured in RPMI 1640 with 10% FBS (2.5.2). 10ul of T lymphocytes at a concentration of 6x10⁶/ml were cultured with 5ul of antigen presenting cells at concentrations of 3x10⁶/ml to 2.9x10³/ml with and without purified protein derivative of tuberculin (PPD). The concentration of PPD was varied in initial experiments using PBMs to determine the dose response curve and optimal concentration for a maximum response and was found to be about 10μg/ml. The control responses were also measured with PBMs as a positive control and T cells and antigen presenting cells alone as negative controls. Cells were cultured in triplicate in 20ul hanging drops in Terasaki plates (O'Brien et al, 1979). The volume in each well was adjusted to 20ul/well by adding tissue culture medium as necessary then the plates were inverted, the lids replaced and the plates put in a plastic box on a rack to keep them suspended above sterile water placed in the base of the box to prevent the cells drying out.

Cells were incubated at 37°C in 5% CO₂ for 6 days, pulsed with 1ul of [³H Thymidine (2Ci/μmol), diluted
to 1ug/ml in RPMI 1640 (Farrant et al, 1980) and harvested 2 hours later onto filter paper using a microharvester (Flow) described by O’Brien et al, 1979. The harvester contains wells over which filter discs (Titertek) were cut. The culture plate, (still inverted) was placed on the harvester and suction applied below the filter discs. The discs were then washed under suction with saline, then 5% trichloroacetic acid, followed by methanol to wash off tritiated thymidine not bound to DNA. The thymidine, but not the DNA (which is much larger), can pass through the paper. The dry discs were transferred into scintillation vials, scintillation fluid was added and the incorporation of tritium was measured using a liquid scintillation counter.

Response of T cells with different antigen presenting cells was expressed as a count per minute from the tritium.

2.5.26 Mitogenic stimulation using Terasaki technique.

Cells were cultured in RPMI 1640 with 10% FBS (2.5.2) in 20ul wells on Terasaki plates. Cell concentrations were adjusted as required and either 5ul of the T cell mitogen Concanavalin A, (at a concentration around 0.5ug/ml RPMI 1640) or a control of RPMI alone was added to the wells. All
combinations of cells were studied in triplicate. The total volume in each well was adjusted to 20ul using RPMI 1640 with 10% FBS. The plates were then inverted, the lids replaced and the plates put in a box on a rack to keep it above sterile water placed in the box to prevent the cells drying out. The cells were then incubated at 37°C in 5% CO₂ for 3 days, pulsed with 1ul of tritiated thymidine (2Ci/mmol) at a concentration of 1ug/ml in RPMI 1640 and harvested 2 hours later onto filter paper using a harvester as described in 2.5.25. Thymidine incorporation was measured using a liquid scintillation counter.

2.5.27 Mixed Lymphocyte Reaction using Terasaki technique

PBMs were prepared as in 2.5.15 and resuspended in RPMI 1640 with 10% FBS (2.5.3). For the subject under investigation T cells were separated by E-rosetting as in 2.5.16, monocytes by immune adherence as in 2.5.18 and dermal cells as in 2.5.13. Epidermal cells were separated as in 2.5.14 after the epidermis was separated from the dermis as in 2.5.13. All cells were suspended in RPMI 1640 with 10% FBS. PBMs and T cells were used at a concentration of 3 x 10⁴/well and monocytes and
epidermal cells at $5 \times 10^3$/well. Dermal cells were used at $2.5 \times 10^3$/well.

PBMs from a normal volunteer at a concentration of $6 \times 10^5$/ml in RPMI with 10% FBS were irradiated at 17.5cGy/min for 2 minutes in a blood irradiation machine to prevent future cell division. 5ul of the irradiated PBMs were added to various combinations of cells from the subject under investigation in triplicate in a Terasaki plate and the volume in each well adjusted to 20ul. The plates were incubated at 37°C for 4 days, pulsed and harvested as in 2.5.26 above.
2.6 Production of monoclonal antibodies

2.6.1 Solutions commonly used

Iscoves medium (Flow) 450ml
+5ml penicillin/ streptomycin (10,000 iu/ml) (Flow)
+50ml FBS (heat-inactivated at 56°C for 30 minutes to destroy Complement components)

Polyethylene glycol 1500 (PEG)
1g of PEG 1500 was melted by autoclaving at
121b/sq in for 15 minutes then 1ml of Hepes buffered
RPMI 1640 (Flow) was added and the solution stored at
4°C.

PBS
2g potassium dihydrogen phosphate
8.56g disodium biphosphate
4.5g sodium chloride
Made up to 1L in distilled water, then the pH was adjusted to
7.4 and the solution filter sterilized

2.6.2 Antigen used to stimulate mice

Cells from a spleen heavily infiltrated with LCH
cells were separated (2.5.12), frozen (2.5.5) and
stored in liquid Nitrogen. Before use they were
thawed (2.5.6), washed three times (2.5.4) in RPMI 1640 (2.5.2) and $10^7$ cells resuspended in 0.5ml of RPMI 1640.

2.6.3 Immunizing mice

Female balb/c mice that were about 6 weeks old were used. The immunizing cells were injected intraperitoneally three times at two week intervals. The mouse was bled using the tail approach and the mouse serum was screened for the presence of antibodies using an APAAP technique (2.4.9) with frozen sections of spleen involved in LCH. The serum was used to replace the primary antibody in this technique. When staining of the LCH cells was seen to occur the fusion was performed 4 days after the last injection.

2.6.4 The fusion

All steps from here were carried out using sterile techniques. The mouse myeloma line NS1 Ag4-1 was used as a fusion partner. These cells were grown in Iscoves medium as above and were split to one quarter of their concentration of cells using fresh medium every three days so they were growing exponentially when
the fusion was performed. The PEG (2.6.1) and Iscoves medium (2.6.1) were heated to 37°C in a waterbath. The mouse was killed by cervical dislocation and the body swabbed with 70% ethanol. The spleen was removed and placed in a petri dish with several mls of PBS to help prevent the spleen cells from clumping. The spleen was passed through a metal sieve and the cells obtained washed three times in Iscoves medium (2.5.4) and counted (2.5.3).

The NS1 cells were washed three times in Iscoves medium and counted. The spleen cells and NS1 cells were then mixed in a 10ml tube at a ratio of 10 spleen cells to one NS1 cell and spun down to a pellet of cells using a centrifuge then resuspended in a minimal volume of medium and placed in a water bath at 37°C.

1ml of warm PEG was added over one minute, stirring continuously. 1ml of serum-free Iscoves medium was added over one minute, then a further 4mls was added over 4 minutes without stirring. The tube was inverted and the contents tipped into a 50ml centrifuge tube.

20ml of Iscoves medium was added followed by 20ml of medium with 15% FBS by running it gently down the side of the tube. The tube was inverted once and left at 37°C for 1-2 hours whilst the feeder plates were prepared.
2.6.5 Preparation of feeder plates

A normal Balb/c mouse was killed by cervical dislocation and the abdomen swabbed with 70% ethanol. The abdominal skin was cut and then pulled apart without puncturing the peritoneum. 5ml of cold Iscoves medium was injected intraperitoneally and the abdomen palpated. The medium was then withdrawn using a 21G needle. The cells in the medium were counted and washed twice. Approximately $2.5 \times 10^6$ macrophages were obtained from each mouse.

2.6.6 Plating out following fusion

Two hours after fusion the cells were spun down and resuspended to $2-5 \times 10^5$/ml in Iscoves medium with 10% FBS and the macrophages were added to a final concentration of $2-5 \times 10^4$/ml in the same medium. The cells were then plated out in 24 well plates at 1ml/well and placed in an incubator at 37°C with 5% CO2 overnight. The next day HAT selection was commenced.

2.6.7 HAT selection

Hypoxanthine phosphoribosyltransferase (HPRT) is an
enzyme that uses exogenous hypoxanthine as a substrate whose products allow the incorporation of thymidine into DNA. This takes place in the presence of aminopterin which would otherwise block thymidine incorporation. The NS1 myeloma cells lack HPRT so die. Spleen cells have HPRT but die in culture because, unlike myeloma cells, they are not immortal. Hybrids are able to grow as the spleen cells provide the HPRT and the myeloma cells confer immortality.

1 ml of 2xHAT (hypoxanthine, aminopterin and thymidine) medium (Flow) with 10% FBS was added to each well. One week later 1 ml of medium was removed from each well and replaced with 1 ml of HAT with 10% FBS. Two weeks later 1 ml of medium was removed and replaced with 1 ml of HT (hypoxanthine and thymidine) in Iscoves medium (Flow) with 10% FBS. At this stage the hybrids started to grow well. Each week after this the medium in the wells was partially replaced with 1 ml of Iscoves medium with 10% FBS. Screening was commenced when the wells were yellow.

2.6.8 Screening using cytospin preparations of cells or frozen tissue sections

Cytospin preparations of the immunizing cells were prepared as in 2.4.3 then 25 ml of clone supernatant was put on each section and incubated in a moist box
at 4°C overnight together with positive (OKT6) and negative (normal mouse serum) controls. The slides were washed 3 times in PBS then 25ul of 1:20 dilution of peroxidase-conjugated anti-Mouse Ig was added to each section. The staining was then continued as in 2.4.7.

2.6.9 Cloning by limiting dilution

Clones of cells in wells which produced staining of LCH cells when the supernatant was screened as in 2.6.8 were grown up to about 1x10⁶/ml and then subcloned into a 96 well flat-bottomed microtitre plate. At the first cloning about 6 cells per well are needed so the cells were diluted to 30 cells/ml and 200ul added to each well. Peritoneal macrophages from a normal mouse were added to each well using a concentration of 5 x 10⁴/ml and volume of 200ul. The cells took about 2 weeks to grow up and as the medium in the wells started to turn yellow they were tested for the presence of antibody by screening as in 2.6.8. The positive wells were transferred to a Linbro plate and cloned again at 0.3-1 cell per well. The screening was repeated and the cells transferred back to the Linbro plate and, when growing well, to flasks. Aliquots of cells were frozen down at intervals as
in 2.5.5 in case of infection or breakdown of the incubator.

2.7 Flow cytometry

2.7.1 Flow Cytometer

The flow cytometer used was a FACS analyser (Becton Dickinson). A single cell suspension stained with appropriate fluorescent reagents was drawn through a constricted channel traversed by a beam of light from a mercury arc lamp. When the cells are stained with 2,4 diamidino 2 phenylindole hydrochloride (DAPI) (Boehringer Mannheim), an excitation wavelength of 360nm is used and the fluorescent light collected at 490nm. The intensity of the fluorescent signals emitted by each cell, which depends on the amount of macromolecules present, is measured and processed by two Z-80 microprocessors.

2.7.2 Paraffin embedded material

Cells were prepared for flow cytometry (FCM) using a method described by Hedley et al (1983) and Newton, Camplejohn and McGibbon (1987). For the skin biopsies five 60µm thick sections were used and for the other specimens a single 60µm section was used.
The sections were dewaxed in xylene, rehydrated through a series of alcohols (100%, 95%, 70% and 50%) into distilled water then incubated in pepsin (Sigma) 5mg/ml at 37°C for 30 minutes at pH 1.5. After centrifugation at 2000 rpm for 3 minutes and resuspension of cell nuclei in Isoton (Coulter) the nuclei were stained with DAPI (2.7.1) 1ug/ml which is considered to be a DNA specific stain. Cells prepared in this way were subjected to FCM. The coefficient of variation (CV) of the peak of normal diploid cells was used to assess the quality of FCM results.

2.7.3 Fresh material

The tissue was cut into 1mm wide strips and incubated in collagenase (0.5mg/ml) and dithiothreitol (1.5mg/ml) (BDH) in MEM tissue culture medium (Gibco) at 37°C for 30 minutes. as described by Epstein, Munderloh and Fukuyama in 1979 to separate epidermis and dermis. The epidermis was then peeled off the dermis using watchmakers forceps and both were then separately incubated in 0.5ml of trypsin (0.5mg/ml) in versene (Gibco) at 37°C for 10 minutes.
0.25 ml FBS and 3.25ml MEM medium were added and the resultant cell suspensions were passed through a 19 and then 21 gauge needle to disaggregate the cells. The cells were then pooled and filtered through 35um pore polyester gauze. The cells were then incubated with OKT6 antibody (Ortho) at a concentration of 10ul/10^6 cells for 30 minutes. The cells were washed in MEM then resuspended in MEM with 5% human serum containing a (Fab)_2 biotinylated rabbit anti-mouse antibody (Dakopatts). The cells were incubated on ice for 30 minutes, washed then resuspended in medium containing avidin-FITC (Dakopatts). After a further incubation on ice for 20 minutes the cells were washed and then fixed with cold 70% alcohol. After fixation the cells were stained with propidium iodide with RNA-ase. Simultaneous measurement of DNA content (red fluorescence), antibody fluorescence (green) and Coulter volume were made using the FACS analyser and the data stored on a Consort 30 computer. DNA content was measured on a linear scale, while green fluorescence and volume were measured using a 3-decade log amplifier. 10,000 cells were measured per sample.
RESULTS

3.1 Clinical study of children with LCH

3.1.1 Introduction

LCH is a rare disease and all the previously published series of patients have collected children presenting over several decades (Sims, 1977), patients seen at a variety of centres (Lahey, 1962, Gadner et al, 1987) or both (Greenberger, 1981). There is a wide variation between centres in classification and treatment of patients. Furthermore supportive treatments, such as antibiotics, change with time and tend to improve results. During the last few decades LCH was regarded as a malignancy and treated with increasingly aggressive chemotherapy regimes but it is now generally considered to be a reactive disorder.

LCH has varied clinical manifestations and there is, as yet, no uniformly accepted classification system of extent of disease. This, combined with its rarity, means that very few adequate comparative treatment studies have been carried out. At the Hospital for Sick Children, Great Ormond Street (GOS) a conservative approach has been uniformly adopted since 1980. Single system bony disease is treated,
where possible, with curettage or injection of intralesional steroid. Multisystem disease is treated only if there is evidence of constitutional upset (fever, pain, immobility), failure to thrive or worsening disease in vital organs. Pulsed high dose prednisolone is the first line drug (Broadbent and Prichard, 1985) given at a dose of 2mg/kg/day for a month, and then tailed off over a two month period. Cytotoxic drugs, initially vinblastine or vincristine and more recently etoposide (150mg/m²/day intravenously or 300mg/m²/day orally, for 3 days every 3 weeks for 18 weeks), are used as the second line of systemic treatment.

The first aim of this study was to look at the outcome of patients with LCH treated at GOS over a relatively short period and to see how their morbidity and mortality compared to those of previously published studies in which more aggressive and prolonged chemotherapy regimes were used.

A uniformly acceptable means of classifying the disease is necessary to enable comparison of results between centres and to set up multi-centre treatment trials. Because LCH is a rare disease and because it can have many different manifestations, then some form of "scoring" system, to group data together, is helpful to enable multivariate analysis of the data.
obtained. Several 'scoring' systems have been devised (Lavin and Osband, 1987) but the most widely used remains that devised by Osband in 1962 and modified in 1975. The Histiocyte Society was founded in 1985 and involves many individuals with an active interest in research into LCH. It was felt that a new scoring system should be devised by members that was universally acceptable and could be used for comparative studies and later multi-centre trials. A preliminary system was devised by Dr V Broadbent (VB) (see 2.1.2).

The second aim of the study was therefore to compare scores obtained by the Broadbent system to the most established scoring system devised by Lahey and see how useful the two were in predicting disease outcome.

3.1.2 Patients and methods

All patients with LCH referred to the Haematology and Oncology Department at The Hospital for Sick Children, Great Ormond Street (GOS) between 1980 and 1987 were studied. These dates were chosen as all children presenting over this period had been seen and assessed by one clinician (VB).

The age of the child at referral, sex, and systems affected were recorded. Patients were allotted a
score using both the system devised by Lahey (2.1.1) and that devised by Broadbent (2.1.2). Scores were calculated at initial assessment and the maximum scores achieved at the time of the study were determined. The type and number of courses of treatment, if any, were noted. Outcome was assessed by both allocating an outcome score (table 3.1.1) and by adding up the number of courses of systemic treatment given up to a maximum of six. Where steroids were given continuously each three month period of treatment was regarded as a separate course. Morbidity was also recorded.
Table 3.1.1 Scoring system for outcome

1. No handicap
2. Minor handicap, not threatening longevity
3. Severe or life-threatening handicap
4. Dead
Statistical analysis

Results were analysed by regression of ordinal (dependent) data using GLIM (Royal Statistical Society, London). The two different methods of assessing outcome were correlated using Spearman Rank Correlation.

3.1.3 Results

Between 1980-1987 60 children were referred, each with biopsy-proven LCH apart from 2 with single system disease of bone in whom the diagnosis was made on radiological appearance. The number of children presenting in each year is shown in Table 3.1.2. The histopathological diagnoses were made before the guidelines produced by the Histiocyte Society (Chu et al, 1987), but the necessary material was not available to make definite retrospective diagnoses according to these criteria. Sufficient follow-up information was available on 58 children. Two children, both from overseas, had been lost to follow-up.
<table>
<thead>
<tr>
<th>Year of presentation</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1980</td>
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<td>1981</td>
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<td>9</td>
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<td>1985</td>
<td>6</td>
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<td>1986</td>
<td>2</td>
</tr>
<tr>
<td>1987</td>
<td>5</td>
</tr>
</tbody>
</table>
Age  The age range at presentation was 0 to 15 years. The distribution was skewed with a median of one year (see Figure 3.1.1). Figure 3.1.2 shows the distribution compared to that in previous studies and shows that the age distribution is similar.

Gender  40 patients were male and 18 were female.

Race  50 patients were white Europeans, 6 were Asians, 1 was Oriental and one was of black African origin.
Figure 3.1.1 Age at presentation. Bar chart showing number of children against the age in years, taken to the nearest year, at which they presented to GOS.
Figure 3.1.2 Age at diagnosis compared to other series. LIT and IBC series previously reported by Lahey, 1962. (IBC represents Iowa, Baltimore and Cincinatti clinics, LIT represents series from published articles). Gre represents series by Greenberger et al, 1981. GOS represents present series.
Extent of disease 14 children had single system disease; in 13 the disease was affecting bone and in one case it affected the skin. Of the 44 with multi-system disease, 22 had 'vital organ dysfunction' (lung, liver or haemopoetic systems) as defined by Lahey in 1975.

Lahey score The number of patients with each Lahey score at the time of study is shown in Figure 3.1.3. The relationship of Lahey score to age is seen in Table 3.1.3 and shows that the younger children tend to have the higher Lahey scores.
Figure 3.1.3 Number of patients against maximum Lahey score obtained
Table 3.1.3 Relationship between age at presentation and maximum Lahey score

<table>
<thead>
<tr>
<th>Age at presentation (years)</th>
<th>No of children</th>
<th>Mean Lahey score at time of study</th>
</tr>
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<tr>
<td>0</td>
<td>7</td>
<td>3.4</td>
</tr>
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<td>1</td>
<td>22</td>
<td>4.3</td>
</tr>
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<td>2</td>
<td>7</td>
<td>2.6</td>
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<td>6</td>
<td>2.3</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>2.0</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
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<td>14</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>3.0</td>
</tr>
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</table>
Treatment. Of the patients with single system disease, 8 had no treatment apart from a diagnostic biopsy. 1 received radiotherapy, 4 had surgery (1 had a skin plaque excised, 2 had curettage of bone lesions and 1 a laminectomy) and 1 had intralesional steroid injection.

Of the 44 patients with multisystem disease 5 had no treatment, 1 had topical mustine to the skin alone and 2 had radiotherapy alone. Of 36 children treated with prednisolone, 21 went on to receive cytotoxic drugs in addition.

The number of courses of systemic treatment are shown in Tables 3.1.4 and 3.1.5. Although the number of steroid courses per patient has remained at about 0.2/patient/year, the number of courses of cytotoxic drugs per patient has declined steadily over the period under study.
Table 3.1.4 Mean number of courses of steroids given to patients presenting since 1980.

<table>
<thead>
<tr>
<th>Year</th>
<th>total no</th>
<th>number of patients under follow up</th>
<th>mean no of courses per given patient under follow up</th>
</tr>
</thead>
<tbody>
<tr>
<td>1980</td>
<td>1</td>
<td>6</td>
<td>0.17</td>
</tr>
<tr>
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<td>1982</td>
<td>7</td>
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</tr>
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<td>36</td>
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</tr>
<tr>
<td>1984</td>
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</tr>
<tr>
<td>1987</td>
<td>11</td>
<td>58</td>
<td>0.19</td>
</tr>
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</table>
Table 3.1.5 Number of courses of cytotoxic drugs

<table>
<thead>
<tr>
<th>Year</th>
<th>total no of courses given</th>
<th>number of patients under follow up</th>
<th>mean no of courses per patient under follow up</th>
</tr>
</thead>
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<td>6</td>
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</tr>
<tr>
<td>1981</td>
<td>4</td>
<td>15</td>
<td>0.27</td>
</tr>
<tr>
<td>1982</td>
<td>6</td>
<td>25</td>
<td>0.24</td>
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<tr>
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<tr>
<td>1984</td>
<td>4</td>
<td>45</td>
<td>0.09</td>
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<tr>
<td>1985</td>
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<tr>
<td>1986</td>
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</tr>
<tr>
<td>1987</td>
<td>3</td>
<td>58</td>
<td>0.05</td>
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</tbody>
</table>
Outcome. Of the 14 children with single system disease, 1, who received radiotherapy in 1981, now has a minor periorbital deformity but 13 have no long-term sequelae.

Of the 44 with multi-system disease, 8 died (18%) of whom 2 were girls and 6 boys. All deaths were of children aged under 2 years at presentation with vital organ dysfunction, accounting for 36% (8 out of 22), of this group and occurred within 2 years of presentation. The gender ratio of deaths did not significantly differ from the ratio in the whole group.

Table 3.1.6 is a Life Table of survival after diagnosis and shows that the mortality occurs within the first two years after diagnosis.

Table 3.1.7 shows the outcome related to age at presentation and Figure 3.1.4 compares the mortality related to age in our series to those published in the Lahey (1962) series. Comparison of actuarial rather than crude survival rates would have been preferable but this information is not given in other series. This shows that survival has greatly improved for patients with LCH, but as these are old series, this could be due to changes in supportive treatments rather than in specific treatment of the disease.
Table 3.1.6 Life Table for patients with multisystem Langerhans cell histiocytosis

<table>
<thead>
<tr>
<th>Interval since diagnosis (years)</th>
<th>No of deaths</th>
<th>No of patients alive at start of interval</th>
<th>Est prob of death</th>
<th>Est prob of survival</th>
<th>% of survivors at end of year</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.8636</td>
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<td>3</td>
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</tr>
</tbody>
</table>
Table 3.1.7 Outcome related to age at presentation

<table>
<thead>
<tr>
<th>Age at presentn (years)</th>
<th>Number of children</th>
<th>Number who died</th>
<th>Number with handicap</th>
<th>Number with no sequelae</th>
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Figure 3.1.4 Mortality related to age compared to LIT and IBC series reported by Lahey, 1962. (LIT represents series from literature review, IBC represents Iowa, Baltimore and Cincinatti series). GOS represents present series.
Figure 3.1.5 compares the percentage surviving in the present, the DAL-HX (Gadner et al, 1987) and the AEIOP studies (Ceci et al) according to the presence or absence of vital organ dysfunction. Figure 3.1.6 shows this data subdivided for the further risk factor of age and compares the present and AEIOP studies (unpublished data; data not available in the DAL-HX 83 paper). These show that our mortality rate is no worse than that in other recent European trials in which more aggressive and prolonged treatment was used.

Table 3.1.8 shows the mortality related to Lahey score and compares this with other published series in Figure 3.1.7. This shows that mortality tends to increase as Lahey score increases, the peak in our series at a score of 5-6 probably being due to the small numbers involved. Table 3.1.9 shows the mortality related to year at presentation; the numbers are too small to be able to show any trend towards change.
Figure 3.1.5 Outcome in different series. Percentage two year survival in present (GOS), AEIOP (Ceci et al), and DAL-HX 83 (Gadner et al, 1987).

% surviving

Results from different studies

- Organ dysfunction
- No organ dysfunction
Figure 3.1.6 Outcome related to risk factors.
Percentage three year survival in present and AEIOP studies subdivided according to risk factors of age and presence or absence of vital organ dysfunction.

OD represents vital organ dysfunction
**Table 3.1.8** Mortality related to Lahey score

<table>
<thead>
<tr>
<th>Lahey score (maximum)</th>
<th>Number of patients</th>
<th>Number who died</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>0</td>
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<tr>
<td>2</td>
<td>11</td>
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<td>2</td>
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<td>8</td>
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</tbody>
</table>
Figure 3.1.7 Mortality related to maximum Lahey score obtained. GOS represents present series, LIT and IBC the series published by Lahey in 1962 and Gre the series by Greenberger et al, 1981.
### Table 3.1.9 Mortality related to year of presentation

<table>
<thead>
<tr>
<th>Year of presentation</th>
<th>Number died</th>
</tr>
</thead>
<tbody>
<tr>
<td>1980</td>
<td>2</td>
</tr>
<tr>
<td>1981</td>
<td>2</td>
</tr>
<tr>
<td>1982</td>
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<td>1984</td>
<td>2</td>
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<td>1985</td>
<td>1</td>
</tr>
<tr>
<td>1986</td>
<td>1</td>
</tr>
<tr>
<td>1987</td>
<td>0</td>
</tr>
</tbody>
</table>
12 patients with multi-system disease (including 6 of the 8 who were not given systemic treatment), had no long-term sequelae. 4 had orthopaedic abnormalities alone, 9 had endocrine dysfunction alone, 1 had hearing deficit alone, 1 had liver fibrosis alone and 9 had more than one of the above groups. 16 patients (36%) had diabetes insipidus which in 11 patients was associated with other disease related morbidity. In all cases chronic morbidity was the result of the disease rather than its treatment.

The two methods of assessing outcome produced results which were closely correlated to each other, \( r = 0.671, \ p < 0.01 \).

**Comparison of Broadbent score and Lahey score**

The Broadbent scores and Lahey scores closely correlated to each other. The initial scores at presentation correlated with an \( r \) value of 0.732, and the final scores with an \( r \) value of 0.841.

The maximum Lahey score was highly significantly related to outcome measured by both outcome score \( (p < 0.001) \) and number of treatment courses \( (p < 0.0001) \). There was no significant association between Lahey score at presentation and outcome measured by either of these means.
The maximum Broadbent score correlated closely to both outcome score \((p<0.0001)\) and number of treatment courses \((p<0.0001)\). The Broadbent score at presentation correlated significantly \((p<0.05)\) to outcome score but not to the number of treatment courses.

There was no significant difference between Broadbent and Lahey scores but the finding that the Broadbent score is better than the Lahey at predicting outcome when the patient is initially assessed means that it may be clinically useful and further study of this is indicated.

3.1.4 Discussion

This study looked at a comparatively large number \((58)\) of children seen over a relatively short time \((seven\ years)\) who had all been assessed by one individual.

The ratio of males to females was approximately 2:1 which has been noted in previous studies \((Lahey, 1962; Greenberger et al, 1981)\). The age distribution had a skewed pattern with a peak at 1 year and corresponded closely with the distribution found in the Lahey \((1962)\) series although the children were younger than in the Greenberger \((1981)\) series, possibly because of different referral patterns. The
racial origins of all children presenting to GOS is not recorded so the significance of the origins of children with LCH is unknown. The extent of disease as indicated by the Lahey score was higher in children under 18 months than in children over this age. The inverse relationship between extent of disease and age has been noted before (Lahey, 1962).

Of the 44 patients with multisystem disease, 36 (82%) received systemic therapy in the form of oral prednisolone. 21 patients went on to receive cytotoxic drugs as the prednisolone did not satisfactorily control their disease. This is, however, 48% of the total number with multisystem disease, all of whom would have received cytotoxic drugs in the DAL-HX (Gadner et al, 1987), AEIOP (Ceci et al) or Toogood et al (1979) series.

Although the number of steroid courses per patient under study per year has remained about the same over the period 1980-87, the number of courses of cytotoxic drugs per patient per year has declined. This could be due to an increasing disinclination to give cytotoxic drugs because of their side effects, to more effective treatment by non-cytotoxic drugs so patients are less likely to require second line systemic treatment, or to more effective cytotoxic drugs (etoposide rather than vinblastine) which require fewer courses of treatment to produce the
desired result. It is unlikely to be just the last, as the trend to decrease started before etoposide was introduced in 1985.

The survival of, and low morbidity in, all patients with single system disease is not unusual but was obtained with minimal intervention which is presumably an advantage to the affected child.

8 children died, comprising 18% of those with multisystem disease, and all deaths were within 2 years of presentation. 10 children presented within the last 2 years and of these 1 died and the others have been followed up for less than two years and are therefore still at risk. Children who died were all under 2 years old at presentation and the risk factor of young age has been documented (Lahey, 1962, Greenberger et al, 1981). Younger children have, on average, more extensive disease with a higher Lahey score than have older children (Greenberger et al, 1981), a finding confirmed in our study, and there were insufficient numbers to see if age was a risk factor independent of extent of disease. Gender was not a risk factor for mortality, as the ratio did not significantly differ from the ratio of those presenting with the disease.

The mortality figure of 18% compares to 36% of the IBC series (Lahey, 1962) which included good-prognosis patients with polyostotic and single
system skin disease excluded from the GOS series. In the series by Greenberger et al (1981) 38% out of 113 patients with multi-system disease died. The patients in these three series (GOS, Lahey and Greenberger et al) had a comparable distribution of Lahey scores indicating extent of disease and the distribution of ages in the Lahey and GOS series was similar although the distribution curve of the Greenberger series was slightly moved to the right i.e. they were older which would give them a better prognosis. The results obtained are therefore comparable for the two most important variables, age and Lahey score, which influence outcome (Greenberger et al, 1981). These previous studies both, however, include patients presenting over several decades and supportive treatments, such as antibiotics, have improved over that time which may be responsible for the decrease in mortality in the present series.

Toogood et al (1979) reported the results of aggressive chemotherapy in 25 children of whom 13 would have been defined by us as having multisystem disease, including 5 with vital organ dysfunction. 5 children (38%) died of overwhelming sepsis, including 2 in whom only bone and gingiva were affected and who would have been unlikely to be given systemic treatment at our centre.

There have been two recent reports of patients
presenting over the last decade who are included in large multicentre studies, one by Gadner et al (1987) of the DAL-HX 83 study, and a preliminary report of the Italian AEIOP study (Ceci et al). Gadner et al (1987) reported a study from 45 centres in Germany, Austria and the Netherlands, which was started in June 1983, and in which 51 children were treated with etoposide, vinblastine and prednisolone and given maintenance therapy of 6-mercaptopurine and pulses of prednisolone, vinblastine, etoposide and methotrexate. The distribution of age and Lahey scores of these patients is not given, but only 12 of the 51 had vital organ dysfunction. The AEIOP study has 143 patients with single system disease and 86 with multisystem disease. 28 had vital organ dysfunction defined by a modified Lahey system. The extent of disease (as measured by Lahey score) in these patients tended to be less than ours presumably because GOS is a tertiary referral centre whilst both the European studies are from multiple centres and so will include patients from primary and secondary referral centres. All patients with disease other than a single bone lesion were treated with cytotoxic drugs. The survival rates in our series are at least as good as the survival rates in the DAL-HX and AEIOP studies. As the patients in these studies have less extensive disease than ours (as indicated by Lahey
scores and numbers with organ dysfunction) this makes our results compare even better.

24 out of 36 (63%) of survivors with multisystem disease suffered long-term sequelae although this was often mild, for example diabetes insipidus requiring replacement with the vasopressin analogue, DDAVP. Lahey (1975b) found that 63% of 59 survivors had a disability and Sims (1977) 54% of 43 patients. This compares to 35% in the AEIOP study, but the criteria for disability are not defined. The rate of diabetes insipidus in the present series (36% of those with multisystem disease) is comparable to the 35% found by Sims (1977) and 4 out of 13 (31%) by Toogood et al (1979), but contrasts to 18% in the AEIOP study and only 4% in the DAL-HX study. The higher incidence of diabetes insipidus could again reflect the more extensive disease in our patients. Alternatively it may be a true consequence of less intensive treatment. Further studies are needed to determine whether aggressive or maintenance treatment prevents the development of diabetes insipidus. Even if it does, it may not be justified to give potentially dangerous prophylactic treatment for an eminently treatable condition.

In this series 44 patients had non-monoostotic disease of whom 22 were in a favourable prognosis group in that they did not have `vital organ
dysfunction. Of this group none died but 13 had sequelae. Of the 24 patients in the unfavourable prognosis group with organ dysfunction 8 died and 11 had long-term sequelae.

Multiagent cytotoxic regimes are immuno- and myelosuppressive and run the risk of causing tumours, especially when combined with radiotherapy (Greenberger, 1981). In Greenbergers series 5 out of 127 patients had already developed cancers that could be attributed to their treatment. The results presented above using conservative pulsed therapy compare favourably with other published series where more aggressive treatment cytotoxic drugs have been used both in terms of mortality and long term sequelae apart, perhaps, from the incidence of diabetes insipidus and are less likely to induce malignancies.

Both maximum Lahey and Broadbent scores had a highly significant correlation with outcome as measured by an outcome score and by the number of courses of systemic treatment required. The initial Broadbent but not the initial Lahey score correlated significantly with the final outcome and it may therefore be of value in assessing prognosis when the patient is initially assessed. This requires
further investigation.
3.2 Production of cell line

3.2.1 Introduction

The main difficulty encountered with in vitro studies of LCH is obtaining material. LCH is a rare disease and is most common in those under the age of 15 (Berry, 1987) so it is unusual to obtain large samples of fresh material for research purposes. The development of cell lines from tumours such as leukaemias (eg HL60, U937) has enabled in vitro study of these cells which helps elucidate the original disease. If a cell line was raised from LCH cells this would enable functional studies of these cells to be performed much more easily. LCH cells have not been grown successfully in long term cultures but LCH cells grown in a methylcellulose clonal culture system showed clonal cell aggregates when harvested after 6 days in culture (Selvaggi et al, 1985).

The aim of this project was therefore to obtain long term culture of LCH cells.

3.2.2 Materials and methods

Part of a spleen which had been removed from a five
year old boy with biopsy proven LCH because of hypersplenism was obtained. Part of the tissue was processed as in 2.5.12 and part was examined histologically by carrying out immunoperoxidase staining for the CD1a antigen using OKT6 antibody (Ortho) (2.4.7), staining for the S100 protein (2.4.4) and electron microscopy (2.5.7). Histological and immunohistochemical examination showed that 50% of the spleen cells were histiocytes which were S100 and OKT6 positive and occasional Langerhans cell granules were seen on electron microscopy.

The spleen cells were put in various culture conditions as in 2.5.21 to try and establish a cell line. A line of morphologically histiocytic cells was established from cells grown in RPMI with 20% FBS in glass petri dishes. This was actively dividing and was HLA Class II and CD1-positive but was lost after three weeks because of infection before it could be further characterized. No line was obtained from cells grown under alternative culture conditions. Subsequent samples obtained were all placed in RPMI with 20% FBS in glass petri dishes alone.

Material was subsequently obtained from three bone marrow aspirates and two skin biopsies from patients with involvement of these systems by LCH. These were processed as in 2.5.11 and 2.5.13 and placed in RPMI with 20% FBS in glass petri dishes.
Two normal bone marrow aspirates were obtained and processed as the LCH bone marrow.

3.2.3 Establishment of a cell line

A cell line was obtained from the bone marrow aspirate obtained from a ten month old child who had histiocytic cells in the marrow which were positive on staining with OKT6 using an immunoperoxidase stain (2.4.7) and showed intense α-mannosidase activity (2.4.12). After 10 days in culture the bone marrow cells had proliferated and approximately 50% of the cells were adherent and 50% non-adherent. Both populations were divided and resuspended in fresh media. The adherent population was mainly fibroblasts but the non-adherent population continued to show prolific growth and needed splitting to one-third of their concentration every 3-4 days.

The line was grown for eighteen months and continued to have the same characteristics. Cells from the other bone marrows with LCH involvement and from the normal bone marrows showed some clonal expansion and survived for about 2 months but gradually all the cells died. Cells from the skin biopsies were overgrown by fibroblast proliferation.
3.2.4 Study of cell line

Some of the cells were used to make cytospin preparations as in 2.4.3 and were stained using an APAAP technique (2.4.9) with the antibodies in Table 3.2.1. They were also examined for non-specific esterase, acid phosphatase, lysozyme, α-mannosidase, S100 and peanut agglutinin as in 2.4. Some of the cells were fixed as in 2.5.7 and examined under the electron microscope and others were examined for the expression of Fc receptors as in 2.5.8.

Cells were tested for the presence of Epstein Barr nucleic antigen to see if they were virally transformed, and for infection by mycoplasma.
Table 3.2.1 Antibodies used to study cell line

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<tr>
<td>HLA-DR (Dakopatts)</td>
<td>Class 2 MHC</td>
</tr>
<tr>
<td>T11 (Dakopatts)</td>
<td>T cells,</td>
</tr>
<tr>
<td>T cell cocktail (Royal Free) T cells</td>
<td></td>
</tr>
<tr>
<td>T4 (Dakopatts)</td>
<td>helper T cells, LCH cells</td>
</tr>
<tr>
<td>T8 (Dakopatts)</td>
<td>suppressor T cells</td>
</tr>
<tr>
<td>OKT6 (Ortho)</td>
<td>thymocyte, LCH cells</td>
</tr>
<tr>
<td>Dako-macrophage</td>
<td>Langerhans cells</td>
</tr>
<tr>
<td>OKM1 (Ortho)</td>
<td>macrophages</td>
</tr>
<tr>
<td>OKM5 (Ortho)</td>
<td>monocyte</td>
</tr>
<tr>
<td>B cell (Royal Free)</td>
<td>monocyte</td>
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<tr>
<td>a-1 antitrypsin</td>
<td>B cells</td>
</tr>
<tr>
<td></td>
<td>macrophages</td>
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</table>
3.2.5 Results of study of cell line

The cells were found to be large cells with frequent mitoses. On electron microscopy they looked histiocytic but no Langerhans cell granules were seen (Figure 3.2.1 and 3.2.2).

Acid phosphatase staining produced a dot-like reaction (3.2.3). Acid phosphatase is present in many types of haemopoietic cell including lymphocytes, polymorphonuclear neutrophils and all the mononuclear phagocyte system. It is usually weak or negative in the B cell disorders and gives a diffuse reaction in monoblasts (Dacie and Lewis, 1984).

The cells were non-specific esterase positive with a fairly diffuse reaction which is typical of monocytes rather than the very small dot-like reaction produced in lymphocytes (Dacie and Lewis, 1984) (figure 3.2.4). This was fluoride sensitive, which is found in monocytes but not lymphocytes (Dacie and Lewis, 1984).

The test for lysozyme activity, which is present in the monocytic series of cells after the promonocyte stage and in neutrophils, but not in lymphocytes was positive.

S100, Peanut agglutinin and a-mannosidase stains were all negative.
Rosettes were formed with sensitized srbc, showing that the cells expressed Fc receptors.

Immunocytochemical staining showed that the cells expressed the Class 1 MHC but all of the other antigens tested for in Table 3.2.1, which included MHC Class II, T lymphocytes, B lymphocytes and monocyte/macrophage markers, were negative.

Tests for infection by Ebstein Barr virus and mycoplasma were negative.
Figure 3.2.1 Electron microscopy of cells from cell line

Figure 3.2.2 Electron microscopy of cells from cell line
Figure 3.2.3 Cells from cell line stained with acid phosphatase.

Figure 3.2.4 Cells from cell line stained with non-specific esterase.
3.2.6 Attempts to differentiate cell line

To attempt to make the cell line more mature and express more markers of cell type, several different culture conditions were used as in 2.5.22. These have been shown to induce differentiation in normal and tumour cells including the promyelocyte HL-60 and monoblast-like U937 cell lines. A range of concentrations of the chemicals was used, with previous publications giving a guide as to what concentrations the chemical produced its effect.

Rubenfeld et al (1981) have demonstrated that in vitro cocultivation of bone marrow cells or circulating T cells with epidermal cells will induce the production of the enzyme terminal deoxynucleotidyl transferase in a percentage of the mononuclear cells. The cell line was therefore cocultivated with epidermal cells to see if this induced maturation.

Phorbol esters induce differentiation of some cell systems but cause proliferation of others (Vandenbark and Niedel, 1984). They induced maturation of human keratinocytes in vitro but caused increased growth of an unidentified subpopulation of cells (Hawley-Nelson et al, 1982). They induce irreversible differentiation of a number of human leukaemia cell lines of myeloid-myelomonocytic lineage including
Calcitriol has been shown to induce the morphological and functional differentiation and inhibit the growth of U937 cells (Rigby et al, 1984).

Retinoids can influence both cellular differentiation and proliferation in normal and some tumour cells (Sporn and Roberts, 1984).

Prostaglandin E2 or cholera toxin markedly increased the extent of retinoic acid differentiation of U937 (Olsson and Breitman, 1982) and HL-60 (Olsson et al, 1982b) cell lines.

Alpha and beta-interferons have been shown to induce the differentiation of U937 cells but not of HL-60 cells (Hattori et al, 1983).

Cells were harvested daily for ten days, cyto spin preparations (2.4.3) made and the cells examined using the same enzymehistochemical and immunocytochemical stains as before (3.2.4). Cells from day 10 were examined under the electron microscope (2.5.7).

3.2.7 Results of attempts to differentiate cell line

Calcitriol and retinoids inhibited the growth rate of the cells but all of the different attempts to induce differentiation in the cell line failed to
produce any change in the morphological, enzyme histochemical and immunohistochemical features examined.

3.2.8 Discussion

A cell line was produced from the bone marrow of a child with histological evidence of marrow involvement by LCH. The cells were morphologically histiocytic and had fluoride-sensitive non-specific esterase and lysozymal activity to support this. They expressed Class 1 but not Class 2 MHC antigens and had Fc receptors. They did not have the characteristic features of LCH cells; ie were negative to S100, Peanut agglutinin and a-mannosidase and did not express CD1 (OKT6) or CD4 antigens or have Langerhans cell granules present on electron microscopy. T cell, B cell and macrophage antigenic markers were all negative.

Attempts to induce maturation of the cell line were all unsuccessful.

Although this was a histiocytic line established from a patient with LCH, its inability to express the characteristic features of LCH cells means that it may be a bone marrow cell which is a precursor of cells other than LCH cells. Unless it can be made to mature to a cell with more characteristics in common
with LCH cells, it is of no value in attempting to elucidate the functional characteristics of LCH cells.

LCH cells, like Langerhans cells, may be unable to proliferate during in vitro cultivation because they are terminally differentiated. If this is so, it would support a reactive rather than a malignant aetiology for the disease.
3.3 Comparison of the functional activities of Langerhans cells, blood dendritic cells and different monocyte populations.

3.3.1 Introduction

It is now recognised that the macrophage series is a heterogeneous population of cells with different phenotypic and functional characteristics. A subpopulation of this series, known as dendritic cells has been identified which, unlike classical macrophages, are poorly phagocytic (Kaye, Chain and Feldman, 1985) and lack Fc and complement receptors (Steinman and Ussenzweig, 1980). Dendritic cells are potent stimulators of allogeneic cells in vitro in the mixed lymphocyte reaction (Steinman and Witmer, 1978) and are also powerful at presenting foreign antigens and initiating immune responses in vitro (Ussenzweig et al, 1980). Studies in animals have suggested that it is dendritic cell contamination that is responsible for antigen presentation by adherent peripheral blood mononuclear cells (PBM), (Ussenzweig and Steinman, 1986) although they make up only about 2% of the PBM population (Knight et al, 1987). Cells from a variety of sites have been classified as dendritic cells on
morphological grounds. These include the dendritic cell of the blood, interdigitating reticulum cells of the lymph node, veiled cells in afferent lymphatics and Langerhans cells in the skin. The relationship of these cells is speculative and although some authors believe that the Langerhans cell becomes a veiled cell and then an interdigitating reticulum cell after antigen challenge in the skin (Hoefsmit et al, 1982), others feel that although the cells are related they mature from a common stem cell rather than one becoming another (Ishii and Wanatabe, 1987).

Langerhans cells are the potent antigen presenting cells of the epidermis and in an epidermal cell suspension they are able to present PPD to T cells (Braathen and Thorsby, 1980. Braathen and Thorsby (1983) found that epidermal cells were more potent than monocytes at presenting nickel and Herpes simplex virus, but not PPD, leading to a T cell blastogenic response. Enriched Langerhans cells have been shown (Bjercke et al, 1984) to be more effective than similar numbers of adherent monocytes in inducing T cell responses to Candida albicans and herpes simplex virus and did not require the presence of significant numbers of keratinocytes to exert this function (Bjercke et al, 1984a,b). The alloantigen presenting capacities of Langerhans cells and blood
dendritic cells are very similar, and more efficient on a cell to cell basis than PBMs in animals (Sontheimer, 1985).

Langerhans cells have substantial phenotypic differences to blood dendritic cells as shown in Table 3.3.1. No study, however, has compared their functional characteristics in presenting recall antigen in man.
Table 3.3.1 Comparison of blood dendritic cells and Langerhans cells.

<table>
<thead>
<tr>
<th></th>
<th>Dendritic cells</th>
<th>Langerhans cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen presentation</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adherence to plastic</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proliferation in culture</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T cell surface receptors</td>
<td>-</td>
<td>+ (CD1, CD4)</td>
</tr>
<tr>
<td>Monocyte</td>
<td>-</td>
<td>+ (Fc IgG, C')</td>
</tr>
<tr>
<td>HLA-Dr expression</td>
<td>+</td>
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</table>
The aim of this study was to compare the abilities of different putative antigen presenting cells to present purified protein derivative of tuberculin (PPD) to autologous memory T cells in man. I compared monocytes separated by two different techniques; a quick method with a relatively new density gradient centrifugation medium, Nycodenz-monocyte, and traditional immune adherence. I compared these to enriched blood dendritic cells and to Langerhans cells in an epidermal cell suspension. It was hoped that these functional studies could then be applied to LCH cells.

Studies were limited by the number of cells that could be generated from each volunteer. In view of this, a 20ul hanging drop technique was used utilising Terasaki plates to culture the cells. The culture of cells in 20ul hanging drops from Terasaki plates was initially applied to lymphocytes (Farrant, 1980; O'Brien, 1979). It has since been used for dendritic cells from synovial fluid (Tyndall et al, 1983) and to measure a mixed leukocyte reaction (MLR) using peripheral blood dendritic cells (Knight et al, 1986). At the time these experiments were performed the Terasaki plates had not been applied to the measurement of presentation of recall antigen by dendritic cells or to the study of Langerhans cell or LCH cell function.
3.3.2 Materials and methods

Epidermal cells and venous blood were obtained from 4 healthy volunteers known to produce a delayed hypersensitivity reaction to tuberculin protein (PPD). Blood mononuclear cells were separated as in 2.5.15, T cells by E-rosetting (2.5.16), monocytes by adherence (2.5.18) and using Nycodenz-monocyte (2.5.17) and dendritic cells were separated as described by Gaudernack and Bjercke (1985) (2.5.19). Adherent monocytes left after removing enriched dendritic cells by panning with Immunoglobulin G were also studied. Epidermal cells were obtained using suction blisters and were separated as in 2.5.14. The cells were suspended in RPMI 1640 with 10% Fetal Bovine Serum (2.5.2) and the viability of the cells was determined as in 2.5.3 and in all cases was above 95%.

Cytospin preparations of the cells were made as in 2.4.3 and stained using an APAAP technique as in 2.4.9. Antibodies used were T cell cocktail (Royal Free) which stains all peripheral blood T cells; Dako-macrophage which reacts with circulating monocytes and tissue macrophages; OKT6 (Ortho) which recognises CD1a expressed on human thymocytes and Langerhans cells and RFD1 (Royal Free) which labels
part of the Dr locus that is preferentially expressed by dendritic cells. (Poulter et al, 1986).

Preliminary experiments were performed to establish a dose response curve for PPD as described in 2.5.25 and the maximum responses were obtained with PPD at a concentration of 10μg/ml (Figure 3.3.1). T cells at a concentration of 6 x 10⁶/ml in the presence of a range of concentrations of different putative antigen presenting cells were placed in the wells of a Terasaki plate with 10μg/ml of PPD of tuberculin as described in 2.5.25. The total volume in each well was adjusted to 20μl and the cells were cultured for 6 days, then pulsed with tritiated thymidine, harvested and the incorporated radioactivity counted (2.5.25).
Figure 3.3.1 Dose-response relationship between concentration of PPD and response of T cells in the presence of antigen presenting cells.

Response of PBMs to different concentrations of PPD

Response as dpm x 10^-3

Final conc PPD ug/ml
3.3.3 Results

**Phenotypic analysis of cell populations**

The results of staining cell populations using different antibodies is shown in Table 3.3.2. This shows that the T cell population is contaminated by 2% Dako-mo positive monocytes; the epidermal cell suspension contains 1% OKT6-positive Langerhans cells and the monocytes are 75-80% pure with 1% RFD1-positive dendritic cells in the Nycodenz monocyte population and 2% in the adherent monocyte population. The enriched dendritic cell population contained only 10% RFD1-positive dendritic cells and the rest of the cells were mainly monocytes.
Table 3.3.2 Percentage of cells in each purified population staining with different antibodies.
Results as mean +/- 1SD for the 4 subjects.

<table>
<thead>
<tr>
<th></th>
<th>RFT</th>
<th>Dako-mo</th>
<th>OKT6</th>
<th>RFD1</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>95+-5</td>
<td>2+-1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EC</td>
<td>0</td>
<td>1+-1</td>
<td>1+-1</td>
<td>0</td>
</tr>
<tr>
<td>NM</td>
<td>0</td>
<td>80+-8</td>
<td>0</td>
<td>2+-1</td>
</tr>
<tr>
<td>MA</td>
<td>0</td>
<td>75+-10</td>
<td>0</td>
<td>1+-1</td>
</tr>
<tr>
<td>DC</td>
<td>0</td>
<td>90+-5</td>
<td>0</td>
<td>10+-2</td>
</tr>
</tbody>
</table>

EC = epidermal cells
NM = Nycodenz monocytes
MA = adherent monocytes
DC = dendritic cells
mo = macrophage
Responses of T cells with different APCs

Dendritic cells, Langerhans cells and monocytes were all capable of presenting PPD to autologous T cells resulting in a blastogenic response. Table 3.3.3 shows the results for each subject as a Stimulation Index (Stimulation Index = response of cells with PPD divided by background counts in cells without PPD). Table 3.3.4 shows the mean results for the four subjects and this is shown graphically in Figure 3.3.2.

With all the cell populations the response tended to increase as the cell concentration increased, but with the Langerhans cells in the epidermal cell suspension the response reached a peak and then began to fall as the cell concentration continued to rise. The reason for the fall in response with higher numbers of epidermal cells was probably due to the number of epidermal cells present which exhausted the nutrients.

Cells separated by Nycodenz-monocyte had similar dose-response characteristics to those separated by immune adherence. Dendritic cells produced a similar response at a cell concentration about one-eighth that of the adherent or Nycodenz monocytes. Adherent mononuclear cells depleted of dendritic cells were unable to present PPD to T cells demonstrating that in man, as in animal models, it is the dendritic cell
that is responsible for antigen presentation to memory T cells. Furthermore the dendritic cell population contained 10% RFD1-positive cells; five times that of Nyodenz monocytes and 10 times that of adherent monocytes which is of a similar order to the relative activity of the dendritic cell compared to the monocyte population.

Epidermal cells contained 1% Langerhans cells. The potency of the epidermal cells suspension in presenting PPD to T cells was greater than that of enriched dendritic cells although the number of antigen presenting Langerhans cells was one-tenth (1% versus 10%) of that of the RFD1-positive dendritic cells. It is possible that Langerhans cells may be more potent than pure dendritic cells at presenting PPD of tuberculin to T cells. However, two other possibilities exist; firstly that the keratinocytes present in the epidermal cell suspension were producing stimulatory cytokines which augmented the response. Keratinocytes are known to elaborate a number of cytokines including interleukin 1 (Luger). Secondly it may be that inhibitory cells were present in the dendritic cell population which reduced the measurable response.
**Table 3.3.3.** Response of T cells to PPD presented by different APCs measured as a Stimulation Index (mean of stimulated cells/ mean of unstimulated cells for the triplicate of wells). All results at 10μg/ml PPD.

APC = antigen presenting cell

ECS = epidermal cell suspension  DC = dendritic cells

MN = Nycodenz-monocytes  MA = adherent monocytes

MA-DC = MA after depletion of DC

**Subject 1**

<table>
<thead>
<tr>
<th>APC</th>
<th>Cell Conc</th>
<th>ECS</th>
<th>DC</th>
<th>MN</th>
<th>MA</th>
<th>MA-DC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3x10^6/ml</td>
<td>2.1</td>
<td>29.5</td>
<td>9.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.5x10^6</td>
<td>10.3</td>
<td>18.3</td>
<td>5.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.5x10^6</td>
<td>13.9</td>
<td>13.8</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.8x10^6</td>
<td>13.8</td>
<td>7.0</td>
<td>1.5</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
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<td>2.5</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.4x10^4</td>
<td>12.5</td>
<td>4.5</td>
<td>0.8</td>
<td>2.5</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>4.7x10^4</td>
<td>8.7</td>
<td>1.9</td>
<td>0.5</td>
<td>1.3</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>2.3x10^4</td>
<td>8.1</td>
<td>3.9</td>
<td>0.4</td>
<td>2.7</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1.2x10^4</td>
<td>2.6</td>
<td>1.2</td>
<td>2.3</td>
<td>1.9</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>5.8x10^3</td>
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<td>1.0</td>
<td>1.5</td>
<td>2.8</td>
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<td>2.9x10^3</td>
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<td>1.0</td>
<td>0.9</td>
<td>1.2</td>
<td>0.8</td>
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</table>
Table 3.3.3 (b).

Subject 2

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<th>Cell Conc</th>
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<th>MN</th>
<th>MA</th>
<th>MA-DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$3 \times 10^5$/ml</td>
<td>1.6</td>
<td>20.2</td>
<td>9.8</td>
<td></td>
<td>1+-1</td>
</tr>
<tr>
<td>$1.5 \times 10^6$</td>
<td>4.6</td>
<td>13.0</td>
<td>10.4</td>
<td>8.4</td>
<td>1+-2</td>
</tr>
<tr>
<td>$7.5 \times 10^6$</td>
<td>7.4</td>
<td>7.5</td>
<td>7.8</td>
<td>5.2</td>
<td>1+-2</td>
</tr>
<tr>
<td>$3.8 \times 10^7$</td>
<td>9.9</td>
<td>2.9</td>
<td>4.4</td>
<td>3.6</td>
<td>2+-1</td>
</tr>
<tr>
<td>$1.9 \times 10^7$</td>
<td>13.3</td>
<td>2.4</td>
<td>3.3</td>
<td>2.2</td>
<td>1+-2</td>
</tr>
<tr>
<td>$9.4 \times 10^4$</td>
<td>17.8</td>
<td>1.8</td>
<td>2.5</td>
<td>1.4</td>
<td>1+-2</td>
</tr>
<tr>
<td>$4.7 \times 10^4$</td>
<td>14.6</td>
<td>1.4</td>
<td>1.2</td>
<td></td>
<td>2+-1</td>
</tr>
<tr>
<td>$2.3 \times 10^4$</td>
<td>7.1</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1.2 \times 10^4$</td>
<td>8.9</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$5.8 \times 10^3$</td>
<td>6.3</td>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$2.9 \times 10^3$</td>
<td>2.3</td>
<td>1.7</td>
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</table>
Table 3.3.3 (c).

Subject 3

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<tr>
<th>Cell Conc</th>
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<th>DC</th>
<th>MN</th>
<th>MA</th>
<th>MA-DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>3x10&lt;sup&gt;-6&lt;/sup&gt;/ml</td>
<td>13.2</td>
<td>29.3</td>
<td>11.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5x10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>20.9</td>
<td>19.2</td>
<td>10.8</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>7.5x10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>17.7</td>
<td>13.8</td>
<td>6.5</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>3.8x10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>17.5</td>
<td>6.2</td>
<td>3.3</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>1.9x10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>15.0</td>
<td>6.8</td>
<td>1.1</td>
<td>0.6</td>
<td>1+-1</td>
</tr>
<tr>
<td>9.4x10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>10.8</td>
<td>4.5</td>
<td>0.6</td>
<td>3.5</td>
<td>1+-2</td>
</tr>
<tr>
<td>4.7x10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>10.2</td>
<td>1.8</td>
<td>3.9</td>
<td>1.1</td>
<td>1+-1</td>
</tr>
<tr>
<td>2.3x10&lt;sup&gt;-7&lt;/sup&gt;</td>
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<td>3.9</td>
<td>0.9</td>
<td>1.4</td>
<td>3+-2</td>
</tr>
<tr>
<td>1.2x10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>2.0</td>
<td>1.2</td>
<td>1.1</td>
<td>3.8</td>
<td>2+-1</td>
</tr>
<tr>
<td>5.8x10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>0.5</td>
<td>0.6</td>
<td>0.3</td>
<td>0.8</td>
<td>1+-1</td>
</tr>
<tr>
<td>2.9x10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>2.1</td>
<td>2.4</td>
<td>2.7</td>
<td>1.0</td>
<td>1+-1</td>
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</table>
Table 3.3.3 (d).

Subject 4

<table>
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<th>Cell Conc</th>
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<th>DC</th>
<th>MN</th>
<th>MA</th>
<th>MA-DC</th>
</tr>
</thead>
<tbody>
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<td>3x10⁶/ml</td>
<td>5.8</td>
<td>15.0</td>
<td>8.3</td>
<td>10.4</td>
<td></td>
</tr>
<tr>
<td>1.5x10⁶</td>
<td>6.6</td>
<td>6.6</td>
<td>8.1</td>
<td>11.1</td>
<td></td>
</tr>
<tr>
<td>7.5x10⁵</td>
<td>18.3</td>
<td>30.4</td>
<td>2.4</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>3.8x10⁵</td>
<td>12.1</td>
<td>18.4</td>
<td>0.8</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>1.9x10⁵</td>
<td>4.1</td>
<td>15.7</td>
<td>2.3</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>9.4x10⁴</td>
<td>3.6</td>
<td>12.9</td>
<td>1.1</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>4.7x10⁴</td>
<td>2.8</td>
<td>10.5</td>
<td>1.7</td>
<td>2.9</td>
<td></td>
</tr>
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<td>2.3x10⁴</td>
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<td>10.4</td>
<td>0.7</td>
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</tr>
<tr>
<td>1.2x10⁴</td>
<td>1.1</td>
<td>7.1</td>
<td>0.6</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>5.8x10³</td>
<td>2.4</td>
<td></td>
<td>2+-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.9x10³</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3.4. Responses of T cells in association with different antigen presenting cells to PPD. Mean results + SEM for the 4 subjects shown as Stimulation index.

<table>
<thead>
<tr>
<th>Cell conc</th>
<th>ECS</th>
<th>DC</th>
<th>MN</th>
<th>MA</th>
</tr>
</thead>
<tbody>
<tr>
<td>(11) 3x10^6/ml</td>
<td>5.7+-3</td>
<td>23.5+-3</td>
<td>8.8</td>
<td>10.6+-1</td>
</tr>
<tr>
<td>(10) 1.5x10^6</td>
<td>10.6+-3</td>
<td>14.3+-3</td>
<td>8.7+-1</td>
<td>9.1+-1</td>
</tr>
<tr>
<td>(9) 7.5x10^5</td>
<td>14.3+-3</td>
<td>16.4+-4</td>
<td>4.9+-1</td>
<td>5.8+-1</td>
</tr>
<tr>
<td>(8) 3.8x10^5</td>
<td>13.3+-2</td>
<td>8.7+-3</td>
<td>2.5+-1</td>
<td>4.8+-2</td>
</tr>
<tr>
<td>(7) 1.9x10^5</td>
<td>11.6+-2</td>
<td>9.5+-3</td>
<td>2.3+-1</td>
<td>2.6+-2</td>
</tr>
<tr>
<td>(6) 9.4x10^4</td>
<td>11.2+-3</td>
<td>5.9+-2</td>
<td>1.3+-1</td>
<td>2.6+-1</td>
</tr>
<tr>
<td>(5) 4.7x10^4</td>
<td>9.1+-2</td>
<td>3.9+-2</td>
<td>1.1+-0</td>
<td>1.8+-1</td>
</tr>
<tr>
<td>(4) 2.3x10^4</td>
<td>4.7+-2</td>
<td>5.1+-2</td>
<td>0.6+-0</td>
<td>2.1+-1</td>
</tr>
<tr>
<td>(3) 1.2x10^4</td>
<td>3.7+-2</td>
<td>2.6+-2</td>
<td>1.4+-1</td>
<td>2.1+-1</td>
</tr>
<tr>
<td>(2) 5.8x10^3</td>
<td>2.8+-1</td>
<td>1.2+-1</td>
<td>0.9</td>
<td>1.8</td>
</tr>
<tr>
<td>(1) 2.9x10^3</td>
<td>1.5+-1</td>
<td>1.7+-1</td>
<td>1.8</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Numbers in brackets denote antigen presenting cell concentration as shown in Figure 3.3.2.
Figure 3.3.2. Responses of T cells combined with different antigen presenting cells to PPD. Mean results for 4 subjects. Concentration of cells corresponds to numbers in brackets in Table 3.3.4.
3.4.4 Discussion

Studies of Langerhans cell function have been limited by difficulties in obtaining autologous Langerhans cells in sufficient quantities to be able to perform valid experiments. Initial experiments in this study using a standard microsystem demonstrated that insufficient cells were generated to enable the antigen-presenting capacity of different cell populations over a wide range of concentrations to be compared simultaneously. The use of the hanging droplet technique enabled one-tenth of the number of cells required in the standard microsystem to be used and responses were obtained with as few as 15 Langerhans cells per well. This technique is therefore particularly well suited to the study of dendritic cells including Langerhans cells.

Monocytes separated by Nycodehz-monocyte were functionally similar to those separated by immune adherence in presenting PPD to autologous T cells. Blood dendritic cells produced the same response as monocytes at approximately one-eighth of the concentration of Nycodehz or adherent monocytes. Dendritic cells had 10% RFD-1 positive cells compared to 2% in the Nycodehz population and 1% in the adherent monocyte population, and differences in the activities of the cells could be due to differences
in numbers of antigen presenting cells or in cells influencing their function. Monocytes depleted of dendritic cells were unable to act as antigen-presenting cells which indicates that in man, as in animals, it is the dendritic cell subpopulation that is responsible for the antigen presenting capacity of monocytes.

Contrary to Braathen and Thorsby (1980), who found that Langerhans cells and adherent monocytes had a similar functional ability to present PPD, the epidermal cell suspension which was composed of 1% OKT-6 positive Langerhans cells were found to be about 16 times more effective than monocytes at presenting PPD to autologous T cells resulting in a blastogenic response. If the Langerhans cells are the only active cells in this population (Bjerke et al, 1984 a,b), and dendritic cells were the only active cells in the monocyte population, then that makes Langerhans cells about 16 times more effective than RFD-1 dendritic cells on a one to one basis. The epidermal cell suspension was at least as effective as the enriched dendritic cell population despite one-tenth of the number of Langerhans cells compared to the number of dendritic cells (1% against 10%). This difference could, however, be due to the contaminating cells present which may have stimulatory or inhibitory effects. There are several
techniques to isolate blood dendritic cells (Knight, 1987; Gaudernack and Bjercke, 1985) but none obtains a pure cell population. Until purer populations of cells are studied the significance of the difference found between Langerhans and dendritic cells remains uncertain.

In conclusion therefore, Nycodenz monocyte produced a population of cells that was functionally similar at presenting PPD of tuberculin to that purified by immune adherence. In man, as in animals, it is the dendritic cell subpopulation that is responsible for the antigen presenting capacity of monocytes as monocytes depleted of dendritic cells were functionally inactive. Langerhans cells and blood dendritic cells are potent antigen-presenting cells compared to monocytes in man. This supports their grouping together as dendritic cells on a functional as well as morphological basis.

The hanging droplet technique can be used for the culture of Langerhans cells to study their antigen presenting capacity. This means that one-tenth of the number of cells are needed than in a conventional microsystem, and reduces one of the main constrictions on the study of Langerhans cell function; that of limited cell number.
3.4 Functional activity of LCH cells

3.4.1 Introduction

Although LCH cells are thought to be closely related to Langerhans cells there remain several phenotypic differences between them. For example the CD4 antigen is strongly positive on LCH cells but only weakly so on Langerhans cells (Harrist et al, 1983). LCH cells also express CR1 antigen and Interleukin-2 receptors which are usually absent from Langerhans cells. CD4 and interleukin-2 receptors are activation antigens and it is possible that LCH cells represent Langerhans cells at a more activated level.

One of the most distinctive features of the in vitro functional activity of Langerhans cells, and one which is probably their main in vivo function, is their ability to act as antigen-presenting cells. There have been no reported studies of the functional activities of LCH cells in this respect. It is difficult to obtain material for study when the disease is rare and affects mainly young children. The latter limits both the availability of specimens for what is a research rather than a diagnostic investigation, and the size of the specimens thus obtained.
The aim of these experiments was to determine whether LCH cells could act as accessory cells and present mitogens and recall antigens to autologous T cells leading to a blastogenic response.

In specimens obtained from children who had not been immunized, the blastogenic response of T cells to mitogens rather than to recall antigens to which they may not have been immunized, was studied. The size of the response was measured as a count of radioactivity using incorporation of tritiated thymidine into DNA.

3.4.2 Methods and results

Normal volunteers - standard microsystem.

100nl of venous blood and specimens of skin were obtained from three healthy volunteers. PBMNs were separated from the blood as in 2.5.15 and some of these were then purified by E-rosetting to obtain T cells (2.5.16) or by immune adherence to obtain monocytes (2.5.18). Epidermis was obtained by inducing one 8mm suction blister and the cells were separated as in 2.5.14. A 4mm punch biopsy of normal forearm skin was taken, the epidermis added to the suction blister roof and processed with it, and the dermal cells separated as in 2.5.13. Aliquots of the
different cell populations were taken, cytospin preparations made (2.4.3) and stained using OKT6 (Ortho), RFT cell cocktail (Royal Free) and Dako-Dr (Dakopatts) antibodies by an APAAP technique (2.4.9).

The responses of different combinations of cells to the mitogens phythaemagglutinin and Concanavelin A were then studied using a standard microsystem as in 2.5.24. The resperter T cells were used at a concentration of $10^6$/well, the epidermal cells were added at a range of $2.5 \times 10^4$ to $10^5$ per well and monocytes at $2.5 \times 10^4$ to $10^5$ per well. Dermal cells were used at a concentration of $10^5$/well. Controls were carried out with cells but excluding the mitogens. All combinations were carried out in triplicate. The results are shown in Table 3.4.1. Table 3.4.2 shows the results of staining different cell populations with different antibodies.
Table 3.4.1 Results for normal subjects when T cells were stimulated by mitogens. Results are given as a Stimulation Index (SI) where SI was equal to the mean counts in the test wells divided by the mean count in the negative control wells without mitogen.

PBM = peripheral blood mononuclear cells
Mono = monocytes x $10^{-5}$/well
Con A = Concanavalin A  PHA = phythaemagglutinin
EC = epidermal cell suspension x $10^{-5}$/well

<table>
<thead>
<tr>
<th>Subject A.</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>cells incubated</td>
<td>PHA</td>
</tr>
<tr>
<td>together</td>
<td></td>
</tr>
<tr>
<td>PBM</td>
<td>15.3</td>
</tr>
<tr>
<td>T cells</td>
<td>0.9</td>
</tr>
<tr>
<td>T cells + mono 2.5</td>
<td>7.6</td>
</tr>
<tr>
<td>&quot; 5</td>
<td>10.5</td>
</tr>
<tr>
<td>&quot; 10</td>
<td>18.4</td>
</tr>
<tr>
<td>T cells + EC 2.5</td>
<td>8.2</td>
</tr>
<tr>
<td>&quot; 5</td>
<td>15.7</td>
</tr>
<tr>
<td>&quot; 10</td>
<td>19.1</td>
</tr>
<tr>
<td>mono 10</td>
<td>0.42</td>
</tr>
<tr>
<td>EC 10</td>
<td>0.85</td>
</tr>
<tr>
<td>T cells + mono 10 + EC 10</td>
<td>19.7</td>
</tr>
<tr>
<td>T cells + mono 10 + dermal cells</td>
<td>15.9</td>
</tr>
</tbody>
</table>
Table 3.4.1(b).

<table>
<thead>
<tr>
<th>Subject B. cells incubated together</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHA</td>
</tr>
<tr>
<td>PBM</td>
<td>12.6</td>
</tr>
<tr>
<td>T cells</td>
<td>0.9</td>
</tr>
<tr>
<td>T cells + mono 2.5</td>
<td>8.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>9.2</td>
</tr>
<tr>
<td>&quot;</td>
<td>11.7</td>
</tr>
<tr>
<td>T cells + EC 2.5</td>
<td>4.5</td>
</tr>
<tr>
<td>&quot;</td>
<td>11.3</td>
</tr>
<tr>
<td>&quot;</td>
<td>13.1</td>
</tr>
<tr>
<td>mono 10</td>
<td>1.1</td>
</tr>
<tr>
<td>EC 10</td>
<td>0.8</td>
</tr>
<tr>
<td>T cells + mono 10 + EC 10</td>
<td>12.9</td>
</tr>
<tr>
<td>T cells + mono 10 + dermal cells</td>
<td>15.9</td>
</tr>
</tbody>
</table>
Table 3.5.1(c).

<table>
<thead>
<tr>
<th>Subject C.</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>cells incubated together</td>
<td>PHA</td>
</tr>
<tr>
<td>PBM</td>
<td>5.6</td>
</tr>
<tr>
<td>T cells</td>
<td>0.7</td>
</tr>
<tr>
<td>T cells + mono 2.5</td>
<td>3.4</td>
</tr>
<tr>
<td>&quot; 5</td>
<td>4.6</td>
</tr>
<tr>
<td>&quot; 10</td>
<td>6.1</td>
</tr>
<tr>
<td>T cells + EC 2.5</td>
<td>4.1</td>
</tr>
<tr>
<td>&quot; 5</td>
<td>7.2</td>
</tr>
<tr>
<td>&quot; 10</td>
<td>7.9</td>
</tr>
<tr>
<td>mono 10</td>
<td>1.0</td>
</tr>
<tr>
<td>EC 10</td>
<td>0.9</td>
</tr>
<tr>
<td>T cells + mono 10 + EC 10</td>
<td>6.9</td>
</tr>
<tr>
<td>T cells + mono 10 + dermal cells</td>
<td>7.9</td>
</tr>
</tbody>
</table>
Table 3.4.2 Percentage of cells in each purified population staining with different antibodies.
Results as mean +/- 1 SD for the three subjects.

<table>
<thead>
<tr>
<th>Antibody used</th>
<th>RFT</th>
<th>Dako-Dr</th>
<th>OKT6</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>95+-5</td>
<td>5+-5</td>
<td>0</td>
</tr>
<tr>
<td>monocytes</td>
<td>1+-2</td>
<td>35+-5</td>
<td>0</td>
</tr>
<tr>
<td>Epidermal cells</td>
<td>0</td>
<td>1+-1</td>
<td>1+-1</td>
</tr>
</tbody>
</table>

T cells in the presence of monocytes were stimulated by mitogens to proliferate and incorporate tritiated thymidine into their DNA, and the size of this proliferative response is indicated by the amount of incorporated tritium, measured as a count per minute of radioactivity. Epidermal cells, which contain about 1% HLA-Dr and CD-1a positive Langerhans cells, can replace monocytes in their action as accessory cells for mitogen-driven T cell proliferative responses. The responses to monocytes and epidermal cells together with T cells was slightly greater than to either accessory cell alone. That these responses are not additive is probably because they were measured at the plateau of the T cell responses.

Dermal cells did not affect the response of T cells.
in the presence of monocytes to mitogens.

**Patients-standard microsystem.**

A variety of tissues was obtained from patients who all had multi-system disease. None was on systemic treatment at the time the specimens were obtained. The experiments performed were limited by the small number of cells obtained from each patient.

**Patient AF.** Part of a spleen that had been removed because of hypersplenism was obtained from a 5 year old boy with well-established LCH. Part of the specimen was snap-frozen in liquid Nitrogen, sections were cut as in 2.4.2 and stained using OKT6 antibody as in 2.4.9.

A single cell suspension was obtained from the rest of the specimen as in 2.5.12, and the constituent cells were then purified. T cells were obtained by E-rosetting part of the cell suspension with sheep red blood cells (2.5.16). Monocytes were separated from the cell suspension by immune adherence (2.5.18) onto plastic petri dishes and LCH cells were enriched by panning using the antibody OKT6 which binds to LCH cells (2.5.20). Samples of the cells were taken, cytospin preparations made (2.4.3) and stained using an APAAP technique (2.4.9) using OKT6, RFT cell cocktail and Dako-Dr antibodies.
The cells were incubated with mitogens using a standard microsystem as in 2.5.24. LCH cells were used at a concentration of $10^5$ per well, T cells at $10^5$ per well and monocytes at $10^5$ per well. All combinations of cells were carried out in triplicate wells. Control wells with cells but without the mitogen were also prepared.

**Results.** Sections of the spleen showed that 50% of the cells had a histiocytic morphology and were OKT6-positive, thus confirming substantial involvement with LCH cells. The staining characteristics of the different cell populations is shown in Table 3.4.3. Table 3.4.4 shows the blastogenic response of cells from patient AF after culture with the mitogens Concanavalin A and Phythaemagglutinin.
Table 3.4.3. Percentage of cells from patient AF showing each purified population staining with different antibodies.

<table>
<thead>
<tr>
<th>Antibody used</th>
<th>RFT</th>
<th>Dako-Dr</th>
<th>OKT6</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>95</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>monocytes</td>
<td>0</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>LCH cells</td>
<td>0</td>
<td>70</td>
<td>70</td>
</tr>
</tbody>
</table>
Table 3.4.4 Response of cells from AF to mitogens

Results are shown as Stimulation Index (SI) where

\[
SI = \frac{\text{mean counts in test wells}}{\text{mean counts in control wells without mitogen}}
\]

<table>
<thead>
<tr>
<th>Cells incubated</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>together</td>
<td>PHA</td>
</tr>
<tr>
<td>T cells</td>
<td>1.1</td>
</tr>
<tr>
<td>T cells + mono</td>
<td>15.2</td>
</tr>
<tr>
<td>T cells + LCH cells</td>
<td>1.0</td>
</tr>
<tr>
<td>Mono</td>
<td>1.1</td>
</tr>
<tr>
<td>Mono + LCH cells</td>
<td>0.9</td>
</tr>
<tr>
<td>T cells + Mono + LCH cells</td>
<td>0.9</td>
</tr>
</tbody>
</table>

PHA = phythaemagglutinin
Con A = Concanavelin A
Mono = adherent monocytes
T cells in the presence of monocytes produced a blastogenic response to mitogens. LCH cells were unable to substitute for monocytes and therefore could not act as accessory cells in this experiment. Furthermore the presence of LCH cells completely inhibited the normal blastogenic response of T cells in the presence of monocytes to mitogens. The concentration of LCH cells (50% of $10^4$/well = 5 x $10^3$/well) was of a similar order of magnitude to the RFD-1 positive cells in the monocyte population ($10^3$/well) and the Langerhans cells in the epidermal cell suspension ($10^3$/well) from the normal volunteers which did produce a response.
Patient EL. Blood and a skin biopsy from the vulva were obtained from a 55 year old woman. Part of the skin biopsy was snap-frozen in liquid Nitrogen, sections cut (2.4.2) and stained with OKT6 antibody (2.4.9).

PBM's were separated from the blood (2.5.15), then T cells were isolated by E-rosetting with sheep red blood cells (2.5.16) and monocytes by immune adherence (2.5.18). LCH cells were separated from the skin as in 2.5.13 and panned using OKT6 antibody (2.5.20) to separate the LCH cells. Cytospin preparations of each cell type were made (2.4.3) and stained using OKT6, RFT cell cocktail and Dako-Dr antibodies with an APAAP technique (2.4.9).

The cells were incubated with Con A as in 2.5.24 using a standard microsystem. There were insufficient cells to study the responses to PHA as well as to Con A. T cells and monocytes were added at concentrations of $10^5$ of each cell type per well, and LCH cells at $10^4$/well. Negative controls without Con A were also set up.

**Results.** Sections of the skin showed an infiltration with histiocytic cells that were OKT6-positive. The staining characteristics of the different cell populations is shown in Table 3.4.5.
Table 3.4.5 Percentage of cells from patient EL showing each purified population staining with different antibodies.

<table>
<thead>
<tr>
<th>Antibody used</th>
<th>RFT</th>
<th>Dako-Dr</th>
<th>OKT6</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>90</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>monocytes</td>
<td>0</td>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>LCH cells</td>
<td>0</td>
<td>50</td>
<td>45</td>
</tr>
</tbody>
</table>

Table 3.4.6 shows the response of T cells after incubation with Concanavalin A.
Table 3.4.6 Response of cells from EL to mitogen

Blastogenic response of cells from Patient EL incubated with Concanavelin A. Results are shown as Stimulation Index of mean result of stimulated cells divided by mean count of unstimulated cells.

<table>
<thead>
<tr>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>cells incubated</td>
</tr>
<tr>
<td>together</td>
</tr>
<tr>
<td>PBM</td>
</tr>
<tr>
<td>T cells</td>
</tr>
<tr>
<td>T cells + mono</td>
</tr>
<tr>
<td>T cells + LCH cells</td>
</tr>
<tr>
<td>Mono</td>
</tr>
<tr>
<td>Mono + LCH cells</td>
</tr>
<tr>
<td>T cells + MA + LCH cells</td>
</tr>
</tbody>
</table>

PBM = peripheral blood monocytes
Mono = adherent monocytes
Con A = Concanavelin A
LCH cells were again unable to substitute for monocytes in presenting Con A to T cells leading to a blastogenic response. They again inhibited the normal response of T cells and monocytes but not completely; the response was reduced to about 20% of that without LCH cells present.
Specimens of blood and biopsies of skin were obtained from two babies with LCH. There were insufficient cells to use in the standard microsystem, so the hanging drop technique was used for cell culture. Because the children had not been immunized recall antigens could not be used so the responses of their cells to the mitogen Concanavalin A. To validate this technique, the responses of cells from normal healthy volunteers were first established.

**Normal volunteers** 20ml of fresh heparinised (10U/ml) venous blood and two 4mm diameter punch biopsies of normal forearm skin were obtained from two volunteers in their twenties. In Section 3.3 I had established that epidermal cells can act as antigen presenting cells in the Terasaki system. LCH cells occur in the dermis, so with the normal subjects I wished to establish whether normal dermal cells could affect mitogen stimulated T cell responses. PBMs were obtained (2.5.15) from the blood, T cells separated by E rosetting (2.5.16) and monocytes by immune adherence (2.5.18). The T cells were adjusted to a concentration of $10^6$/ml and the monocytes to $10^6$ and $5 \times 10^5$/ml in RPMI 1640 with 10% FBS (2.5.2). The skin was treated as in 2.5.13, the epidermis removed and the dermal cells adjusted to a concentration of $5 \times 10^5$/ml in RPMI 1640 with 10% FBS. An epidermal cell
suspension was made as in 2.5.14 and adjusted to a concentration of $10^5$ ml.

To study the response to mitogens, 5ul of the T cells were then placed in Terasaki plates (2.5.26) with 5ul of the monocytes, or 5ul of the dermal cells, or with both these cells together. The T cells were combined with the epidermal cells without the dermal cells. T cells alone, monocytes alone and dermal cells alone were used as controls. Triplicate wells of all combinations of cells were incubated with either 5ul of Concanavelin A (0.5 or 2.5ug/ml in RPMI 1640 with 10% FBS), or in RPMI 1640 with 10% FBS alone, as a control. The total volume in each well was adjusted to 20ul using RPMI 1640 with 10% FBS. The cells were incubated, pulsed and harvested as in 2.5.26. Uptake of tritium into DNA was measured as counts per minute (cpm) +/− 1 standard deviation for the triplicate readings.

Results for normal subjects These are shown in Tables 3.4.7 and 3.4.8.
Table 3.4.7. Subject JK. Response to Con A of various cell combinations. Blastogenic response of T cells measured as incorporation of thymidine into DNA measured as counts per minute +/- 1 standard deviation for each triplicate of wells.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Conc 0</th>
<th>Conc 0.5</th>
<th>Conc 2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>16+-5</td>
<td>57+-9</td>
<td>97+-28</td>
</tr>
<tr>
<td>T cells + mono (2.5x10³)</td>
<td>32+-6</td>
<td>106+-18</td>
<td>612+-82</td>
</tr>
<tr>
<td>T cells + Epi</td>
<td>47+-9</td>
<td>130+-9</td>
<td>954+-72</td>
</tr>
<tr>
<td>T cells + mono (5x10³) + epi</td>
<td>24+-12</td>
<td>ND</td>
<td>1020+-42</td>
</tr>
<tr>
<td>T cells + dermal cells</td>
<td>35+-7</td>
<td>ND</td>
<td>90+-16</td>
</tr>
<tr>
<td>T cells + mono (5x10³) + dermal</td>
<td>43+-7</td>
<td>ND</td>
<td>1151+-120</td>
</tr>
<tr>
<td>mono (5x10³)</td>
<td>20+-4</td>
<td>ND</td>
<td>25+-8</td>
</tr>
<tr>
<td>dermal cells</td>
<td>18+-4</td>
<td>ND</td>
<td>22+-5</td>
</tr>
</tbody>
</table>

mono = monocytes with conc / well
epi = epidermal cell suspension (5x10³/well)
ND = not done
Table 3.4.8. Subject AC. Response to Con A of various cell combinations. Response shown as thymidine uptake as counts per minute mean ± SD for triplicate wells

<table>
<thead>
<tr>
<th>Conc Con A ug/ml</th>
<th>0</th>
<th>0.5</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>85±-17</td>
<td>247±-40</td>
<td>271±-73</td>
</tr>
<tr>
<td>T cells + mono (2.5x10^3)</td>
<td>34±-21</td>
<td>173±-33</td>
<td>733±-104</td>
</tr>
<tr>
<td>T cells + mono (5x10^3)</td>
<td>38±-33</td>
<td>205±-35</td>
<td>1553±-256</td>
</tr>
<tr>
<td>T cells + epi</td>
<td>48±-6</td>
<td>ND</td>
<td>1770±-90</td>
</tr>
<tr>
<td>T cells + mono (5x10^3) + epi</td>
<td>42±-8</td>
<td>ND</td>
<td>2020±-210</td>
</tr>
<tr>
<td>T cells + dermal cells</td>
<td>49±-11</td>
<td>ND</td>
<td>179±-16</td>
</tr>
<tr>
<td>T cells + mono (5x10^3) + dermal</td>
<td>43±-7</td>
<td>ND</td>
<td>1151±-120</td>
</tr>
<tr>
<td>mono (5x10^3)</td>
<td>20±-3</td>
<td>ND</td>
<td>43±-14</td>
</tr>
<tr>
<td>dermal cells</td>
<td>20±-5</td>
<td>ND</td>
<td>19±-3</td>
</tr>
</tbody>
</table>

mono = monocytes at conc/ well

epi = epidermal cells at 5 x 10^3/ well
The uptake of tritiated thymidine into DNA of T cells alone without the presence of a mitogen was negligible, as the counts for these cells was about the same as for the controls of RPMI or Con A alone, without any cells present. T cells in the presence of monocytes produced a blastogenic response to Con A, as measured by increased tritium uptake producing an increased count. Maximal responses were produced with Con A at 2.5ug/ml and a larger response was produced when there were 5000 rather than 2500 monocytes per well showing that over this range of monocyte concentration an increase in accessory cells leads to a bigger blastogenic response in T cells.

An epidermal cell suspension could replace monocytes and act as accessory cells in mitogen driven T cell responses. Epidermal cells and monocytes together did not have an additive effect, probably because the T cell response is at the plateau of its dose response curve. Addition of dermal cells showed that normal dermal cells did not significantly alter the response of T cells in the presence of monocytes to Con A.

Results for patients.
Patient K: 5ml of venous blood and a 3mm punch biopsy of involved skin were obtained from a one year old girl. The blood was heparinized, PBM's obtained
and T cells separated by E-rosetting. Because of the limited number of cells available, the non-rosetted PBMs were not further purified for monocytes by adherence onto plates, but were taken as the T lymphocyte depleted (E-ve) population. The T cells were adjusted to a concentration of $10^6$/ml RPMI with 10% FBS (2.5.2) and the E-ve cells to $10^6$/ml RPMI with 10% FBS. The skin was treated as in 2.5.13 and the dermal cells, which included the LCH cells, adjusted to concentrations of $10^5$ and $5 \times 10^5$/ml RPMI 1640 with 10% FBS. Further purification was not attempted because of limited cell number. An aliquot of each cell population was taken, cytospin preparations made (2.4.3) and these were stained using an APAAP technique (2.4.9) using OKT6, RFT cell cocktail and Dako-Dr antibodies. The results are shown in Table 3.4.9.

The cells were used to study their responses to the mitogen Concanavelin A. 5ul of the T cells was placed in wells on a Terasaki plate (2.5.26) with 5ul of the T lymphocyte depleted (E-ve) cell population or with the LCH cells, and with both these cell populations together. T cells alone, E-ve cells and PBMs were incubated as controls. Triplicate wells of all combinations of cells were incubated with either 5ul of Concanavelin A (2.5ug/ml in RPMI 1640 with 10%
FBS), or with RPMI 1640 with 10% FBS as a control.
The total volume in each well was adjusted to 20ul
using RPMI 1640 with 10% FBS.

The cells were incubated as in 2.5.26 for 3 days,
pulsed with tritiated Thymidine and harvested 2 hours
later as in 2.5.26.
Table 3.4.9 Percentage of cells in each enriched cell population staining with different antibodies.

<table>
<thead>
<tr>
<th>Antibody used</th>
<th>RFT</th>
<th>Dako-Dr</th>
<th>OKT6</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>80</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>monocytes(E-ve)</td>
<td>5</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>LCH cells</td>
<td>0</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>
**Table 3.4.10 Response of cells from K to Con A**

This Table shows the blastogenic response of cells from Patient K when incubated in the presence of Concanavelin A compared to controls without Concanavelin A. Response is shown as mean counts per minute +/- 1 standard deviation of tritiated thymidine incorporated into DNA of cells.

<table>
<thead>
<tr>
<th>cells incubated together</th>
<th>control with RPMI</th>
<th>Con A</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBM s</td>
<td>42+-15</td>
<td>1890+-160</td>
</tr>
<tr>
<td>T cells</td>
<td>40+-20</td>
<td>234+-66</td>
</tr>
<tr>
<td>E-ve (monocytes)</td>
<td>76+-40</td>
<td>644+-32</td>
</tr>
<tr>
<td>T cells + E-ve</td>
<td>82+-18</td>
<td>2689+-140</td>
</tr>
<tr>
<td>T cells + D</td>
<td>76+-21</td>
<td>125+-21</td>
</tr>
<tr>
<td>T cells + E-ve + D</td>
<td>40+-38</td>
<td>3821+-176</td>
</tr>
<tr>
<td>T cells + E-ve + D/2</td>
<td>92+-21</td>
<td>2799+-52</td>
</tr>
</tbody>
</table>

**PBMs = peripheral blood mononuclear cells**

**E-ve = T cell depleted PBMs**

**D = dermal cells (5x10^6/well) including LCH cells**

**D/2 = dermal cells (2.5x10^6/well) including LCH cells**
Because the T cells were contaminated by 15% Dr-positive monocytes, a blastogenic response was obtained with the T cell population alone. Unlike the previous two patients with LCH, with this patient the LCH cells did not inhibit the response of T cells in the presence of monocytes to Con A.
Patient KB. 5ml of venous blood and a 3mm punch biopsy of involved skin were obtained from an 18 month old boy. This was separated and cell concentrations adjusted as for Patient K. Dermal cells were at a concentration of $10^5$/ml.

The results of staining cytoospin preparations of the cell populations with antibodies using an APAAP technique is shown in Table 3.4.11.
Table 3.4.11. Percentage of cells in each enriched cell population staining with different antibodies.

<table>
<thead>
<tr>
<th>Antibody used</th>
<th>RFT</th>
<th>Dako-Dr</th>
<th>OKT6</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>80</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>monocytes</td>
<td>0</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>LCH cells</td>
<td>0</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

The results of incubation with Con A are shown in Table 3.4.12.
Table 3.4.12. Response of cells from KB to Con A This table shows the blastogenic response of cells from Patient KB after incubation with Concanavelin A compared to after incubation with a control of RPMI alone. Results are shown as mean counts per minute +/- 1 SD of tritiated thymidine incorporated into DNA.

<table>
<thead>
<tr>
<th>cells incubated</th>
<th>control</th>
<th>con A</th>
</tr>
</thead>
<tbody>
<tr>
<td>together</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBM</td>
<td>35+-5</td>
<td>2050+-60</td>
</tr>
<tr>
<td>T cells</td>
<td>30+-3</td>
<td>150+-20</td>
</tr>
<tr>
<td>mono</td>
<td>34+-6</td>
<td>45+-4</td>
</tr>
<tr>
<td>T cells + mono</td>
<td>21+-6</td>
<td>1060+-110</td>
</tr>
<tr>
<td>T cells + LCH cells</td>
<td>28+-4</td>
<td>130+-30</td>
</tr>
<tr>
<td>T cells + mono + LCH</td>
<td>32+-5</td>
<td>1200+-200</td>
</tr>
<tr>
<td>T cells + mono + LCH/2</td>
<td>38+-6</td>
<td>1100+-90</td>
</tr>
</tbody>
</table>

PBM = peripheral blood mononuclear cells

Mono = E negative cells (monocytes)
The T cells were again impure so produced some blastogenic response without the addition of monocytes but again a greater response was produced in the presence of monocytes. LCH cells could not act as accessory cells and did not inhibit the response of T cells in the presence of monocytes to Con A.

3.4.4 Discussion

There have been no functional studies of LCH cells mainly because of the difficulty in obtaining material. The disease is rare and affects predominately children so biopsies cannot easily be performed just for research purposes, and samples that are obtained are small.

In patient AF the LCH cells from the spleen were unable to replace monocytes and present mitogens to T cells. Furthermore they abolished the normal response of T cells in the presence of monocytes to mitogens. With patient EL, LCH cells from a skin biopsy were also unable to present the mitogen Con A to T cells and inhibited the response by T cells in the presence of monocytes to about 25% of that without LCH cells. With cells from patients K and KB the LCH cells were also unable to replace monocytes in inducing a T cell response to mitogen.
However the inhibition of the normal response of T cells in the presence of monocytes to mitogen was not observed with LCH cells again isolated from dermis. In these two patients the LCH cells were not panned using OKT6 antibody because of insufficient cell numbers and this difference, either because of the different cell populations present in a less purified population, or the effects of panning, may account for the difference in results between patients. There was no difference in disease activity between the first two and the latter two patients so the difference is not due to this. Cells from the first two patients were incubated in a standard microsystem rather than using a hanging droplet technique. Comparable results have been obtained using the two systems for cell culture with other cells and the change to the hanging droplet technique was made only as it enabled more tests to be carried out on the small numbers of cells obtained. It seems unlikely that the difference is results is due to the different techniques used to culture the cells. These experiments have given some conflicting results but it seems that LCH cells cannot replace monocytes in their role as accessory cells in mitogen driven T cell responses. They therefore differ functionally from Langerhans cells which do act as accessory cells. Further experiments need to be carried out to
investigate the functional activities of LCH cells. The hanging droplet technique has been used successfully for the in vitro study of LCH cells and enables the main problem with this work, that of obtaining sufficient material to carry out these studies, to be reduced.
3.5 Comparison of S100 and PNA stains in the paraffin tissue diagnosis of LCH.

3.5.1 Introduction

When establishing a diagnosis of LCH, LCH cells, which are the characteristic pathological finding, must be distinguished from other histiocytic cells. LCH cells characteristically have Langerhans cell granules in their cytoplasm (Basset and Turiaf, 1965), show intense α-mannosidase activity (Elleder et al, 1977) express the CD1 antigen on their cell surface (Chollet et al, 1982) and have the S100 protein present (Wanatabe et al, 1983). When looking for these features to make or exclude the diagnosis of LCH there are, however, technical problems. Langerhans cell granules can be seen only on electron microscopy of suitably fixed material and α-mannosidase and anti-CD1 antibody (OKT6) stains require snap-frozen material. The only one of these tests that can be performed on routine paraffin-embedded material is the S100 stain.

S100 protein is not exclusive to Langerhans cells and LCH cells but is present in melanocytes, glial tissue and a variety of normal and tumour cells (Kahn et al, 1983). It has also been reported to be present in some cases of Malignant Histiocytosis.
Peanut agglutinin has been reported to produce dense cell surface and paranuclear staining of LCH cells (Jaffe, 1984). It produces diffuse staining of a large number of cells including keratinocytes, erythrocytes, macrophages (Leathem and Atkins, 1983a) and some lymphoid precursors (Ponder, 1983). However the only other cell reported to produce the same characteristic binding as LCH cells are interdigitating reticulum cells of the lymph node and Reed-Sternberg cells in Hodgkin's lymphoma. Peanut agglutinin staining in cases suspected of being LCH is not, however, often performed particularly outside the United States.

The aim of this study was to assess the value of the peanut agglutinin stain by comparing S100 and peanut agglutinin stains in establishing the diagnosis of LCH, and differentiating it from other histiocytic disorders.

3.5.2 Material and methods

Formalin fixed and paraffin embedded material was available from five skin biopsies, two lymph nodes, two spleens and one lung biopsy all from different patients with well established LCH who had histological involvement of these organs. For
comparison sections from a benign melanocytic naevus, a juvenile xanthogranuloma and progressive nodular histiocytosis of the skin, T cell lymphoma and Hodgkins lymphoma of lymph nodes, B cell lymphoma involving a spleen, infection associated haemophagocytic syndrome involving liver and sections of liver and spleen from a patient with malignant histiocytosis were used. Sections from normal organs were also stained.

Sections were cut as in 2.4.1 and sections from each specimen were stained with S100 and peanut agglutinin stains as in 2.4.4 and 2.4.5.

Several of the skin sections were also stained with a one-step antibody technique as in 2.4.6.

3.5.3 Results

The S100 stain produced uniform cytoplasmic staining of the LCH cells in every section of skin (Figure 3.5.1; 3.5.2), lung, spleen and lymph node (Figure 3.5.3) involved in LCH. S100 protein was also present in melanocytes and in Langerhans cells in the skin and interdigitating reticulum cells in lymph node.

Peanut agglutinin produced dense paranuclear and cell surface staining of the LCH cells in every section of skin (Figure 3.5.4), lung, spleen and
lymph node (Figure 3.5.5) involved in LCH. Identical results were obtained using the one-step as in the two-step antibody technique. Reed-Sternberg cells in lymph nodes involved in Hodgkin's lymphoma, and interdigitating reticulum cells in normal and diseased lymph nodes produced the same dense cell surface and paranuclear staining. A diffuse staining of erythrocytes and keratinocytes occurred but melanocytes and normal Langerhans cells were not stained.

Cells in the sections from juvenile xanthogranuloma, T and B cell lymphomas, infection-associated haemophagocytic syndrome and malignant histiocytosis were otherwise negative to both S100 and peanut agglutinin stains.

Cells in the dermis of the skin biopsy from the patient with progressive nodular histiocytosis were positive to the S100 stain but negative to peanut agglutinin stains. As these cells were also CD1 positive, it seems likely that they were Langerhans cells which may migrate into the dermis in some reactive conditions.
Figure 3.5.1
Skin with LCH involvement stained for S100 protein
Single arrow indicates LCH cell, double arrow indicates melanocyte, triple arrow indicates Langerhans cell (Original x200).

Figure 3.5.2
Lymph node with LCH involvement stained for S100 protein. Arrows indicate LCH cells (original x800).
Figure 3.5.3
Skin with LCH involvement stained with Peanut agglutinin. Single arrows indicate characteristic cell surface and paranuclear staining of LCH cells, double arrow indicates cell surface staining alone and triple arrow indicates perinuclear staining alone (original x100).

Figure 3.5.4
Skin with LCH involvement stained with Peanut agglutinin. Arrows indicate LCH cells showing perinuclear staining (original x800).
Figure 3.5.5

Lymph node with LCH involvement stained with Peanut agglutinin. Arrows indicate LCH cells (original x800).
3.5.4 Discussion

Any test for LCH should ideally be sensitive, specific and easy to perform by a laboratory that does not often have to do it. It is preferable that it should be able to be performed on routine formalin fixed paraffin embedded material as the diagnosis can then be confirmed in retrospect or in cases that were not clinically suspected.

The S100 protein is not exclusive to LCH cells. It is present in some neurones, glial and Schwann cells of the nervous system (Moore, 1965), interdigitating reticulum cells of the lymph nodes (Takahashi et al, 1981) and chondrocytes (Stefansson et al, 1982). It is also present in melanocytes and normal Langerhans cells and this latter may cause confusion as to whether a collection of S100 positive cells is a normal reactive process or an abnormal collection of LCH cells. S100 protein has also been demonstrated in myoepithelial cells of sweat glands, salivary glands and breast, serous glands of the lung and sustentacular cells of the adrenal medulla. Among neoplasms S100 protein has been reported in neurogenic tumours, melanomas, sweat and salivary gland tumours, bronchoalveolar carcinomas, phaeochromocytomas and tumours of cartilage (Kahn et al, 1983). S100 has also been reported (Wanatabe et
al, 1983) to be positive in some cases of Malignant Histiocytosis although it was not in the sections studied here.

In the case of progressive nodular histiocytosis, the peanut agglutinin stain, but not the S100 stain, was able to distinguish between the normal presence of Langerhans cells and the abnormal presence of LCH cells. The peanut agglutinin was therefore superior to the S100 in distinguishing progressive nodular histiocytosis from LCH.

Peanut agglutinin stains a wide variety of cells including macrophages, erythrocytes and the distal collecting tubule cells of the kidney (Leathem and Atkins, 1983a). However the distinctive dense cell surface and paranuclear staining of the LCH cells has additionally been described only of Reed-Sternberg cells in Hodgkins lymphoma (Ree and Kadin, 1984) and interdigitating reticulum cells of the lymph node (Ree and Kadin, 1986).

Both the S100 and peanut agglutinin stains identified LCH cells in skin, spleen, lymph node and lung. The techniques for each stain are comparable in terms of time, cost and simplicity of performance and can both be performed on paraffin embedded material. The dense cell surface and paranuclear staining of LCH cells by peanut agglutinin is easily recognised and is more specific
than the S100 stain. It is particularly valuable in distinguishing malignant histiocytosis and normal Langerhans cells from LCH cells in non-Langerhans cell histiocytosis, and should therefore be used routinely in these cases.
3.6 Production of monoclonal antibodies

3.6.1 Introduction

B lymphocytes can be activated to produce antibody specific for an antigen of interest, but are very short-lived in culture. In contrast there are myeloma lines which grow indefinitely in vitro but which have lost their ability to produce immunoglobulin. Hybrids can be made between these cells which give rise to a cell line that possess both the property of immortal growth in culture and the ability to produce and secrete an antibody of a pre-defined specificity. This was first described by Kohler and Milstein (1975) when they raised antibodies to sheep red blood cells.

No antibody has been produced that is specific to LCH cells. If an LCH specific antigen exists and could be identified by an antibody, the production of such an antibody would be valuable both in the diagnosis of the disease and, potentially, in targeting drugs against these cells in vivo.

3.6.2 Materials and methods
A spleen known to be heavily infiltrated with LCH cells was used. The cells were separated as in 2.5.12 and stored in liquid Nitrogen (2.5.5) until required. Three mice were injected with these cells as in 2.6.3. Cell fusions were then performed as in 2.6.4 to 2.6.7. The supernatants from the clones of cells in the culture plates were screened for the production of antibodies against cytospin preparations of the immunizing cells (2.6.8). If these were positive the supernatants were screened for antibody production against LCH cells using sections of the same spleen and of skin which were cut as in 2.4.2 and stained using an APAAP technique as in 2.4.9. Clones of cells in wells which produced staining of LCH cells were divided as in 2.6.9 and then screened again against sections of skin and spleen to look for staining of LCH cells.

3.6.3 Results

Several antibody-producing clones were produced, but these either stained cells other than LCH cells or were not specific to LCH cells and stained many other cell types too.
3.6.4 Discussion

Antibodies other than the one required were produced partly because a homogenate of spleen cells rather than pure LCH cells were used. If a plentiful source of LCH cells were obtained in future it would be preferable to purify them first before injecting them to try and limit the number of antigens against which antibodies would be produced. This would make screening procedures easier as there would be fewer antibodies against cells other than LCH cells.

It may also be that there are no antigens that are specific to LCH cells and therefore any antibody raised against LCH cells would also react with other cells. Few antigens are totally specific to one cell type. This may not be important diagnostically if the cells other than the LCH cells are unlikely to be confused with them because of morphological or distribution differences. It would, however, limit any potential use of the antibody as a targeting agent for drugs, as the drugs would be directed against the other cells as well.
3.7 Flow cytometry of LCH

3.7.1 Introduction

The nature of LCH has been a controversial issue. For many years Letterer-Siwe disease was thought to behave in a similar matter to malignancies and to be invariably fatal (Aronson, 1951). However the spontaneous resolution of most cases of single-system LCH and the occasional spontaneous regression of cases of multi-system disease (Broadbent and Pritchard, 1985) has led most clinicians to regard it as a non-malignant disease although this is not supported by any scientific evidence.

DNA aneuploidy has been demonstrated in 80-90% of solid tumours (Barlogie et al, 1980) and also in a number of premalignant conditions such as chronic atrophic gastritis (Teodori et al, 1984 and lichen sclerosis et atrophicus (Newton et al, 1987). Except for tetraploid stemlines, normal or reactive tissue is not associated with aneuploidy (Barlogie et al, 1980) so that aneuploidy is thought to be implicit of neoplasia (Barlogie, 1984).

Goldberg et al (1986) reported a case of LCH in a 76 year old man with single system disease of the skin consisting of numerous cutaneous nodules. A
biopsy specimen of the skin revealed an infiltration of histiocytes which expresses CD1 on the cell surface, contained S100 protein in the cytoplasm whilst on electron microscopy Birbeck granules were seen. Analysis of the DNA content by flow cytometry revealed an aneuploid peak in addition to the normal diploid one, suggesting that the patient had a malignant condition.

This patient was atypical in many respects so the aim of this study was to examine tissue from more typical patients with LCH, particularly those with multisystem aggressive disease in whom aneuploidy might be more likely to occur than in single system disease which carries an excellent prognosis. The DNA was obtained from both formalin-fixed paraffin-embedded tissue and from a fresh tissue biopsy.

3.7.2 Material and methods

Formalin-fixed paraffin-embedded material was obtained from nine patients with well-established LCH (see Table 3.7.1). Only those blocks with a substantial infiltration of LCH cells were used. One sample was post-mortem material and was autolysed. Two samples were processed but insufficient cells were available for examination. Results were
obtained from the remaining six patients by the method described in 2.7.2.

Dysplastic skin lesions (38 solar keratoses and 20 lesions of Bowens disease) and 7 samples of normal skin were processed simultaneously as controls.

In addition fresh material was obtained from a gingival biopsy of one of the above six patients. This was divided into two. One half was embedded in OCT embedding medium and snap-frozen in liquid Nitrogen. Sections were then cut as in 2.4.2 and stained using an anti-CD1 monoclonal antibody OKT6 (Ortho) by an APAAP technique (2.4.9). This showed that the dermis was infiltrated with characteristic LCH cells which were stained by the CD1 antibody. The second half was placed in antibiotic-containing RPMI at 4°C and processed for flow cytometry as in 2.7.3 within two hours.
Table 3.7.1. Patients from whom material was obtained

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age</th>
<th>Site of biopsy</th>
<th>Systems involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>54</td>
<td>mouth, ear</td>
<td>bone, skin, palate</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>4</td>
<td>lymph node</td>
<td>lung, lymph node, skin bone marrow, spleen. Died</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>2</td>
<td>lymph node</td>
<td>liver, lymph node</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>2</td>
<td>pituitary</td>
<td>skin, lung, pituitary, 2 lymph nodes liver, lymph node. Died</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>19</td>
<td>skin</td>
<td>skin</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>1/12</td>
<td>skin</td>
<td>bone, skin</td>
</tr>
</tbody>
</table>

Cases for which results not obtained

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age</th>
<th>Site of biopsy</th>
<th>Systems involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>M</td>
<td>1</td>
<td>lymph node</td>
<td>lymph node, skin, spleen, pituitary. Died.</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>26</td>
<td>skin</td>
<td>skin, pituitary</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>22</td>
<td>skin</td>
<td>skin</td>
</tr>
</tbody>
</table>


3.7.3 Results

All DNA histograms of the six patients were diploid. The mean coefficient of variation of the G1 peak was 5.4% and none was greater than 6%. The CD1-positive cells from the fresh sample, shown on frozen tissue section to be the LCH cells in the dermis and occasional Langerhans cells in the epidermis, comprised 4% of the total number of cells obtained from the specimen and were also diploid (Figure 3.7.1).
Two scatter diagrams are seen. The x axis (FL2) on a linear scale represents the DNA content of the cells. The y axis (FL1), on a 3 decade log scale represents the intensity of fluorescence emitted by OKT6 labelled cells. The upper figure is the control scattergram; cells stained for DNA only. Examination of the lower figure shows a population of cells (X) which are the OKT6 positive cells absent from the control scattergram alone. These represent 4% of the total number of cells.
Due to the small size of the fresh biopsy it was not possible to perform flow cytometry on the dermis and epidermis separately. However previous experience has shown (Norris et al, In press) that using Coulter volume it is possible to make a good distinction between most dermal cells and keratinocytes. Figure 3.7.2a shows a plot of DNA versus volume. A population of small non-proliferative cells can be seen (population A, Figure 3.7.2a) and a distinct band of proliferative keratinocytes is apparent (population B) with $G_1$, $S$ and $G_2$ DNA contents. A population of still larger cells (C) is present. These cells can be extremely large, non-proliferative and show a degree of non-specific fluorescence; they are differentiated keratinocytes. In Figure 3.7.2b volume is plotted against green fluorescence for the control sample stained in the absence of the anti-CD1 antibody. Dermal cells (A) and keratinocytes (B) can be distinguished. An identical plot is given for the anti-CD1 stained sample (Figure 3.7.2c) and in this a small additional population of antibody positive cells can be seen. These cells have a volume equivalent to the proliferative keratinocytes (Figure 3.7.2a, population B) and cannot be distinguished from them without the use of the anti-CD1 antibody. From Figure 3.7.2c by analogy with earlier work it can be suggested that 30% of the total cells are
non-proliferative dermal elements, 66% are keratinocytes and 4% are CD1-positive LCH cells and Langerhans cells.
**Figure 12.** Panel a is a plot of DNA content on the x axis versus Coulter volume on the y axis. Volume is plotted on a 3 decade log scale. Population A is small, non-proliferative putative dermal cells, whilst population B consists of proliferative keratinocytes and population C is very large differentiated keratinocytes.

Panels b and c are plots of green (OKT6) fluorescence versus volume. Both parameters are plotted on a 3 decade log scale. Population A represents small putative dermal cells and population B consists of larger predominantly keratinocyte cells. There is a non-specific increase in fluorescence with cell size in this population. Panel b represents the control sample and panel c the results for the OKT6 sample. In panel c a distinct small population (population C) of OKT6-positive cells is apparent. These cells are larger than dermal cells and have a volume corresponding to proliferative keratinocytes.
Figure 3.7.3 is an overlap histogram showing fluorescence intensity in the control and CD1-positive samples excluding putative keratinocytes (population B in Figure 3.7.2c).

Aneuploidy was demonstrated in 16 out of 17 lesions of Bowens disease and 7 out of 35 solar keratoses but not in any of the normal skin studied. 5 of the dysplastic lesions were unclassifiable due to the presence of too broad a G1 peak or to a skewed G1 peak.
Figure 3.7.3 A fluorescence histogram of green fluorescence (OKT6 linked fluorescence). The solid line represents a control sample processed without addition of the primary OKT6 monoclonal antibody. The dotted line represents the result for the OKT6 stained sample. A clear positively stained population is apparent in this sample.
3.7.4 Discussion

The theoretical basis of DNA flow cytometry is that the dyes used stain the cell nuclei. In a normal population of cells in a benign lesion, all the cells would have the same quantity of nuclear DNA and would thus emit the same amount of fluorescent light when stained with the DNA stain and so would appear at the same channel number on the DNA histogram. In practice, small variations in DNA staining occur so fluorescence is normally emitted about this channel number and a narrow peak is seen. The coefficient of variation of this peak is a measure of the variation in staining and thus the quality of the results.

The definition of aneuploidy necessitates the detection of a distinct G1 peak of aneuploid cells. Near diploid aneuploidy, especially of small numbers of cells, is possible only if the G1 peak of the normal cells has a very small coefficient of variation.

The study reported by Goldberg et al (1986) was of a single patient with LCH and the presentation of the disease was atypical. The patient was elderly ie 76 years old and presented with generalized skin nodules of 0.5-1.5cm diameter. Over 50% of patients with LCH are children under the age of 15 (Berry and Becton,
1987) and LCH in the elderly is unusual. LCH of the skin is most often maculopapular or seborrhoeic eczema-like in appearance although a solitary nodular plaque may occur (Berry and Becton, 1987). The presence of generalized nodules is more suggestive of malignant histiocytosis or progressive nodular histiocytosis. Cells were found in the biopsy of one of the skin lesions in this patient which showed the characteristic morphological and immunohistochemical features of LCH cells. However in malignant histiocytosis the histiocytes may have the S100 protein and Birbeck granules present in their cytoplasm (Henderson and Sage, 1973; Imamura et al, 1971) and rare Langerhans cell malignant histiocytoses have been recognized (Chu et al, 1987). Both malignant histiocytosis and progressive nodular histiocytosis may have normal, reactive Langerhans cells which are S100, Birbeck granule and CD1-positive, present in the lesions which may cause diagnostic confusion. It is possible that the case reported by Goldberg et al would be better classified as one of the non-Langerhans cell histiocytoses or as malignant LCH. It is also not certain that the aneuploid cells reported were the histiocytes as, unlike our fresh tissue specimen, the histiocytic cells were not separated from other cells in the biopsy specimen. Other cells from the skin of this
elderly patient may comprise for the aneuploid subpopulation of cells that was found. For example, keratinocytes from an actinic keratosis may demonstrate aneuploidy (Newton et al, 1987).

This study was of six more typical cases of LCH, all with well established disease and five with multisystem involvement. Flow cytometric analysis of fixed tissue from all these patients showed a normal diploid DNA content. The examination of OKT6-labelled fresh tissue from one of these enabled the specific examination of the DNA content of the LCH cells and these were also shown to be diploid. Flow cytometry can miss true aneuploidy and up to three chromosomes may be duplicated or deleted whilst DNA-ploidy is normal (Kirkham et al, 1987). Furthermore, near diploid aneuploidy may be missed as the peak is too near the normal diploid one to be detected. This study does not, therefore prove that LCH is a non-malignant condition but provides no evidence to support malignancy. This is therefore in accordance with the current view that LCH is a non-malignant disease.
3.8 Conclusions

1. A series of 58 children presenting over the last 7 years to the Oncology Department at The Hospital for Sick Children, Great Ormond Street was studied. The ratio of males to females was 2:1 and the age distribution was skewed with a median of 1 year. Younger children had more extensive disease.

18% of those with multisystem disease died. All were under 2 years at presentation, died within two years of the onset of their disease and had dysfunction of the liver, lung or haemopoietic systems by Lahey's definition.

13 out of 14 with single system disease had no long-term sequelae but 63% of those with multisystem disease did. 36% of those with multisystem disease had diabetes insipidus.

These results using a conservative approach to treatment compare well to other series using a more aggressive approach and do not carry the same risks of treatment-induced malignancies.

A new scoring system was assessed and, unlike the established Lahey system, the score at initial assessment correlated significantly with outcome so it was a better predictor of prognosis.
2. A cell line was produced from the involved bone marrow of a patient with LCH. These were large cells with frequent mitoses which looked histiocytic on electron microscopy, but did not contain Birbeck granules. They produced a diffuse fluoride-sensitive positive reaction when stained for non-specific esterase. They showed a dot-positive pattern with acid phosphatase, and were lysozyme-positive. Stains for S100, peanut agglutinin and a-mannosidase were all negative.

The cells expressed Class 1 MHC antigens and Fc receptors, but antibodies against Class 2 MHC, T cell, B cell, monocyte-macrophage and CD1 antigens were all negative. Attempts to make the cell line more mature were unsuccessful.

3. The hanging droplet cell culture technique was applied to monocytes, blood dendritic cells and, for the first time, to Langerhans cells.

Monocytes separated by Nycoprenz-monocyte were functionally similar to those separated by immune adherence in presenting PPD of tuberculin to autologous T cells. Blood dendritic cells produced the same response as monocytes at much lower cell concentrations. Monocytes depleted of dendritic cells were unable to function as antigen presenting cells.

An epidermal cell suspension, containing 1%
Langerhans cells, was 16 times more potent than monocytes and as potent as enriched dendritic cells at presenting PPD to T cells.

4. In vitro studies using material from four patients with LCH showed that LCH cells were unable to replace monocytes in their role as accessory cells in mitogen driven T cell responses. In two cases they seemed to inhibit the normal response of T cells in the presence of monocytes to mitogens.

5. Peanut agglutinin and S100 stains were compared in the paraffin-tissue diagnosis of LCH. They were comparable in terms of time, cost and ease of performance. The distinctive cell surface and paranuclear staining with Peanut agglutinin was easier to see than the diffuse cytoplasmic staining with the S100 stain. Furthermore Peanut agglutinin was more specific than the S100 stain and is therefore a preferable stain to use in making the diagnosis of LCH.

6. Antibodies were produced using splenic extracts containing LCH cells, but none was specific to LCH cells.

7. Flow cytometry of nine formalin fixed paraffin embedded specimens from six patients with LCH was
carried out. In addition fresh material from one, where the LCH cells were specifically labelled with an anti-CD1 antibody was performed. In all cases the DNA was found to be diploid, so providing no evidence that LCH is a malignant condition.
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