THE HISTOLOGY AND IMMUNOPATHOLOGY

OF VITILIGO

Khalifa Ebeid Sharquie, M.B., Ch.B., (Baghdad)

A

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THE HISTOLOGY AND IMMUNOPATHOLOGY OF VITILIGO

SUMMARY

Twenty-nine Caucasoid patients with 'common' vitiligo were studied. The sera of these patients were tested for autoantibodies. Shave biopsies from uninvolved, marginal and involved areas were studied by DOPA method, direct immunofluorescence tests, indirect immunofluorescence complement fixation technique, Epon embedded tissue for light microscopy and by electron microscopy.

All the patients had symmetrical vitiligo apart from one. About 50% of these patients had the onset of their vitiligo before the age of 20 years. The incidence of autoimmune disorders that are commonly associated with vitiligo seemed to be increased. These patients frequently had a positive family history of vitiligo and other autoimmune disorders. The organ specific autoantibodies were also increased in the sera of these patients. There was no significant deposition of immunoglobulins in all areas biopsied apart from the presence of cytoid (colloid/amyloid) bodies. However, in many patients, there was deposition of fibrin in the papillary dermis and at the dermo-epidermal junction of the marginal and involved areas. There was no complement fixing antibody to the melanocytes in the sera of all the patients tested.

On light and electron microscopy, inflammatory changes were seen in the skin of these patients, mostly in the marginal areas. This consisted of spongiosis of the epidermis with a mononuclear cell infiltrate. Many of the intraepidermal lymphocytes were found in direct contact with melanocytes and Langerhans cells. The degree of mononuclear cell infiltrate of the dermis did not parallel that seen in the epidermis. Sometimes, there was a massive lymphocytic infiltrate in the epidermis, even forming Pautrier-like micro-abscess, but only a few lymphocytes in the upper dermis. It is suggested that the primary inflammatory reaction occurs in the epidermis where an antigen is present.

Langerhans cells were found to be increased in the epidermis of uninvolved areas of patients with vitiligo compared to that of normal controls. These cells were also increased in the involved depigmented areas. Some of these cells were seen to be in direct contact with lymphocytes.

The melanocytes were markedly reduced in the marginal areas and only a few residual cells were found in the involved skin. The melanosomes had the tendency to be singly dispersed in the keratinocytes rather than in membrane bounded complexes.

There was no obvious pathological changes in the cutaneous nerves. However, there appeared to

be some evidence of regeneration of axons. In a third of the patients intra-epidermal nerves were found in the basal layer. Some of these nerves were adrenergic in type and observed occasionally in direct contact with a secretory melanocyte.

Colloid/amyloid bodies were demonstrated in the papillary dermis, by a variety of techniques. They had the tendency to occur in the skin of patients with vitiligo compared to that of normal controls. These colloid/amyloid bodies had many features that would suggest these bodies were derived from degenerating melanocytes.

Three patients with occupational vitiligo due to paratertiary butyl phenol were studied. The findings were similar to that found in idiopathic vitiligo.

The light microscopic ultrastructural and immunopathological findings in vitiligo do support the hypothesis that this condition is an 'auto-immune' disorder.

INTRODUCTION

I. HISTORICAL NOTES

Vitiligo is a disease which has been known from time immemorial. The earliest information concerning it comes from Egyptian writing, the time of the Pharaos and it is contained in the Ebers Papyrus (Ebbell 1937). In this two types of diseases are described, one that is probably leprosy and the other vitiligo. Also in the Indian Sacred book, Antharva Veda, which dates back to 1400 BC, there is mentioned a disease, Schwetakustha, which was almost certainly vitiligo (Whitney 1905).

The arabic word *bohak* as well as *baras* was used for vitiligo. The word *baras* "white skin" is mentioned in the Koran in relation to Jesus being able to cure patients with the condition (chapter 3,V48 and chapter 5,V109).

The name vitiligo is derived from latin word vitelius, which means a calf. It was first used by Roman physician Celsus, of second century AD. The white patches of the disease resembled those of a calf (Fitzpatrick 1964).

It has been known for many thousands of years in the ancient Indian and ancient Egyptian writings about certain plants that could be used for the treatment of vitiligo. The historical aspects of the treatment of vitiligo with psoralens has been reviewed by Fitzpatrick and Pathak (1959). Psoralea Coryli-

folia was the most widely used plant for the treatment of vitiligo which has subsequently been found to contain psoralen. Ibn El Bitar in the thirteenth century used a plant in Egypt called Ammi Majus Linn which also contains psoralen, for the treatment of vitiligo. Psoralens are still the most effective treatment for vitiligo.

II. CLINICAL ASPECTS

(a) Incidence

Vitiligo affects all races and seems to affect the sexes equally. It has been estimated that it occurs in about 1% of the world population (Lerner 1959; El Mofty 1968). Although there is preponderance of females in most large series, this is because they are based on out-patient figures rather than on the actual incidence in the population. The incidence is similar in both sexes (Howitz et al 1977). Genetic factors are undoubtedly involved and between 30-40% of patients have a positive family history of the condition (Lerner 1959; Fitzpatrick 1964; Copeman, Lewis and Bleehen 1974). Some pedigrees suggest that inheritance is determined by an autosomal dominant gene of variable penetrance.

Histocompatibility antigen studies have shown that HLA antigens were found with similar frequency in ninety patients with vitiligo as in 341 controls (Retornaz et al 1976). A second study also did not

show significant association among general population but increased frequency of HLA B13 and HLA BW35 in certain "ethnic" groups (Metzker et al 1980). A third study showed that twenty-two out of thirty-three Japanese patients with Vogt-Koyanagi-Harada syndrome had the histocompatibility antigen HLA-LD-Wa (Yakura 1976).

(b) Clinical Features

Vitiligo can start at any age, but in 50% of patients it develops before the age of twenty (Lerner 1959). It is an acquired pigmentary disorder in which there are variously sized patches of depigmentation of the skin that enlarge and coalesce together to form larger areas. These are white in colour, the borders of the lesions often being somewhat hyperpigmented. In lightly pigmented individuals these white patches are first apparent in the summer months where they fail to tan. Sometimes these white areas burn as a result of sun exposure, but in most patients no symptoms are noted other than the cosmetic disabilities.

The sites most commonly affected are the face, particularly around the eyes and mouth (fig 1), the backs of the hands and fingers, the axillae and groins, umbilicus and genitalia. The parts which are subject to friction and trauma, such as the elbows and knees, are often affected. Vitiligo also occurs in sites of

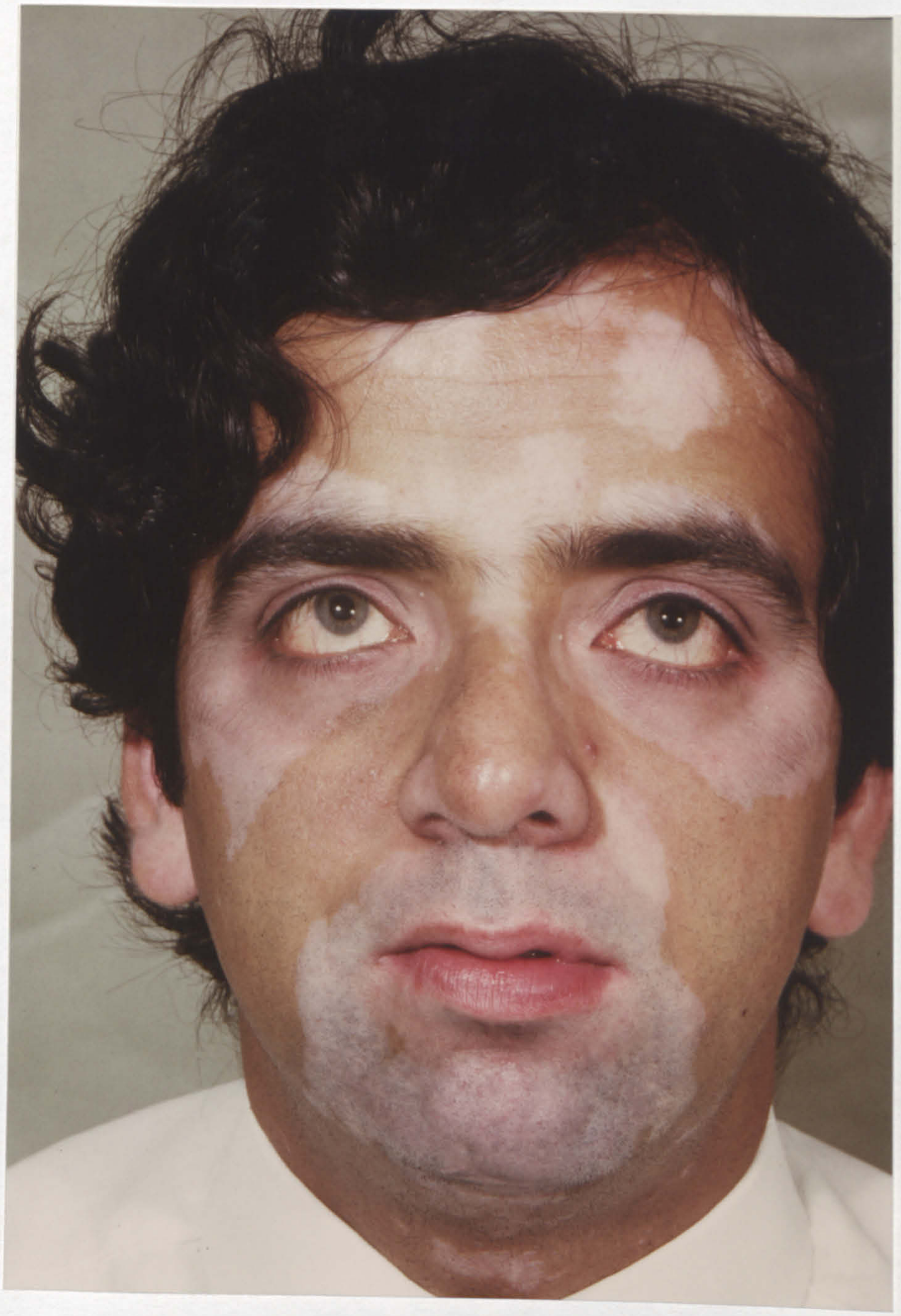


Fig 1. Face of a patient with vitiligo showing symmetrical areas of depigmentation, mainly around the mouth and eyes.

the skin that have been damaged, this being an isomorphic or Koebner phenomenon, (El Mofty 1968; Lerner and Nordlund 1978; Sweet 1978; Bleehen and Ebling 1979). Vitiligo can develop in linear scratch on the skin (fig 2) and the Koebner phenomenon can be of diagnostic value (Fitzpatrick 1964).

Occasionally, the marginal area in the lesion of vitiligo may be erythematous and elevated (Habermann 1933; Becker and Obermayer 1937; Garb and Wise 1948; Buckly and Lobitz 1953; Shukla 1959; Pinkus 1959, Lerner 1959, Fregert, Möller and Roseman 1959; Allende and Reed 1964; Michaelsson 1968; MacMillan and Rook 1971). Similarly few patients have noticed that the loss of pigment in an area has been preceeded by erythematous and itchy rash (Nordlund et al 1980).

Careful examination of the depigmented areas of skin in patients with vitiligo show that there are some varying degrees of loss of pigmentation in the macules. These range from completely white to light brown to a darker brown. This has been described as trichrome vitiligo by Fitzpatrick (1964) and El Mofty (1968). This variable pigmentation of the skin in the areas of vitiligo can be confirmed by examination of patients under a Wood's lamp.

(c) <u>Types of Vitiligo</u>

Vitiligo may assume various forms.

Fig 2. Isomorphic (Koebner) phenomenon at site of scratches.

Complete Vitiligo

The depigmentation of the skin can become so extensive that little or no pigment remains except in the eyes. Even in these patients there are usually some areas of residual pigmentation of the skin. Not only the pigment of skin is lost, but also that of the hair.

Segmental Vitiligo

Not infrequently vitiligo has a dermatomal or quasidermatomal distribution. This is not infrequently seen on the face, particularly in the distribution of one of the divisions of the trigeminal nerve.

Halo Naevi

In this condition a halo of depigmentation occurs around a pigmented naevus. This is most commonly a compound naevus. Not infrequently these halo naevi are multiple and antedate the onset of vitiligo (Kopf, Morrill and Silberberg 1965; Frank and Cohen 1964). Many patients with vitiligo have a previous history of halo naevi and in one quoted series this was the case with 50% of patients (Lerner and Nordlund 1978). Halo naevi also occurs, not infrequently, with other auto-immune disorders in which one can demonstrate organ specific auto-antibodies (Bleehen and Ebling 1979). The central naevus in these lesions usually disappears almost completely, and after a number of years the depigmented halo around it may repigment.

Grey Hair

It is not uncommon in patients with vitiligo for them to develop premature greyness of hair. Many with vitiligo give a positive family history of premature greyness (greyness of hair before the age of thirty). This is almost certainly vitiligo affecting the melanocytes of the hair bulbs. Greyness of the hair is part of the disease of vitiligo (Nordlund et al 1978).

Ocular Vitiligo

Even in patients with extensive vitiligo the colour of the eyes do not change. However, changes have been noted in the uveal and retinal pigment epithelium and this can be detected by slit-lamp examination. In one series of patients 50% had discrete areas of depigmentation in the choroidal and retinal epithelium (Albert, Nordlund and Lerner 1979; Nordlund and Lerner 1979). It is possible that these ocular changes are the result of uveitis from other diseases. However, there is a well known association of vitiligo with inflammatory disease of the eye as in the Vogt Koyanagi-Harada syndrome. This disease is characterised by acute uveitis, alopecia, vitiligo, halo naevi, poliosis and dysacusis with an aseptic meningitis (Nordlund et al 1980, Nordlund and Lerner 1979).

Occupational (Chemical) Vitiligo

A number of chemical compounds can induce cutaneous depigmentation. A number of substituted phenolic compounds can produce a picture that is indistinguishable from vitiligo. Vitiligo can be induced by contact with the mono-benzyl ether of hydroquinone (Oliver, Schwartz and Warren 1940). In recent years, workers in contact with para tertiary butyl phenol (Malten et al 1971; James, Mayes and Stevenson 1977) have developed vitiligo.

The clinical and histological features of those patients who have either their vitiligo, due to occupational contact with those substituted phenols, or as a result of therapy are identical to that observed in idiopathic vitiligo (Kahn 1970, Malten et al 1971; Dogliotti et al 1974; Calnan 1973, Bleehen and Sharquie 1982).

(d) Clinical Association of Vitiligo

Vitiligo is not infrequently clinically associated with a number of disorders that are considered to be autoimmune in aetiology (Lerner 1959; Cunliffe et al 1968; Dawber 1968; Bor, Feiwel, Chanarin 1969, Howitz and Schwartz 1971; McGregory, Katz and Doe 1972; Dawber 1970).

Increased incidence of vitiligo has been reported in the following disorders (see Table 1).

Diabetes mellitus (Dawber 1968, Macaron et al 1977)

Hyperthyroidism (Lerner 1959, Cunliffe et al 1968, Ochi and DeGroot 1969)

Hypothyroidism (Lerner 1955; Morgans 1964; Doniach et al 1972; McGregor et al 1972)

Hashimoto's thyroiditis (McGregor et al 1972)

Pernicious anaemia (Francis 1931; Dawber 1970; Howitz and Schwartz 1971)

Addison's disease (Addison 1855; Dunlop 1963; Lerner 1971; McGregor et al 1972)

Hypoparathyroidism (Fisher and Fitzpatrick 1970; Fields, Fragola and Hadley 1971, Hertz et al 1977)

Alopecia areata (Lerner 1971; Fields et al 1971, Tan 1974, Hertz et al 1977)

Scleroderma (Hertz et al 1977)

Morphoea(Hertz et al 1977, Lerner and Nordlund 1978)

Lichen sclerosus et atrophicus (Wallace 1971)

Myasthenia gravis (Durance 1971; Tan 1974)

Auto-immune haemolytic anaemia (Durance 1971; Walters, Lerner and Nordlund 1978)

Chronic thrombocytopenia (Walters et al 1978)

Melanoma (Lerner 1971; Milton, McCarthy and Carlon 1971)

Table 1. Clinical associations of vitiligo

(e) Emotional Factors

It is sometimes claimed by patients that the onset of their vitiligo coincides with emotional stress (Lerner 1959). However, what is more common is that vitiligo produces emotional distress as a result of the cosmetic disabilities that are all too often apparent. In some countries, vitiligo is thought to be the same as leprosy and this leads to considerable social difficulties.

III. PATHOGENESIS OF VITILIGO

Three major theories have been proposed to explain the pathogenesis of vitiligo. In one, melanocytes are destroyed by an autocytotoxic process. In another, an immune mechanism is involved in the destruction of the melanocytes and in the third a neural factor is also implicated.

(a) Auto-destructive Theory

It has been suggested (Lerner 1971) that melanocytes in the lesion of vitiligo are destroyed as a result of accumulation of toxic melanin precursors. The melanocytes "work themselves to death". In the areas that are most likely to be affected with vitiligo the skin is usually hyperpigmented. It is possible that these melanocytes have been stimulated to produce more melanin and they are more susceptible to destruction as a result of an accumulation of toxic melanin precursors. There is evidence that some of

the intermediate metabolites in the biosynthesis of melanin are highly toxic to melanocytes in vitro (Hochstein and Cohen 1963; Pawelek 1976; Wick et al 1977; Graham et al 1978). Many phenolic compounds have selective lethal effects to melanocytes, both in vitro (Riley 1970; Mansur et al 1978 and Bleehen 1976) and vivo (Bleehen et al 1967, 1968 and Riley 1969) and contact with these chemical compounds has been the cause of occupational and therapeutic vitiligo (Oliver et al 1940, Kahn 1970; Malten et al 1971; Dogliotti et al 1974; Calnan 1973 and James et al 1977).

(b) Auto-immune Theory

It has been suggested that vitiligo is an auto-immune disorder and this has been mainly based on the clinical association of vitiligo with a number of other disorders that are considered to be auto-immune. Patients with vitiligo have increased incidence of organ specific auto-antibodies compared with the general population (Brostoff, Bor and Feiwel 1969; Betterle et al 1976). Organ specific auto-antibodies to thyroid tissue, gastric parietal cells and adrenal tissue are frequently found among patients with vitiligo (Cunliffe et al 1968; Bor, Feiwel and Chanarin 1969; Brostoff et al 1969; Howitz and Schwartz 1971; Dobmeier and Sams 1971; Betterle et al 1976; Macaron et al 1977). However, at the present time the majority

of patients with vitiligo do not have specific anti-melanocyte antibodies. One report by Langhof et al (1965) reported the presence of precipitating antimelanin antibodies in the sera of the majority of patients with vitiligo that they studied. However, this has not been confirmed by other workers (Woolfson et al 1975). Immunofluorescent studies, both direct and indirect, have also failed to demonstrate an antimelanocyte antibodies (Dobmeier and Sams 1971, Betterle 1976, Bleehen 1979). In a recent study by Hertz et al (1977) demonstrated in the sera of two patients with vitiligo, alopecia areata, muco-cutaneous candidiasis and other endocrine disorders, the presence of a complement fixing serum factor against melanocytes. The factor in the serum was bound to melanocytes in normal skin, melanoma and naevus cells. This study was confirmed by Betterle et al 1979 who found that one of two patients with vitiligo and multiple endocrine disorders had circulating antibody against melanin producing cells using this indirect immunofluorescent complement technique. These recent studies have renewed interest that vitiligo could be autoimmune disorders in which antibodies are formed that react with melanin producing cells leading to their destruction. However, it could also be an epiphenomenon and that the antibodies are the result of melanocytic damage.

A further possibility is that cell mediated immune mechanisms are also involved. This is supported by the high lymphocyte microcytotoxicity against melanoma cells in vitro, in cases of regressing halo naevi and Vogt-Koyanagi-Harada syndrome (Roenigk et al 1975; Tagawa 1978; Nordlund et al 1980). Also the lymphocytic cell infiltrate among the naevus cells in cases of actively regressing halo naevi (Frank and Cohen 1964; Stegmaier, Becker and Medenica 1969; Swanson, Wayte and Helwig 1968; Rowden and Lewis 1975) as observed both by light and electron microscopy, suggests further the possibility of cell-mediated immune mechanisms in vitiligo.

(c) Neurogenic Theory

It has been implicated that the cutaneous nerves are involved in vitiligo and that neurochemical factors are released at peripheral nerve endings that have a damaging effect on the melanocytes. This hypothesis was suggested by Lerner (1971). Electron microscopic studies on vitiliginous skin have shown that there are minor degenerative changes in the nerves (Breathnach, Bor and Wyllie 1966, Bleehen 1979).

CHAPTER ONE

PATIENTS AND METHODS

PATIENTS AND METHODS

A total of twenty-nine Caucasian vitiligo patients were studied. Twenty-three were females and six males. The mean age of the patients was 39.2 years, with a mean age of onset of their vitiligo being twenty-six years. (Summary of patients shown in table II). All the patients were examined under a Wood's lamp to determine the extent of the disease and all were assessed for any associated diseases. The following investigations were carried out on all the patients: Full blood count and sedimentation rate and full immunological screen for the following auto-antibodies was carried out; immunofluorescent nuclei (ANF); antibody to mitochondria; antibody to smooth muscle; antibody to gastric parietal cell; antibody to thyroid microsomes and antibody to thyroglobulin.

Multiple shave biopsies were taken from all the patients.

Skin biopsy: The areas of vitiligo were intensified by examining the patients under a Wood's lamp light. The skin was swabbed with alcohol and the biopsy sites were anaesthetised with 2% plain xylocaine. Shave biopsies were taken from the centre of white vitiligo areas, from marginal pigmented skin and from the adjacent uninvolved pigmented skin, as shown in fig 3. The rectangular shave biopsy from

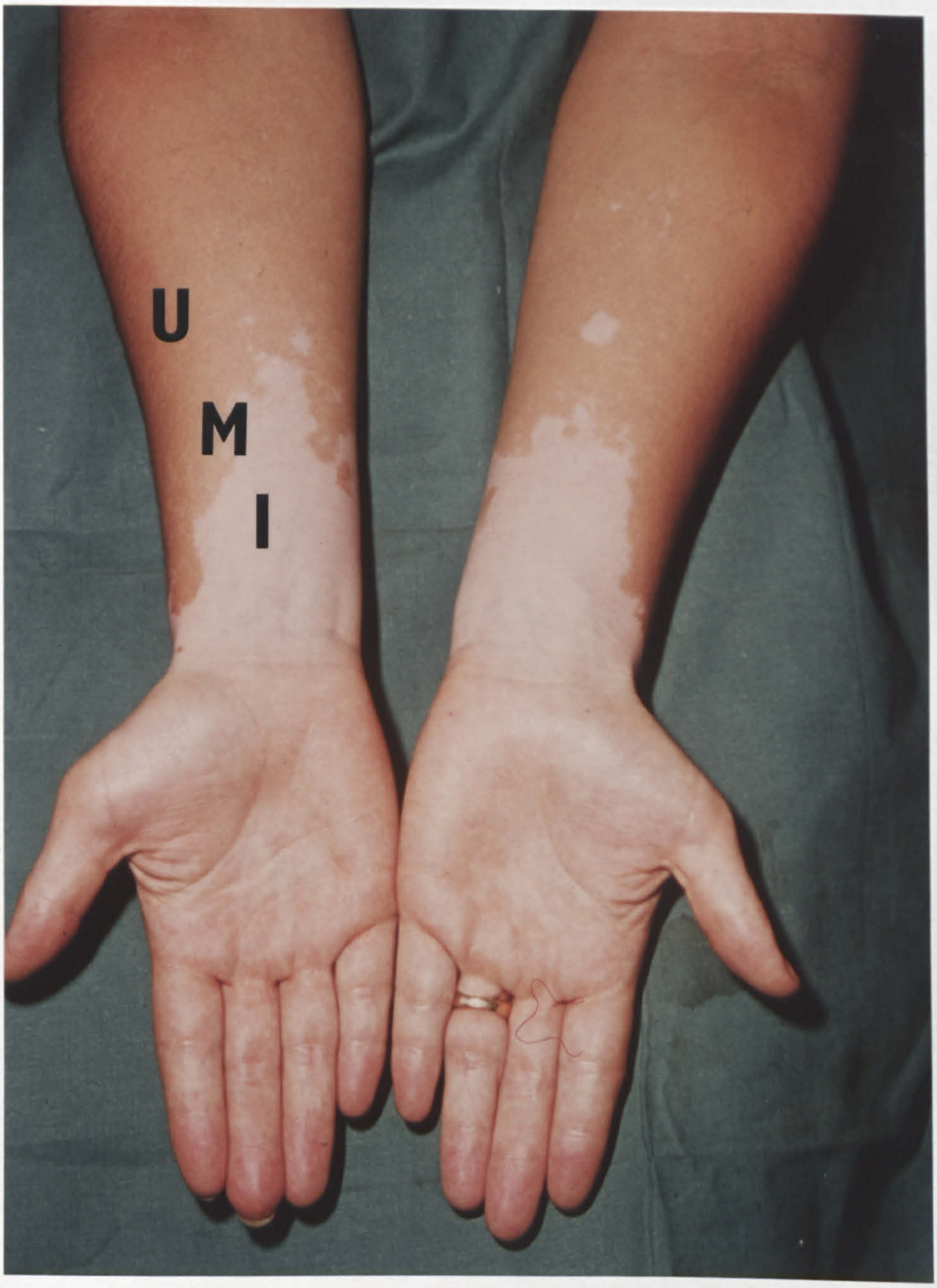


Fig 3. Forearms of a patient with vitiligo showing the sites of shave biopsies from uninvolved (U), marginal (M) and involved (I) areas.

each area was immediately divided into three pieces with a sharp blade. One piece processed for direct immunofluorescence studies, the second for histochemistry (DOPA) studies and the third portion for plastic embedded sections for light and electron microscopy. Ten millilitres of blood was taken, left at room temperature for twenty minutes to clot and then centrifuged at about 2,000 r.p.m. for about twenty minutes. The serum collected was kept at temperature of -30^{o}C until used for the experiments.

Three patients with occupational vitiligo, due to paratertiary butyl phenol, were also studied.

I. DIRECT IMMUNOFLUORESCENCE STUDIES

The skin biopsies were wrapped immediately in normal saline impregnated gauze and immersed in cold saline for immediate transportation to the laboratory. The biopsies were then snap frozen using a cryospray and the tissue mounted on a chuck with O.C.T. compound at -30^{o}C. Vertical cryostat sections 6-8μ in thickness were cut and placed on glass slides. Before staining, the sections were washed in diluted veronal buffer at pH 7.2 for fifteen minutes. Commercially available fluorescein isothiocyanate - conjugated rabbit antihuman antisera (Behringwerke) for IgA, IgG, IgM, fibrin and complements C3, C4 in a 1:15 dilution were pipetted onto the labelled specimens

and left in a moist box for thirty minutes. The slides were then thoroughly washed with buffer for fifteen minutes. The sections were mounted in a Sodium barbitone buffered and glycerol mounting solution and covered with coverslips. The stained sections were examined using a Leitz orthoplan fluorescent microscope. For full details of the method see appendix I.

Biopsies from nineteen normal individuals as controls were studied by direct immunofluorescence using the same techniques and to assess the deposition of immunoglobulins, fibrin and complement. Also twenty further patients with different pathological skin conditions were studied by direct immunofluorescence for the presence of cytoid bodies.

II. INDIRECT IMMUNOFLUORESCENCE COMPLEMENT FIXATION TEST

Sera samples obtained from vitiligo patients were stored at temperature -30°C. These samples were tested for the presence of a complement binding factor capable of reacting with melanin producing cells in the skin. The method for this technique has been fully described by Jordan, Sams and Beutner (1969), Katz, Hertz and Yaoita (1976) and used in relation to vitiligo by Hertz et al (1977).

Biopsies were taken from normal skin, involved

areas of vitiligo and also a pigmented naevus and a blue naevus were used as substrates. The tissues were snap frozen and mounted as described previously. The sections were then washed in phosphate buffered saline (PBS) for fifteen minutes and treated with patients and control sera at different dilutions for forty-five minutes in a moist box at room temperature. Then the sections were washed with PBS for fifteen minutes and dried with a fan. The sections were then treated with complement (human serum as a source of complement) for forty-five minutes at 37^{o}C temperature and washed again in PBS for fifteen minutes. The sections were then covered with fluorescein labelled antibody for C3 for thirty minutes and washed for thirty minutes in PBS. Finally, the sections were mounted in the sodium barbitone/ glycerol buffer. The sections were then examined using the fluorescent microscope.

As a further control sera from patients with bullous pemphigoid and from normal individuals were used. All patients and controls sera were diluted with PBS in dilutions of 1:1, 1:2, 1:4 and 1:8. The human sera as source of complement were diluted with either complement diluent or PBS in 1:5. For full details of the method see appendix II.

III. HISTOCHEMISTRY STUDY (DOPA METHOD)

Portions of the biopsy specimens were incubated in 2N NaBr solution at 37°C for one hour (Staricco and Pinkus 1957). The epidermis was separated off from dermis using dissecting microscope and fine forceps. The epidermal sheets were preliminarly fixed in 5% formaldehyde saline for five minutes, washed several times with distilled water and then incubated in 1:1,000 L-Dopa solution at 37°C for four to five hours. The epidermal sheets were then fixed in 10% formaldehyde saline at 37°C overnight and then dehydrated in graded concentration of ethanol. The sheets were cleared in cedar wood oil and then xylene and finally the epidermal sheets were mounted in depex on microscope slides. The stained tissues viewed with a Leitz orthoplan microscope using a X40 objective and a X8 eyepiece. The melanocytes were counted in approximately 7-10 areas with an eyepiece graticule in the microscope. The final number of melanocytes were expressed in terms of melanocytes per one square millimeter. For full details of the methods see appendix III.

IV. EPON EMBEDDED TISSUES FOR LIGHT MICROSCOPE (HUBER STAIN)

(a) Processing Technique

The tissues were immediately fixed in phosphate buffered glutaraldehyde solution for twenty-four hours.

After washing with distilled water and fixed with osmium tetroxide for two hours the tissues were washed again in distilled water and dehydrated in graded concentrations of ethanol. The tissues were then embedded in epon (see appendix IV for details).

(b) Staining Technique

Sections one micron thick were cut with glass knives on Reichert OMU_3 ultramicrotome. The sections were stained with basic fuchsin and methylene blue (Huber, Parker and Odland 1968; Chu, Smith and MacDonald 1980). A small drop of water was place on a clean glass slide and with the wire loop four to five sections were placed on the surface of water and the sections were dried on hot plate at 80°C for five minutes. The sections were first stained with filtered 3% basic fuchsin on a hot plate at 80°C and dried. Then they were stained with 2% alkaline methylene blue at pH 12, at various intervals of time until the desired effect was obtained. Finally, the sections were dried at room temperature and mounted in lenzol. The edges of the coverslips were sealed with glyceal. For details see appendix IV.

(c) Quantitative Analysis of Cells

The sections were viewed using a Leitz orthoplan light microscope with a X100 objective oil immersion lens and a X8 eyepiece. The clear cells

of the epidermis melanocytes and Langerhans cells, as well as the mononuclear cell infiltrate of the epidermis and the cells in the dermis, were counted using an eyepiece graticule.

The clear cells lying in the basal layer and those in the whole epidermis were counted (fig 4). The number of cells counted were related to in length at the dermo-epidermal junction. The counts, at least 7-10, were finally expressed in terms of clear cells per one millimeter of the epidermis.

Dermal cells, including endothelial cells, were counted along the size of one graticule in vertical lines from the dermo-epidermal junction and at least 7-10 counts were performed in different sections. The counts were then expressed in terms of cells per one square millimeter of the dermis (fig 4).

The mast cells were counted in the same way as dermal cells and expressed as per one square millimeter of dermis.

Biopsies from thirteen normal individuals were also processed and epon-embedded sections stained using the Huber method.

V. ELECTRON MICROSCOPIC STUDY

After biopsy, the tissues were immediately fixed in 3% phosphate buffer glutaraldehyde for

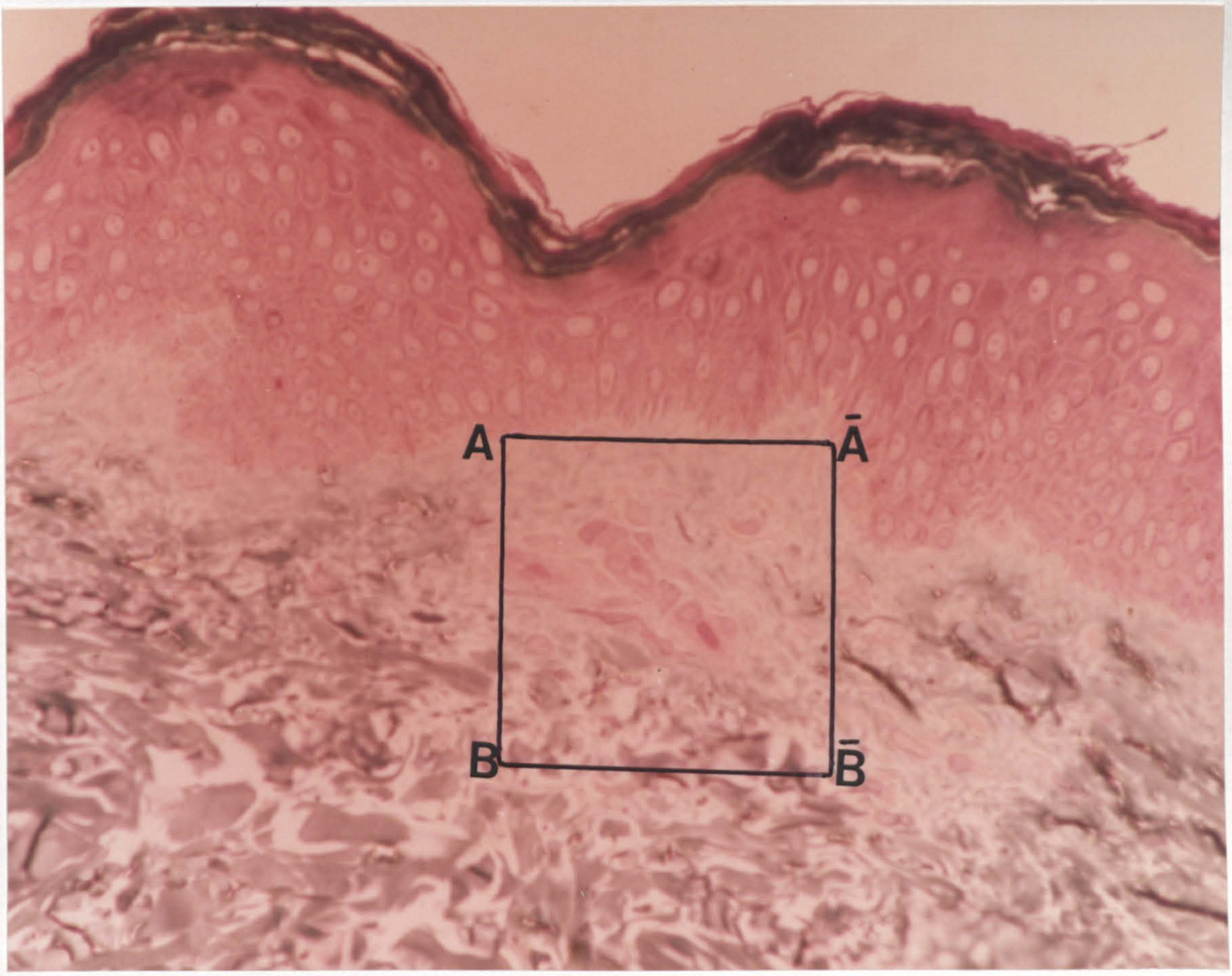


Fig 4. Light micrograph of a normal skin showing the method of counting of epidermal clear and dermal cells. 1μ thick epon-embedded tissue, stained with methylene blue and basic fuchsin. A-Ā represents the epidermal clear cells per one millimeter along the dermo-epidermal junction. While the whole graticule (A-Ā, B-B̄) represents the dermal and mast cells per one square millimeter of the dermis.

twenty-four hours and stored in phosphate buffer to be processed. The tissues were then washed with distilled water several times and post-fixed with 2% osmium tetroxide for one and a half to two hours in a fridge. The tissues were washed with distilled water and stained with uranyl acetate for two hours in a dark place at room temperature. The tissues were dehydrated in graded concentrations of ethyl alcohol. After the tissues were immersed in epoxy propane they were then embedded in araldite using the appropriate moulds. The moulds were placed in $60^{o}C$ oven for forty-eight hours and the tissues were then ready for cutting. Ultra-thin sections 60-70nm in thickness were cut with glass knives on Reichert OMU_3 ultramicrotome and stained with lead citrate. Stained sections were observed in AEI 801 electron microscope and photographs taken with attached camera. For details of the method see appendix V.

The biopsies from nine normal individuals were also processed as above and were studied.

VI. STATISTICAL METHODS

For comparison of melanocytes, mast cell, dermal cell and epidermal clear cell counts in different areas of patients with vitiligo, the Wilcoxon signed rank test for paired differences

was used (Swinscow 1978). The Wilcoxon two sample rank test was used to compare all these cells between vitiligo patients and normal control skin.

CHAPTER TWO

RESULTS

I. CLINICAL SUMMARY OF PATIENTS AND AUTOANTIBODY TEST

(a) Common Vitiligo

Patients

A total of twenty-nine patients with common idiopathic vitiligo were studied. A summary of these patients is shown in table II. There were twenty-three females and six males. The age of onset of these vitiligo patients ranged from one year to sixty-five years, with a mean of twenty-six years. In 44.8% of patients, the onset of the disease was before the age of twenty years. The clinical picture closely resembled to what has been described as to the morphology of the lesions, their symmetrical distribution and the sites most commonly affected. There was one patient with segmental vitiligo, where the depigmentation was localised to the left side of neck, left shoulder, left upperarm and forearm. A few patients had a trichrome appearance to their lesions of vitiligo. Many patients developed areas of depigmentation at the site of the biopsy from the uninvolved area. This represented an isomorphic or Koebner phenomenon.

Associated Diseases

Many of the patients had one or more of the diseases that are commonly clinically associated (table III). 51.7% of patients had these disorders; thyroid diseases, alopecia areata, lichen sclerosus,

No.	Name	Hosp. Number	Sex	Age	Age of onset	Extent of disease	Associated diseases	Family History	Site of biopsy
1	DB	AF3108	M	37	17	Moderate	DLE	Neg	Forearm
2	RY	AP9079	M	28	12	Extensive	Atopic eczema	Neg	Upperarm
3	SD	AI6056	F	33	25	Extensive	Eczema	Hyperthyroidism (Sister)	Forearm
4	MO	AO6051	F	35	25	Moderate	Neg	Neg	Forearm
5	JTR	AR3867	F	42	37	Extensive	Neg	Neg	Back
6	CH	AS7959	F	33	28	Moderate	Neg	Pernicious anaemia (Grandfather)	Forearm
7	JC	AT0586	F	18	14	Extensive	Atopic eczema	Vitiligo (Father)	Forearm
8	JA	-	M	60	30	Extensive	Addison disease	Diabetes mellitus	Face, forearm
9	JT	AW8150	F	23	16	Moderate	AA psoriasis Halo naevus	Diabetes mellitus (Aunt)	Forearm
10	TM	CA4278	F	18	1	Segmental	Neg	Neg	Forearm
11	EM	AR0475	F	52	23	Moderate	Hyperthyroidism	Vitiligo (Father) Thyroid disease (Aunt) Diabetes mellitus (Uncle)	Forearm

continued . .

No.	Name	Hosp. Number	Sex	Age	Age of onset	Extent of disease	Associated diseases	Family History	Site of biopsy
12	JW	AG9788	F	46	20	Extensive	Lichen sclerosus	Diabetes mellitus (Uncle) Vitiligo (sister)	Leg
13	LG	AY9808	F	55	53	Moderate	Lichen sclerosus	Diabetes mellitus (Uncle)	Buttock
14	BR	AR7677	F	50	43	Extensive	Neg	Neg	Back
15	MF	AI6846	F	48	37	Moderate	Lichen planus chronic vasculitis	Pernicious anaemia (Father)	Back
16	DH	AT0045	F	51	44	Extensive	Neg	Thyroid disease	Upperarm
17	AB	CA2980	F	45	27	Extensive	Neg	Vitiligo (Mother) Diabetes mellitus (Brother)	Forearm
18	EMA	AT9276	M	60	15	Moderate	Myxoedema psoriasis	Neg	Back
19	JR	AC9390	F	45	35	Extensive	Rheumatic heart disease	Thyroid disease (Mother)	Forearm
20	PL	AF8882	M	47	40	Extensive	Addison's disease Alopecia areata	Alopecia areata	Forearm

continued . .

No.	Name	Hosp. Number	Sex	Age	Age of onset	Extent of disease	Associated diseases	Family History	Site of biopsy
21	GG	AT3760	F	32	30	Moderate	Halo naevus	Neg	Back
22	SE	CC5976	F	32	20	Extensive	Neg	Vitiligo (Father)	Forearm
23	CL	AP3024	F	38	33	Moderate	Melasma	Neg	Axilla
24	TB	AV3037	M	40	13	Extensive	Pernicious anaemia	Vitiligo (Aunt) Pernicious anaemia (Aunt)	Forearm
25	CE	CC4865	F	24	7	Extensive	Neg	Neg	Forearm
26	JM	AL0557	F	47	30	Extensive	Neg	Goitre (Mother)	Forearm
27	JH	CB7612	F	15	3	Extensive	Neg	Neg	Forearm
28	MB	AZ1409	F	65	65	Moderate	Halo naevus	Neg	Forearm
29	MT	CD3844	F	19	13	Moderate	Neg	Vitiligo (Cousin)	Upperarm

Table II Clinical summary of patients with vitiligo. AA Alopecia areata

	Males	Females	Total %
Alopecia areata	1	1	6.8
Lichen sclerosus	0	2	6.8
Atopic eczema	1	1	6.8
Halo naevi	0	3	10.3
Pernicious anaemia	1	0	3.4
Addison disease	2	0	6.8
Thyroid diseases	1	1	6.8
Lichen planus	0	1	3.4
Discoid lupus erythematosus	1	0	3.4

Table III Incidence of "autoimmune" diseases in 29 patients with vitiligo.

atopic eczema and Addison's disease. All these conditions occurred in 6.8% of the twenty-nine patients studied. Pernicious anaemia, lichen planus and discoid lupus erythematosus occurred each in one patient in the series representing a percentage of 3.4%. Halo naevi were associated with vitiligo in 10.3% representing three of the twenty-nine patients.

Family History

A study of the families of patients showed a high incidence of diseases that are commonly associated with vitiligo. 51.7% (fifteen out of twenty-nine) had a positive family history of these autoimmune disorders in their close relatives (table IV). 24.1% of patients had a family history of vitiligo. There was an increased incidence of diabetes mellitus in families of the patients with vitiligo (20.6%).

Immunological Screen

The results of immunological screening for auto-antibodies are shown in table V. 26.3% of patients had auto-antibodies against gastric parietal cells, while 21% had positive auto-antibodies to thyroid microsomes and 30% for thyroglobulin antibodies. The test for antinuclear factor (ANF) was positive in 12.5%. Only one patient had positive mitochondrial antibodies (4.1%) while non of the patients had antibodies to smooth muscle.

	Number of vitiligo patients with positive family history	%
Vitiligo	7	24.1
Diabetes mellitus	6	20.6
Thyroid diseases	5	17.2
Pernicious anaemia	3	10.3
Alopecia areata	1	3.4

Table IV Incidence of "autoimmune" diseases in the relatives of 29 patients with vitiligo.

No.	Name	Antibody to mitochondria	Antibody to smooth muscle	Antibody to gastric parietal cells	Antibody to thyroid microsomes	Antibody to thyroglobulin	Antinuclear antibodies (ANF)
1	DB	0	0	ND	ND	ND	0
2	SD	0	0	ND	0	0	0
3	RY	0	0	0	0	0	500
4	MO	0	0	0	0	25	0
5	JTR	Pos	0	0	0	0	0
6	JC	0	0	ND	ND	ND	0
7	JT	0	0	0	0	0	0
8	EM	0	0	20	ND	0	20
9	JW	0	0	0	0	0	0
10	LG	0	0	0	0	0	0
11	BR	0	0	Pos	0	250	0
12	MF	0	0	0	0	0	0

continued . .

No.	Name	Antibody to mitochondria	Antibody to smooth muscle	Antibody to gastric parietal cells	Antibody to thyroid microsomes	Antibody to thyroglobulin	Antinuclear antibodies (ANF)
13	DH	0	0	0	0	0	0
14	AB	0	0	0	0	400	0
15	JR	0	0	20	6.25(IU)	0	0
16	PL	0	0	0	0	5	0
17	GG	0	0	20	0	0	20
18	SE	0	0	0	25(IU)	500(IU)	0
19	CL	0	0	0	0	0	0
20	TB	0	0	20	10	25	0
21	JM	0	0	0	Pos	0	0
22	JH	0	0	ND	ND	ND	0
23	MB	0	0	0	0	0	0
24	MT	0	0	ND	ND	ND	0
		4.1	**0**	**26.3**	**21**	**30**	**12.5**

Table V Showing the results of screening for auto-antibodies in patients with vitiligo. The total percentages of positive results for different auto-antibodies are also shown. Results reported with reciprocal of their titre unless otherwise indicated. ND not done.

(b) Occupational Vitiligo

Three male patients with occupational vitiligo were studied (table VI). All three patients were handling at work paratertiary butyl phenol. Two of the patients had noticed that some of their workmates had also developed white areas of depigmentation. The clinical picture was exactly similar to that seen in the common idiopathic vitiligo, both in distribution and the morphology of the lesion and their symmetrical arrangement. Two of the patients had involvement of the anogenital areas that is also seen in common vitiligo.

In all the patients the blood picture was normal and screening for auto-antibodies was negative in two of these patients with occupational vitiligo.

No.	Name	Hosp. Number	Sex	Age	Occupation	Sites involved	Asscciated disease	Family history	Auto-antibodies
1	AG	CD7644	M	30	Coalite worker	Face, trunk, limbs and penis	Neg	Neg	0 for ANF, mitochondria and smooth muscle
2	AD	CE1413	M	43	Coalite worker	Face, trunk, limbs and anogenital area	Extensive alopecia areata of scalp	Neg	0 for ANF, mitochondria smooth muscle, thyroglobulin, thyroid microsome and gastric parietal cells
3	KR	-	M	40	Analytical chemist	Hands and anogenital area	Neg	Neg	-

Table VI Clinical summary of patients with occupational vitiligo.

II. DIRECT IMMUNOFLUORESCENCE

(a) Controls

A total of nineteen normal controls (mean age twenty-five years) were studied. All the biopsies were taken either from the upperarm or forearms. A summary of the findings are shown in table VII.

Most of the controls had a moderately strong diffuse reticulate fluorescence of the papillary dermis with the IgG conjugate and a faint fluorescence at the dermo-epidermal junction zone. In some of the controls there was a weak fluorescence of the dermis with the fibrin conjugate. Immunofluorescence for IgA, IgM and C3 were negative apart from cytoid bodies.

Cytoid (Colloid/Amyloid) Bodies

Globular bodies varying in size were seen, mainly in the papillary dermis at the dermo-epidermal junction. Some of these were in the basal layer of the epidermis and they were dispersed along the dermo-epidermal junction either in regular fashion or in clusters. The shape of these bodies was either oval or round and sometimes the fluorescence appeared more intense around the periphery than the centre. These cytoid bodies were stained with IgA, IgG, IgM, fibrin and complement. However, they were more frequently stained with IgM and IgA (table VIII).

No.	IgG	IgA	IgM	Fibrin	Complement (C3)
1	Neg	Neg	Neg	Blood vessel	Neg
2	Dermis	Neg	Neg	Neg	Neg
3	Dermis	Neg	Neg	Neg	Neg
4	Neg	Neg	Neg	Neg	Neg
5	Dermis	Neg	Neg	Dermis	Neg
6	Dermis	Neg	Neg	Dermis	Faint DEJ papillae
7	Dermis	Neg	Neg	Neg	Neg
8	Dermis	Neg	Neg	Neg	Neg
9	Dermis	Neg	Neg	Neg	Neg
10	Faint, DEJ	Neg	Neg	Neg	Neg
11	Dermis	Neg	Neg	Neg	Neg
12	Dermis, Faint DEJ	Neg	Neg	Neg	Neg
13	Dermis	Neg	Neg	Neg	Neg
14	DEJ	Neg	Neg	Neg	Neg
15	Dermis	Neg	Neg	Neg	Neg
16	Dermis, Faint DEJ	Neg	Neg	Papillary dermis	Neg
17	Dermis	Neg	Neg	Neg	Neg
18	Neg	Neg	Neg	Neg	Neg
19	Dermis	Neg	Neg	Neg	Neg

Table VII. Direct immunofluorescence of normal controls showing distribution of immunoglobulin fibrin and complement deposition. DEJ Dermo-epidermal junction.

No.	IgG	IgA	IgM	Fibrin	Complement (C3)
1	Neg	Neg	Neg	Neg	Neg
2	Neg	Neg	Neg	Neg	Neg
3	Neg	Neg	Neg	Neg	Neg
4	Neg	Neg	Pos	Neg	Neg
5	Neg	Neg	Neg	Neg	Neg
6	Neg	Neg	Neg	Neg	Neg
7	Neg	Neg	Neg	Neg	Neg
8	Neg	Neg	Neg	Neg	Neg
9	Pos	Pos	Pos	Neg	Neg
10	Pos	Pos	Neg	Neg	Pos
11	Pos	Pos	Neg	Neg	Pos
12	Pos	Pos	Pos	Pos	Neg
13	Neg	Pos	Neg	Neg	Neg
14	Neg	Pos	Pos	Neg	Neg
15	Neg	Pos	Pos	Pos	Neg
16	Pos	Pos	Pos	Neg	Neg
17	Pos	Neg	ND	ND	Neg
18	Neg	Neg	Pos	Neg	Neg
19	Neg	Pos	Pos	Neg	Neg

Table VIII Direct immunofluorescence of control skin showing distribution of immunoglobulin, fibrin and complement deposition in cytoid bodies. ND not done.

Biopsies from twenty patients with various dermatoses were also examined using immunofluorescent techniques. The distribution and number of cytoid bodies in these disorders were much the same as in the normal controls (table IX).

(b) Common Vitiligo

A total of twenty-eight patients were studied. The direct immunofluorescence results are shown in tables X,XI and XII. Weak fluorescence for IgG was found in the dermis in a diffuse reticulate pattern. It was, however, more intense in the papillary dermis. This pattern was seen in the uninvolved, marginal and involved areas of most of the patients. The fluorescence was much stronger in the involved and marginal areas when compared with the uninvolved areas. Faint fluorescence at the dermo-epidermal junction was also noticed using IgG conjugate in many of the patients, mainly in the involved skin and also some marginal areas (fig 5). With IgA, IgM conjugates no significant immunofluorescence was observed apart from the presence of cytoid bodies. Deposits of fibrin were found in the dermis in nearly two-thirds of the patients either in the involved, marginal or uninvolved areas. A moderate fluorescence was observed mainly in the papillary dermis. In some specimens a faint fluorescence was present at dermo-epidermal junction, but a strong band of fluorescence (fig 6A,B) was seen at

No.	Diagnosis	IgG	IgA	IgM	Fibrin	Complement (C3)
1	Lichen planus pilaris	Neg	Neg	Neg	Neg	Neg
2	Bullous pemphigoid	Pos	Pos	Neg	Neg	Neg
3	Pyoderma gangrenosum	Neg	Neg	Neg	Neg	Neg
4	Bullous pemphigoid	Neg	Neg	Neg	Neg	Neg
5	Herpes gestationis	Neg	Pos	Neg	Neg	Neg
6	Bullous pemphigoid	Neg	Neg	Pos	Neg	Neg
7	Psoriasis (post-PUVA)	Neg	Pos	Pos	Neg	Pos
8	Psoriasis	Neg	Neg	Neg	Neg	Neg
9	Dermatitis Herpetiformis	Neg	Neg	Pos	Neg	Neg
10	Nodular prurigo	Neg	Pos	Pos	Neg	Neg
11	Pityriasis rubra pilaris	Neg	Neg	Neg	Neg	Neg
12	Pemphigus vegetans	Neg	Neg	Neg	Neg	Neg
13	Bullous pemphigoid	Neg	Pos	Pos	Neg	Neg
14	Dermatitis Herpetiformis	Neg	Neg	Pos	Neg	Neg
15	Dermatitis Herpetiformis	Neg	Neg	Neg	Neg	Neg
16	Pityriasis lichenoides	Neg	Neg	Neg	Neg	Neg
17	Porphyria cutanea tarda	Neg	Neg	Pos	Neg	Neg
18	Pemphigus Foliaceus	Neg	Neg	Neg	Neg	Neg
19	Pemphigus	Neg	Neg	Neg	Neg	Neg
20	Discoid Lupus Erythematosus	Neg	Neg	Neg	Neg	Neg

Table IX Direct immunofluorescence in various dermatoses showing distribution of immunoglobulin, fibrin and complement deposition in cytoid bodies.

No.	Pt. Name	IgG	IgA	IgM	Fibrin	Complement (C3)	Complement (C4)
1	JTR	Neg	Neg	Neg	Neg	Neg	Neg
2	MO	Neg	Neg	Neg	Neg	Neg	Neg
3	CH	Dermis	Neg	Neg	Dermis	Neg	Neg
4	JC	Neg	Neg	Neg	Faint DEJ	Neg	Neg
5	EM	Neg	Neg	Neg	Dermis, BV	Neg	Neg
6	JW	Dermis	Neg	Neg	Dermis	Faint DEJ	Neg
7	LG	Dermis, Faint DEJ	Neg	Neg	Faint DEJ	Neg	Neg
8	BR	Dermis	Neg	Neg	Dermis	Neg	Neg
9	MF	Dermis	Neg	Neg	Neg	Neg	Neg
10	DH	Dermis	Neg	Neg	Dermis	Granular (papillae)	Neg
11	AB	Neg	Neg	Neg	Neg	Neg	Neg
12	EMA	Dermis	Neg	Neg	Neg	Neg	Neg
13	PL	Dermis	Neg	Neg	Dermis	Neg	Neg
14	GG	Neg	Neg	Neg	Neg	Neg	ND
15	SE	Neg	Neg	Neg	Neg	Neg	ND
16	CL	Dermis	Neg	Neg	Neg	Neg	ND
17	TB	Dermis	Neg	Neg	Neg	Neg	ND
18	CE	Neg	Neg	Neg	Neg	Neg	ND
19	JM	Dermis	Neg	Neg	Neg	Neg	ND
20	JH	Dermis	Neg	Neg	Neg	Neg	ND
21	MB	Neg	Neg	Neg	Neg	Neg	ND
22	MT	Dermis	Neg	Neg	Neg	Neg	ND

Table X Direct immunofluorescence of uninvolved skin of patients with vitiligo showing distribution of immunoglobulin, fibrin and complement deposition. DEJ Dermo-epidermal junction, BV Blood vessel, ND not done.

No.	Pt. name	IgG	IgA	IgM	Fibrin	Complement (C3)	Complement (C4)
1	DB	Neg	Neg	Neg	Faint DEJ	Neg	Neg
2	SD	Neg	Neg	Neg	Neg	Neg	Neg
3	RY	Dermis	Neg	Neg	Neg	Neg	ND
4	JTR	Neg	Neg	Neg	Neg	Neg	Neg
5	CH	Dermis Faint DEJ	Neg	Neg	Dermis Faint DEJ	Neg	Neg
6	JC	Neg	Neg	Neg	Neg	Neg	Neg
7	JA	Dermis	Neg	Neg	Faint DEJ & papillae	Neg	Neg
8	JT	Dermis	Neg	Neg	Dermis	Neg	Neg
9	EM	Faint DEJ	Neg	Neg	Dermis	Neg	Neg
10	JW	Dermis	Neg	Neg	Dermis	Faint DEJ	Neg
11	LG	Dermis	Neg	Neg	Neg	Neg	Neg
12	BR	Dermis	Neg	Neg	Neg	Neg	Neg
13	MF	Dermis	Neg	Neg	Neg	Faint DEJ	Faint DEJ
14	DH	Dermis, Faint DEJ	Neg	Neg	Neg	Neg	Neg
15	AB	Dermis	Neg	Neg	Neg	Neg	Neg
16	EMA	Dermis	Neg	Neg	Neg	Neg	Neg
17	JR	Neg	Neg	Neg	Neg	Neg	Neg
18	PL	Dermis	Neg	Neg	Neg	Neg	Neg
19	GG	Neg	Neg	Neg	Dermis	Neg	ND
20	SE	Neg	Neg	Neg	Neg	Neg	ND
21	CL	Dermis	Neg	Neg	Neg	Neg	ND
22	TB	Dermis	Neg	Neg	Neg	Neg	ND
23	CE	Neg	Neg	Neg	Neg	Neg	ND
24	JM	Dermis	Neg	Neg	Neg	Neg	ND
25	JH	Dermis	Neg	Neg	Neg	Neg	ND
26	MB	Neg	Neg	Neg	Neg	Neg	ND
27	MT	Dermis	Neg	Neg	Neg	Neg	ND

Table XI Direct immunofluorescence of marginal skin of patients with vitiligo showing distribution of immunoglobulin, fibrin and complement deposition. DEJ dermo-epidermal junction, ND not done.

No.	Pt. Name	IgG	IgA	IgM	Fibrin	Complement (C3)	Complement (C4)
1	DB	Faint DEJ	Neg	Neg	Neg	Neg	Neg
2	SD	Neg	Neg	Neg	Neg	Neg	Neg
3	RY	Dermis	Neg	Neg	Neg	Neg	Neg
4	JTR	Faint DEJ	Neg	Neg	Neg	Neg	Neg
5	MO	Dermis	Neg	Neg	Neg	Neg	Neg
6	CH	Dermis	Neg	Neg	Dermis	Neg	Neg
7	JC	Dermis DEJ	Neg	Neg	DEJ & papillae	Neg	Neg
8	JA	Neg	Neg	Neg	Neg	Neg	Neg
9	JT	Dermis	Neg	Neg	Dermis & blood vessel	Neg	Neg
10	EM	Neg	Neg	Neg	Neg	Neg	Neg
11	LG	Faint DEJ	Neg	Neg	Neg	Neg	Neg
12	BR	Faint DEJ	Neg	Neg	Dermis DEJ	Neg	Neg
13	MF	Dermis	Neg	Neg	Band DEJ	Neg	Neg
14	DH	Dermis, Faint DEJ	Neg	Neg	Neg	Neg	Neg
15	AB	Dermis, Faint DEJ	Neg	Neg	Neg	Neg	Neg
16	EMA	Dermis	Neg	Neg	Band DEJ	Neg	Neg
17	JR	Dermis	Neg	Neg	Band DEJ blood vessel	Neg	Neg
18	PL	Dermis	Neg	Neg	Neg	Neg	Neg
19	GG	Neg	Neg	Neg	Dermis	Neg	ND
20	SE	Neg	Neg	Neg	Neg	Neg	ND
21	CL	Dermis	Neg	Neg	Dermis	Neg	ND
22	TB	Dermis	Neg	Neg	Neg	Neg	ND
23	CE	Neg	Neg	Neg	Neg	Neg	ND
24	JM	Neg	Neg	Neg	Neg	Neg	ND
25	JH	Dermis	Neg	Neg	Neg	Neg	ND
26	MB	Neg	Neg	Neg	blood vessel	Neg	ND
27	MT	Faint DEJ	Neg	Neg	Neg	Neg	ND

Table XII Direct immunofluorescence of involved skin of vitiligo patients showing distribution of immunoglobulin, fibrin and complement deposition. ND not done. DEJ dermo-epidermal junction,

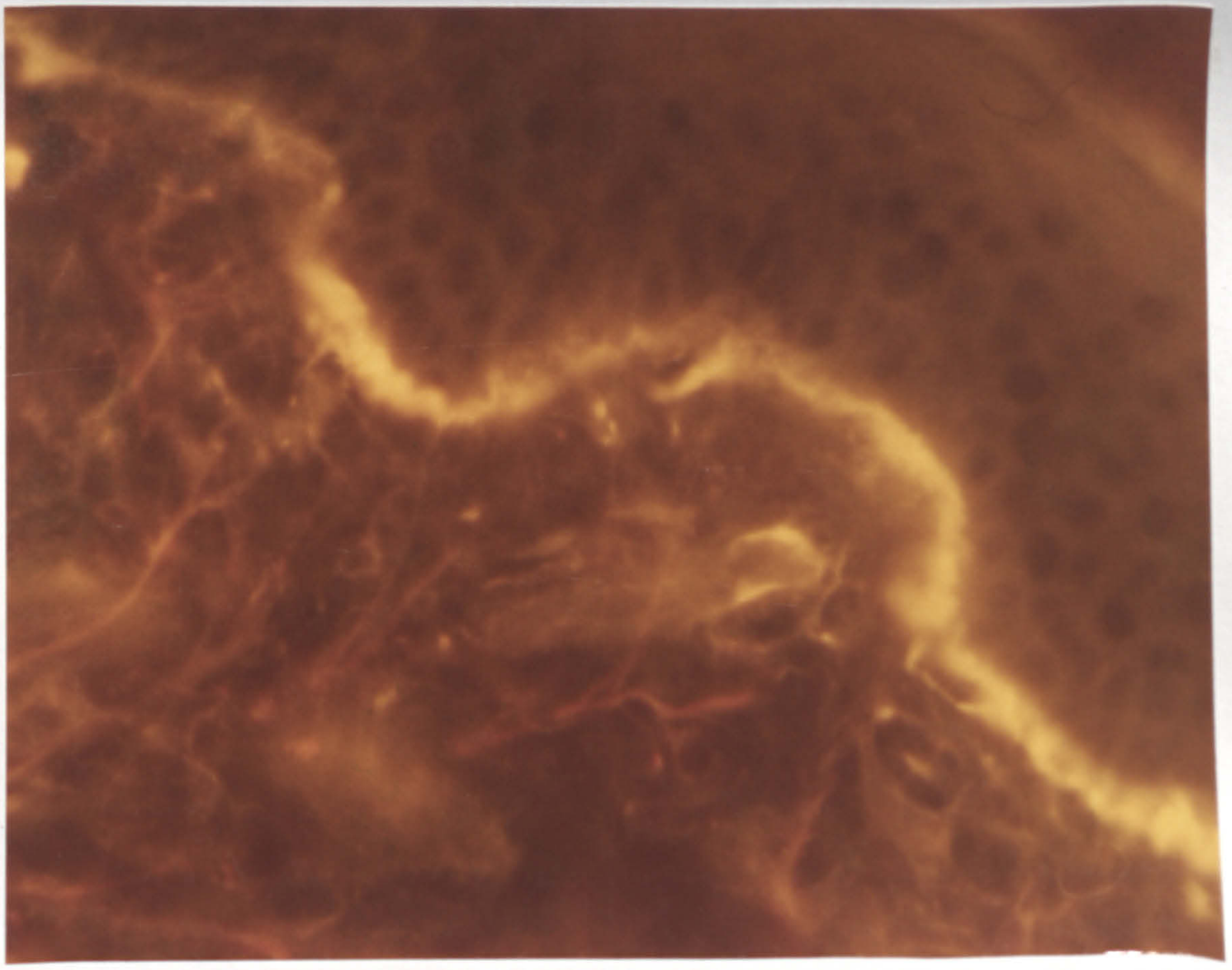


Fig 6A. Direct immunofluorescence study of an involved area from a patient with vitiligo showing intense band of fibrin deposition along the dermo-epidermal junction. (X 80)

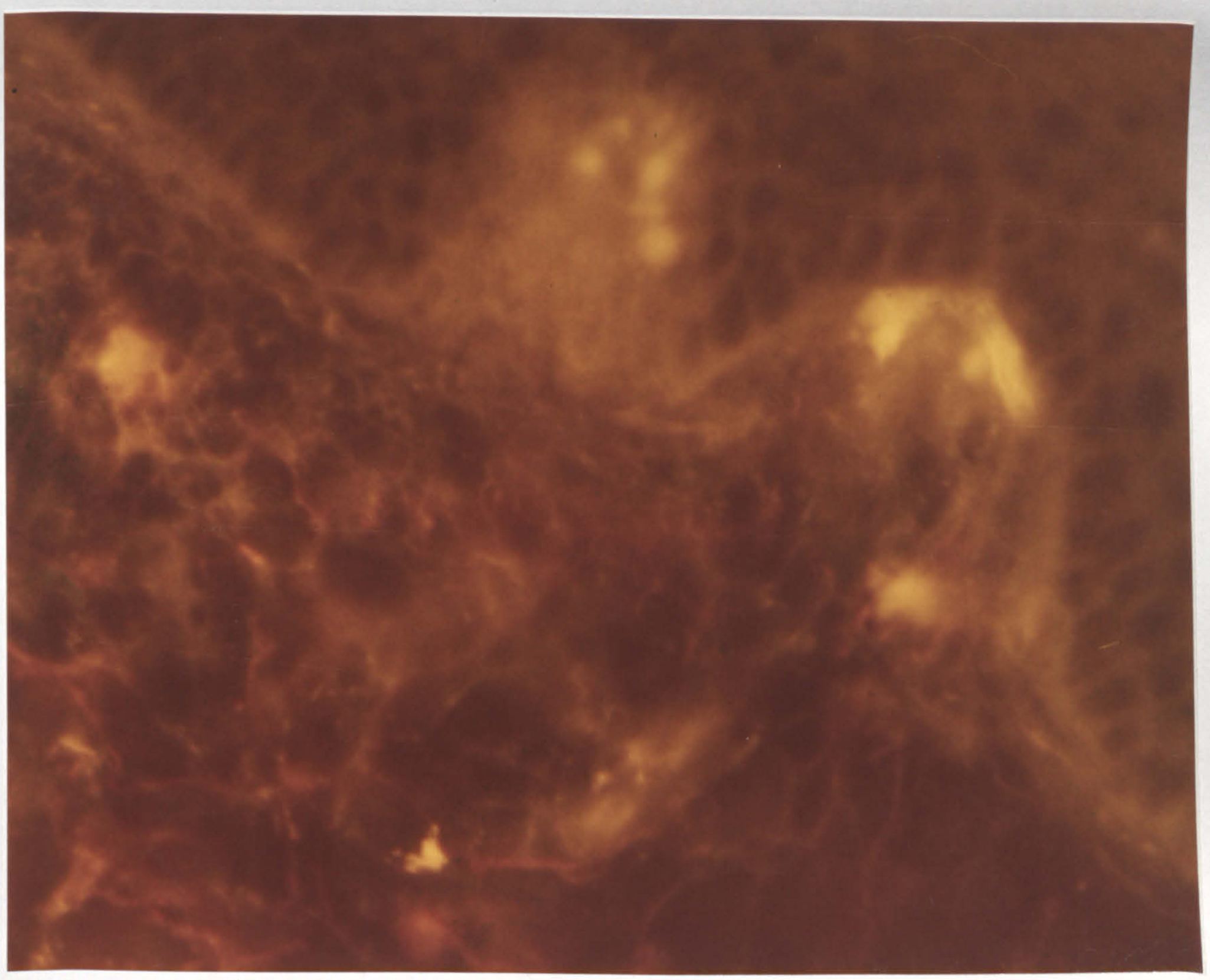


Fig 6B. Direct immunofluorescence study of an involved area from a patient with vitiligo showing intense fibrin deposition along the dermo-epidermal junction, mainly in the region of the dermal papillae. (X 80)

dermo-epidermal junction in three patients only in the involved areas. Some deposition of fibrin was present around the blood vessels. As regards deposition of complement, a faint fluorescence was seen at the dermo-epidermal junction in the marginal areas of a few patients, and in one patient a granular fluorescence was present in the papillary dermis of the uninvolved skin.

Cytoid (Colloid/Amyloid) Bodies

These globular bodies were located in the papillary dermis (fig 7), similar to the controls, in the uninvolved, marginal and involved areas (table XIII, XIV and XV). They were more numerous in the marginal areas as compared to the involved skin. In the involved areas, although cytoid bodies were present in many of the patients, they were less numerous when compared with those found in the uninvolved and marginal sites. In general cytoid bodies have a tendency to occur in vitiligo skin more than in controls. These bodies were stained with immunoglobulins, complement and fibrin conjugates; more with IgA, IgM and IgG, but less with fibrin and complement.

(c) Occupational Vitiligo

Two patients were studied by direct immunofluorescence, the results being similar to those of the common vitiligo patients (table XVI). In the first patient there was diffuse reticulate fluores-

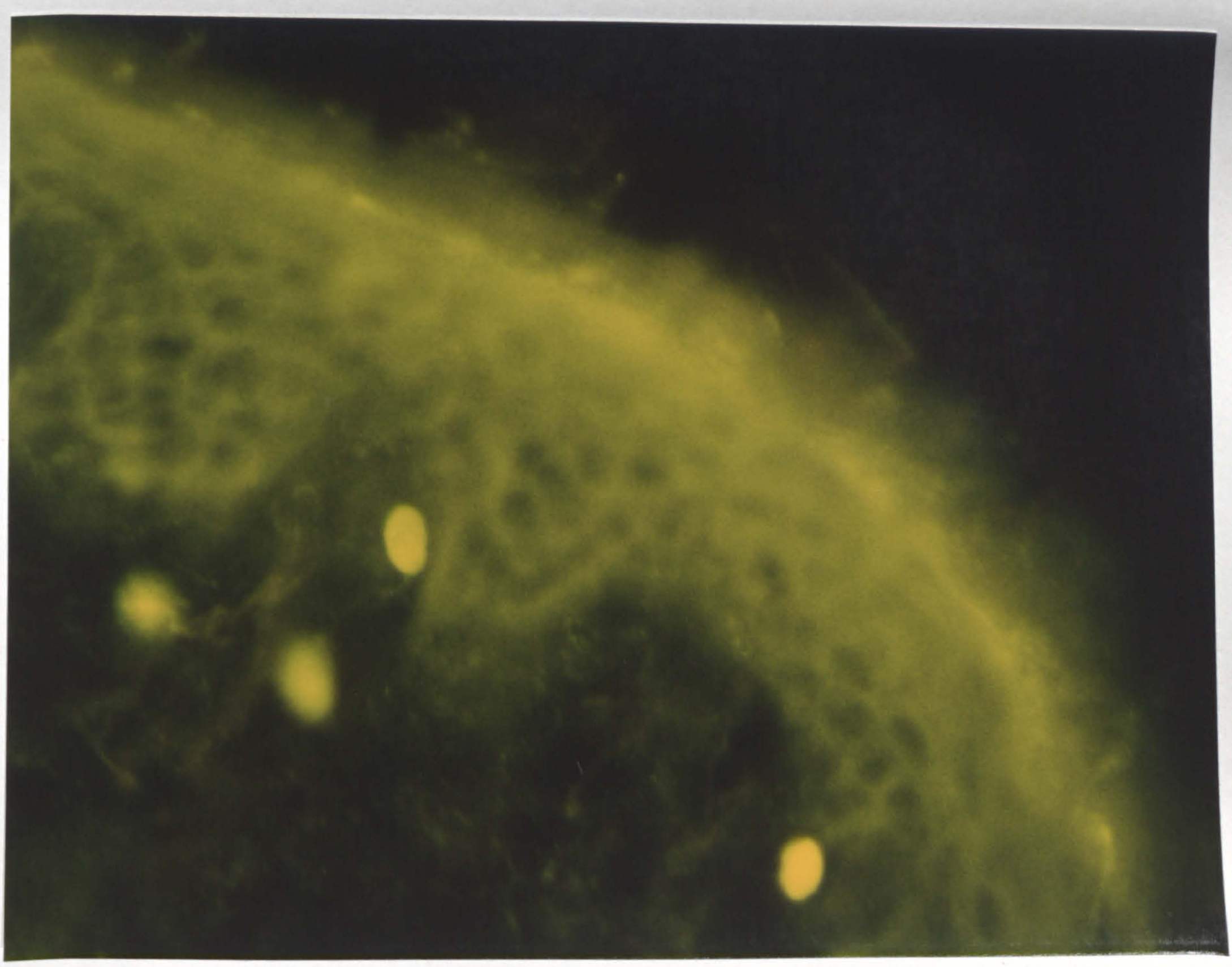


Fig 7. Direct immunofluorescence study of a marginal area from a patient with vitiligo showing IgA stained cytoid bodies in the papillary dermis. (X 80)

No.	Name	IgG	IgA	IgM	Fibrin	Complement (C3)
1	JTR	Neg	Neg	Neg	Neg	Neg
2	MO	Neg	Neg	Neg	Neg	Neg
3	JC	Neg	Neg	Neg	Neg	Neg
4	EM	Neg	Neg	Neg	Neg	Neg
5	LG	Neg	pos	pos	Neg	Neg
6	BR	pos	pos	Neg	Neg	Neg
7	MF	pos	Neg	pos	Neg	Neg
8	DH	pos	pos	pos	Neg	Neg
9	AB	Neg	Neg	Neg	Neg	Neg
10	EMA	Neg	Neg	Neg	Neg	Neg
11	PL	Neg	Neg	Neg	Neg	Neg
12	GG	Neg	Neg	Neg	Neg	Neg
13	SG	Neg	pos	pos	pos	pos
14	CL	Neg	pos	pos	Neg	Neg
15	TB	Neg	Neg	Neg	Neg	Neg
16	CE	pos	pos	pos	Neg	pos
17	JM	pos	Neg	pos	Neg	Neg
18	JH	pos	pos	pos	pos	Neg
19	MB	pos	pos	pos	pos	pos
20	MT	pos	pos	Neg	Neg	pos

Table XIII Direct immunofluorescence of uninvolved skin of vitiligo patients showing the distribution of immunoglobulin, fibrin and complement deposition in cytoid bodies.

No.	Pt. Name	IgG	IgA	IgM	Fibrin	Complement (C3)
1	DB	Neg	Neg	Pos	Neg	Neg
2	SD	Neg	Neg	Pos	Neg	Neg
3	RY	Neg	Neg	Pos	Neg	Neg
4	JTR	Neg	Neg	Neg	Neg	Neg
5	CH	Neg	Neg	Neg	Neg	Neg
6	JC	Neg	Neg	Neg	Neg	Neg
7	JA	Pos	Pos	Neg	Neg	Neg
8	JT	Neg	Neg	Neg	Neg	Neg
9	EM	Neg	Neg	Neg	Neg	Neg
10	JW	Neg	Neg	Neg	Neg	Neg
11	LG	Pos	Neg	Neg	Neg	Neg
12	BR	Neg	Neg	Neg	Neg	Neg
13	MF	Neg	Pos	Pos	Neg	Neg
14	DH	Pos	Pos	Pos	Neg	Pos
15	AB	Neg	Neg	Pos	Neg	Neg
16	EMA	Neg	Neg	Pos	Neg	Neg
17	JR	Pos	Neg	Neg	Neg	Neg
18	PL	Neg	Neg	Neg	Neg	Neg
19	GG	Neg	Neg	Neg	Neg	Neg
20	SE	Pos	Pos	Pos	Pos	Pos
21	CL	Neg	Pos	Pos	Neg	Neg
22	TB	Neg	Neg	Neg	Neg	Neg
23	CE	Neg	Pos	Pos	Neg	Neg
24	JM	Pos	Pos	Pos	Neg	Neg
25	JH	Neg	Pos	Pos	Pos	Pos
26	MB	Pos	Pos	Pos	Pos	Pos
27	MT	Neg	Neg	Pos	Neg	Pos

Table XIV Direct immunofluorescence of marginal skin of vitiligo patients showing distribution of immunoglobulin, fibrin and complement deposition in cytoid bodies.

No.	Pt. Name	IgG	IgA	IgM	Fibrin	Complement (C3)
1	DB	Neg	Neg	Neg	Neg	Neg
2	SD	Neg	Neg	Neg	Neg	Neg
3	RY	Neg	Neg	Pos	Neg	Neg
4	JTR	Neg	Neg	Neg	Neg	Neg
5	MO	Neg	Neg	Pos	Neg	Neg
6	CH	Neg	Neg	Neg	Neg	Neg
7	JC	Neg	Neg	Neg	Neg	Neg
8	JA	Neg	Neg	Neg	Neg	Neg
9	JT	Neg	Neg	Neg	Neg	Neg
10	EM	Neg	Neg	Neg	Neg	Neg
11	JW	Neg	Neg	Neg	Neg	Neg
12	LG	Pos	Pos	Pos	Neg	Pos
13	BR	Pos	Neg	Pos	Neg	Neg
14	MF	Neg	Pos	Pos	Neg	Neg
15	DH	Neg	Pos	Pos	Neg	Neg
16	AB	Neg	Neg	Neg	Neg	Neg
17	EMA	Neg	Neg	Neg	Neg	Neg
18	JR	Neg	Neg	Pos	Neg	Neg
19	PL	Neg	Neg	Neg	Neg	Neg
20	GG	Neg	Neg	Neg	Neg	Neg
21	SE	Neg	Pos	Pos	Pos	Neg
22	CL	Neg	Pos	Pos	Neg	Neg
23	TB	Neg	Neg	Neg	Neg	Neg
24	CE	Neg	Pos	Pos	Neg	Neg
25	JM	Neg	Neg	Neg	Neg	Neg
26	JH	Neg	Pos	Pos	Pos	Pos
27	MB	Pos	Pos	Pos	Pos	Pos
28	MT	Neg	Pos	Neg	Neg	Pos

Table XV Direct immunofluorescence of involved skin of vitiligo patients showing distribution of immunoglobulin, fibrin and complement deposition in cytoid bodies.

Name	IgG	IgA	IgM	Fibrin	Complement (C3)
AG - uninvolved	Dermis	Neg	Neg	CB	Neg
- marginal	Dermis	Neg	Neg	Neg	Neg
- involved	Faint DEJ	CB	Neg	Neg	Neg
AD - uninvolved	CB	Neg	CB	ND	Neg
- marginal	CB	Neg	Neg	Neg	Neg
- involved	Neg	Neg	Neg	Neg	Neg

Table XVI Direct immunofluorescence of patients with occupational vitiligo showing distribution of immunoglobulin, fibrin and complement deposition. CB cytoid body, ND not done.

cence of uninvolved and marginal area, with a moderate fluorescence at dermo-epidermal junction of the involved area when stained with an IgG conjugate. Cytoid bodies were also seen in the papillary dermis in both patients.

III. INDIRECT IMMUNOFLUORESCENCE COMPLEMENT FIXATION TEST

Sera from twenty-three vitiligo patients were tested using the indirect immunofluorescence complement fixation method and all gave negative results on the normal skin and on several melanocytic naevi. Sera from three patients with bullous pemphigoid were tested using the same method and all gave an intense linear band of immunofluorescence along the dermo-epidermal junction and on all the skin substrates used.

Sera from two patients with occupational vitiligo were tested using the same method and both samples gave similar negative results.

IV. DOPA METHOD

(a) Common Vitiligo

Twenty-nine patients with common 'idiopathic' vitiligo were studied (table XVII). The sites of the shave biopsies from these patients, the number of melanocytes per mm^2 in the uninvolved, marginal and involved areas of skin are recorded in the table XVII.

Uninvolved Skin

The melanocytes in this area were generally smaller in size when compared with those cells in the marginal skin (fig 8). The cells had smaller dendrites, but not much in the way of secondary branching. They were dopa-positive, but their reaction was weaker than those cells found in the marginal areas. In many of the biopsies the melanocytes were larger than normal with many primary and secondary dendrites. The dendrites were full of melanin granules and frequently appeared to be beaded. In a number of patients the melanocyte size was very variable even in the same small area.

The number of melanocytes was variable not only for the region, but even in different sites of the same area biopsied. The mean number of melanocytes for the forearms was 1890 $\pm$ 167 SEM, while the total mean for the backs was 1464 $\pm$ 236 SEM. It was also noticed in these 'uninvolved' areas, there were small foci in

No.	Name	Uninvolved ±SEM	Marginal ±SEM	Involved ±SEM	Site of biopsy
1	MO	1850±124	1567±70	0	Back
2	JTR	2093±168	1471±128	ND	Back
3	JT	ND	833±55	28±12	Back
4	LG	423±33	265±26	129±37	Back
5	BR	838±84	360±49	79±30	Back
6	MF	1325±65	899±70	400±116	Back
7	EMA	1889±131	479±106	87±16	Back
8	GG	1835±56	774±131	39±17	Back
9	DB	ND	ND	359±60	Forearm
10	SD	ND	ND	0	Forearm
11	CH	2145±81	2188±120	175±79	Forearm
12	JC	2150±154	-	53±12	Forearm
13	EM	1366±51	507±80	74±30	Forearm
14	AB	2106±174	1306±195	137±48	Forearm
15	JR	1476±111	926±143	92±29	Forearm
16	PL	982±114	675±122	62±21	Forearm
17	SE	1874±233	1106±200	6±4	Forearm
18	TB	2201±195	880±179	67±21	Forearm
19	CE	1467±81	1094±86	65±31	Forearm
20	JM	2667±124	1103±134	143±25	Forearm
21	JH	2922±114	1054±160	175±56	Forearm
22	MB	1325±52	352±79	69±20	Forearm
23	TM	ND	ND	138±41	Forearm
24	DH	1682±151	941±115	111±35	Upperarm
25	CL	1326±50	888±59	54±20	Axilla
26	JA	ND	567±75	0	Face
27	JW	2009±137	1239±149	184±78	Leg
28	MT	1916±44	1267±105	77±18	Upperarm
29	RY	ND	ND	0	Upperarm
Total Mean±SEM		1733±118	948±89	100±18	

Table XVII Showing the population density of melanocytes/mm^2 of epidermal sheets taken from patients with vitiligo.

which there was marked reduction in the density of melanocytes (fig 9). Some of the melanocytes in these areas had lost their dendrites and were rather small in size.

Marginal Areas

The sites biopsied were pigmented margins between involved and uninvolved areas of skin. In these sites there was marked reduction in the number of melanocytes, when compared with uninvolved skin from these patients ($P<0.001$). In general, there was a gradual reduction in the population density of melanocytes from the uninvolved to the involved areas (fig 10). Often there was a sudden drop in the number of melanocytes in this area of vitiligo (fig 11). The mean population density of melanocytes on the sites biopsied from the forearms was 1017 $\pm$ 145 SEM, while from the number on the back was 831 $\pm$ 170 SEM.

The morphology of the melanocytes in this area was variable. Many of the cells were much larger than normal and in addition to being strongly dopa-positive also contained more melanin granules. These cells had large, thick and long primary dendrites with many smaller secondary dendrites coming off them. The dendrites had a rather beaded appearance resembling a rosary. Some melanocytes in several of the biopsies from the marginal areas had more spindle-shaped appearance without many dendrites. In the marginal

areas there were three types of remaining melanocytic cells. The first, being strongly dopa-positive, with either a large or a small cell body, but with thick dendrites, some of which appeared truncated (fig 11). The second type of cell, (fig 12) also large, with thick large dendrites that was weakly dopa-positive and as a consequence the dendrites were hard to visualise. These cells also contained only a few melanin granules. Some of these cells had lost most of their dendrites leaving weakly dopa-positive cell bodies. The third type of residual melanocyte was small and rounded and was weakly dopa-positive. This last type was similar to what was found in the involved areas of vitiligo.

Involved Skin

Shave biopsies were taken from the central portions of established lesions of vitiligo that when examined under a Wood's lamp prior to biopsy appeared to be completely depigmented. In these areas there was a marked reduction in the number of melanocytes; in many sites a complete absence. The melanocytes that remained were around hair follicles, but were also found in the inter-follicular areas (fig 13). These melanocytes were small and had rounded cell bodies. Most of them had lost their dendrites and in general they were weakly dopa-positive. In a few patients though there was a marked reduction in the

number of melanocytes, the residual cells were much larger than normal and were very dendritic (fig 14).

(b) Occupational Vitiligo

Shave biopsies were taken from three patients with occupational vitiligo due to contact with para-tertiary butyl-phenol. Quantitative studies on the population density of melanocytes in these epidermal sheets showed a marked reduction in the number of melanocytes in the marginal area and a complete absence of dopa-positive cells in the involved vitiliginous areas (table XVIII). These changes were similar to what was found in ordinary 'idiopathic' vitiligo.

(c) Study of Hair Follicles

During separation of epidermis from dermis the hair follicles remained usually intact and attached to the dermal surface of the epidermis. The external hair root sheaths were as buds on the dermal surface of epidermis when viewed under a dissecting microscope and hair follicles contained shaft of hairs.

In the uninvolved and marginal areas the dopa-positive melanocytes could clearly be seen in the external root sheath, mainly in the upper third, but particularly around the follicular orifices. They were very few in the lower two-thirds of the follicles and also occasionally seen in the hair bulbs. In the involved areas, few residual melanocytes could be seen

in the hair follicles. This later finding was always associated with a few residual melanocytes in the inter-follicular sites. However, they were completely absent in the hair follicles when melanocytes were completely lacking in the epidermis.

		Number of melanocytes/mm^2±SEM		
Name	Site	Uninvolved	Marginal	Involved
AG	Forearm	2057±2.57	1047±146.55	0
AD	Forearm	1574±28.9	1097±134.2	0
KR	Hand	2105±70	595±50	0

Table XVIII Showing the population density of melanocytes in areas of patients with occupational vitiligo.

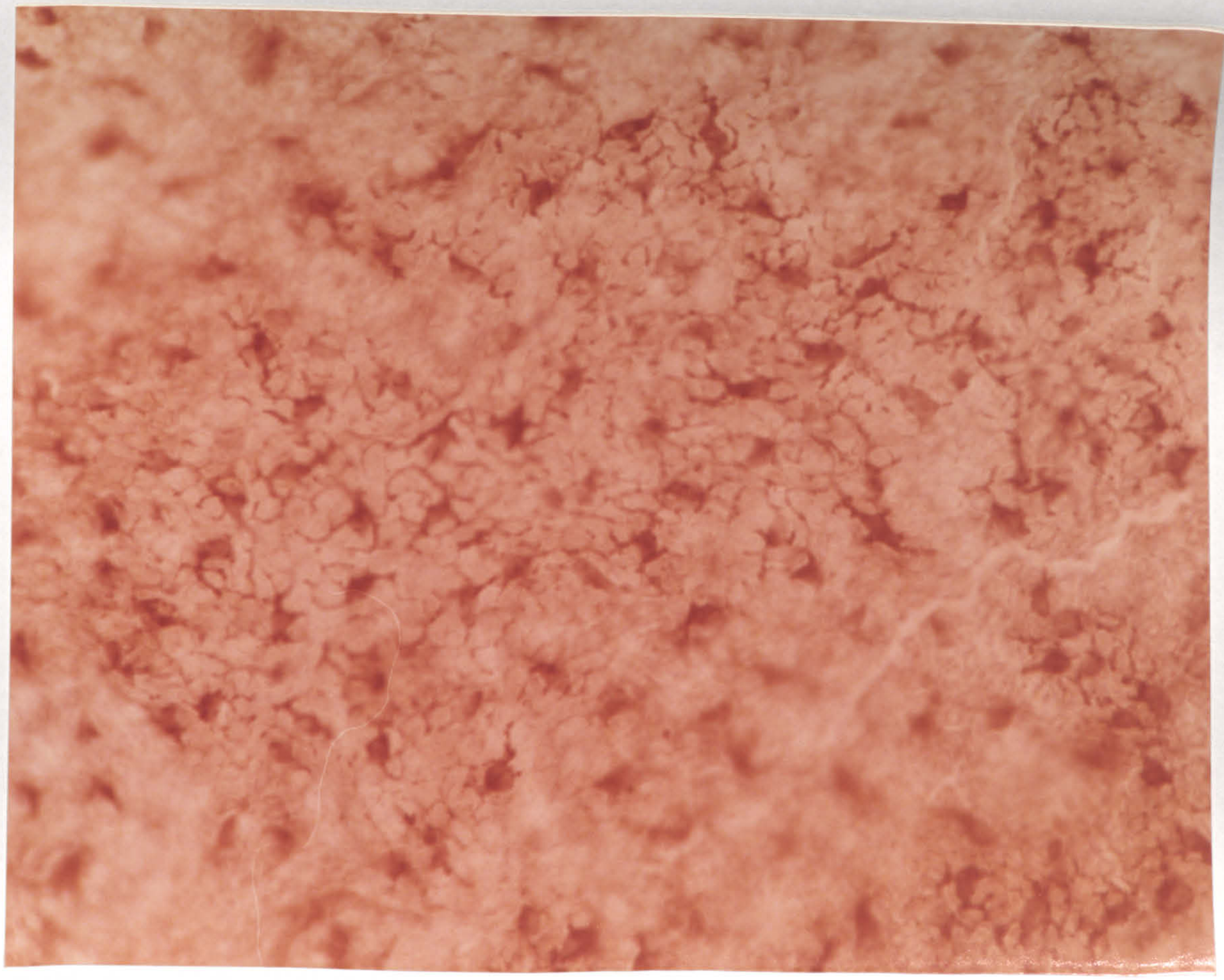


Fig 8. Dopa - incubated epidermis of an uninvolved area from a patient with vitiligo showing normal melanocytes. (X 128)

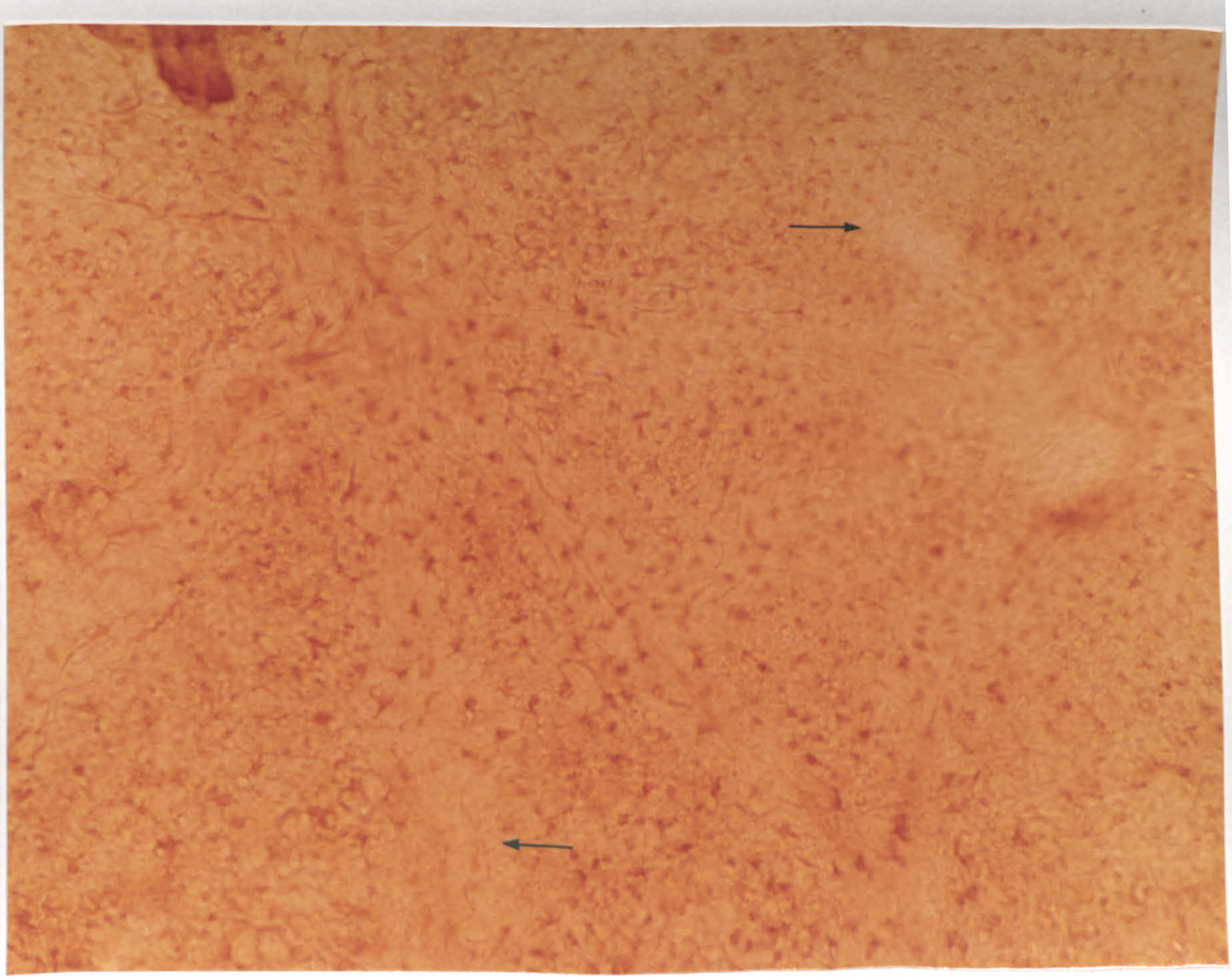


Fig 9. Dopa - incubated epidermis of an uninvolved area from a patient with vitiligo showing an early focus (arrow) of loss of melanocytes. (X 32)

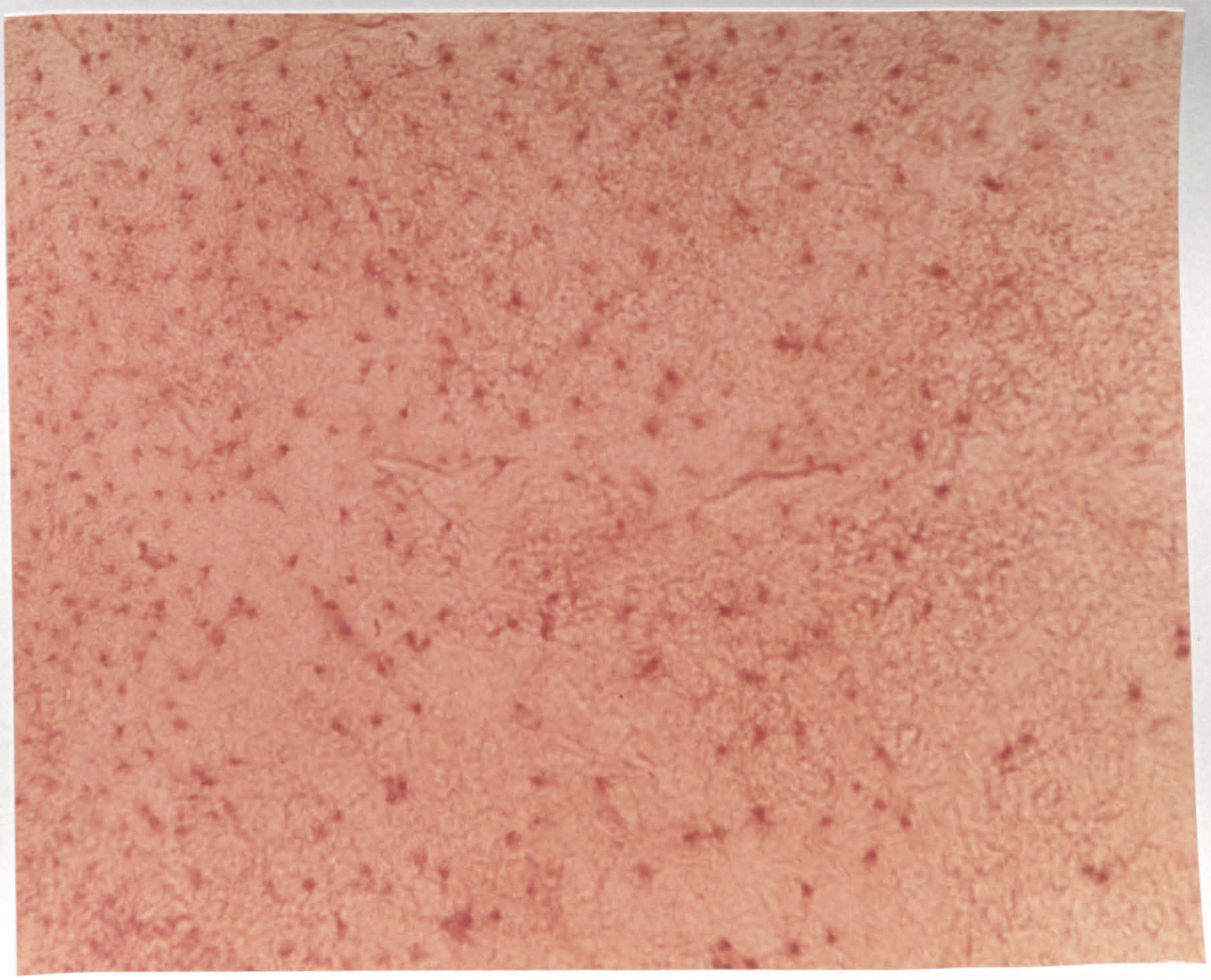


Fig 10. Dopa - incubated epidermis of a marginal area from a patient with vitiligo showing gradual reduction in the number of melanocytes. (X 32)

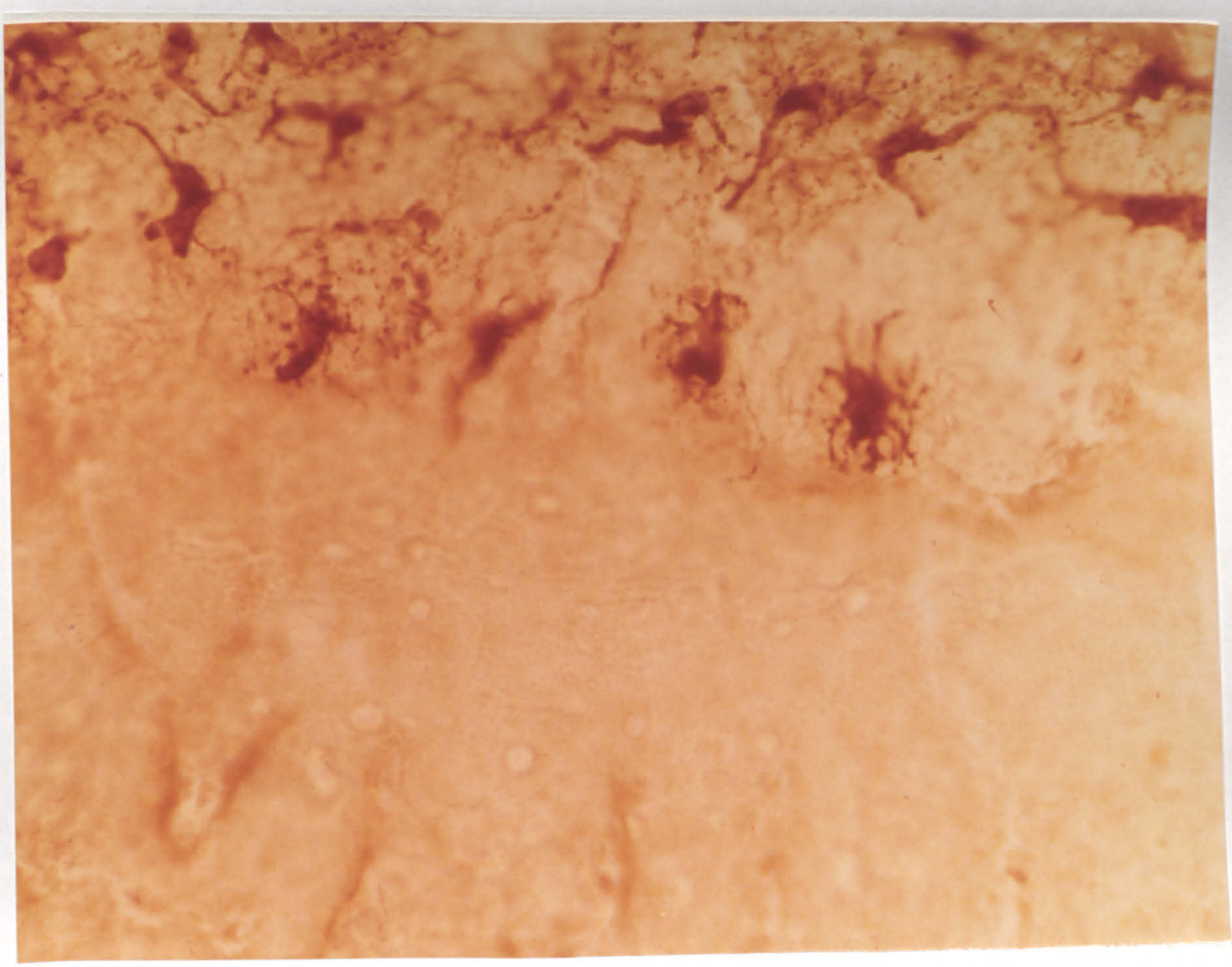


Fig 11. Dopa - incubated epidermis of a marginal area from a patient with vitiligo showing sudden reduction in the number of melanocytes. (X 80)

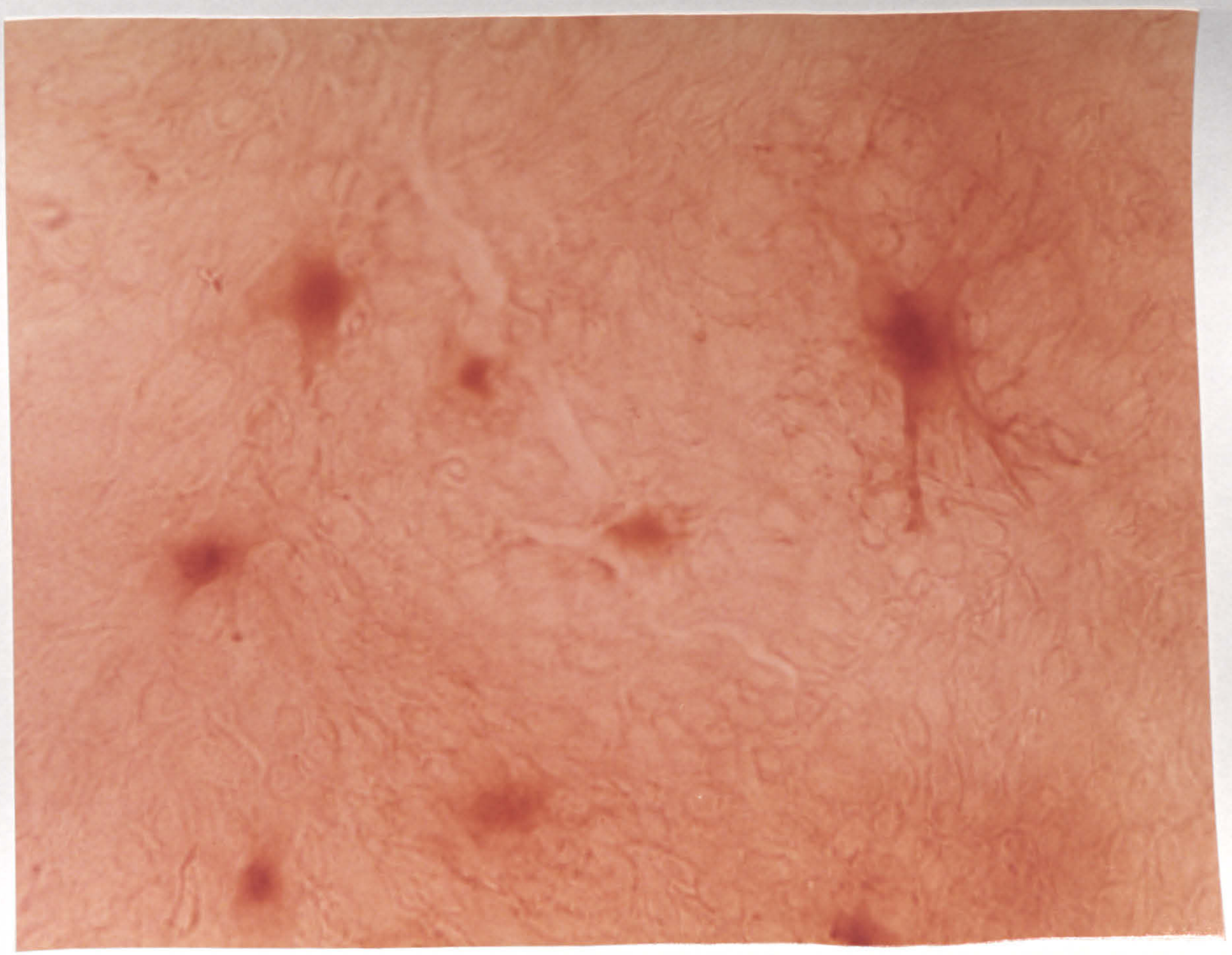


Fig 12. Dopa - incubated epidermis of a marginal area showing few residual melanocytes. The cells are very large but weakly dopa-positive. Some of the cells have lost their dendrites leaving a shadow of cell bodies. (X 80)

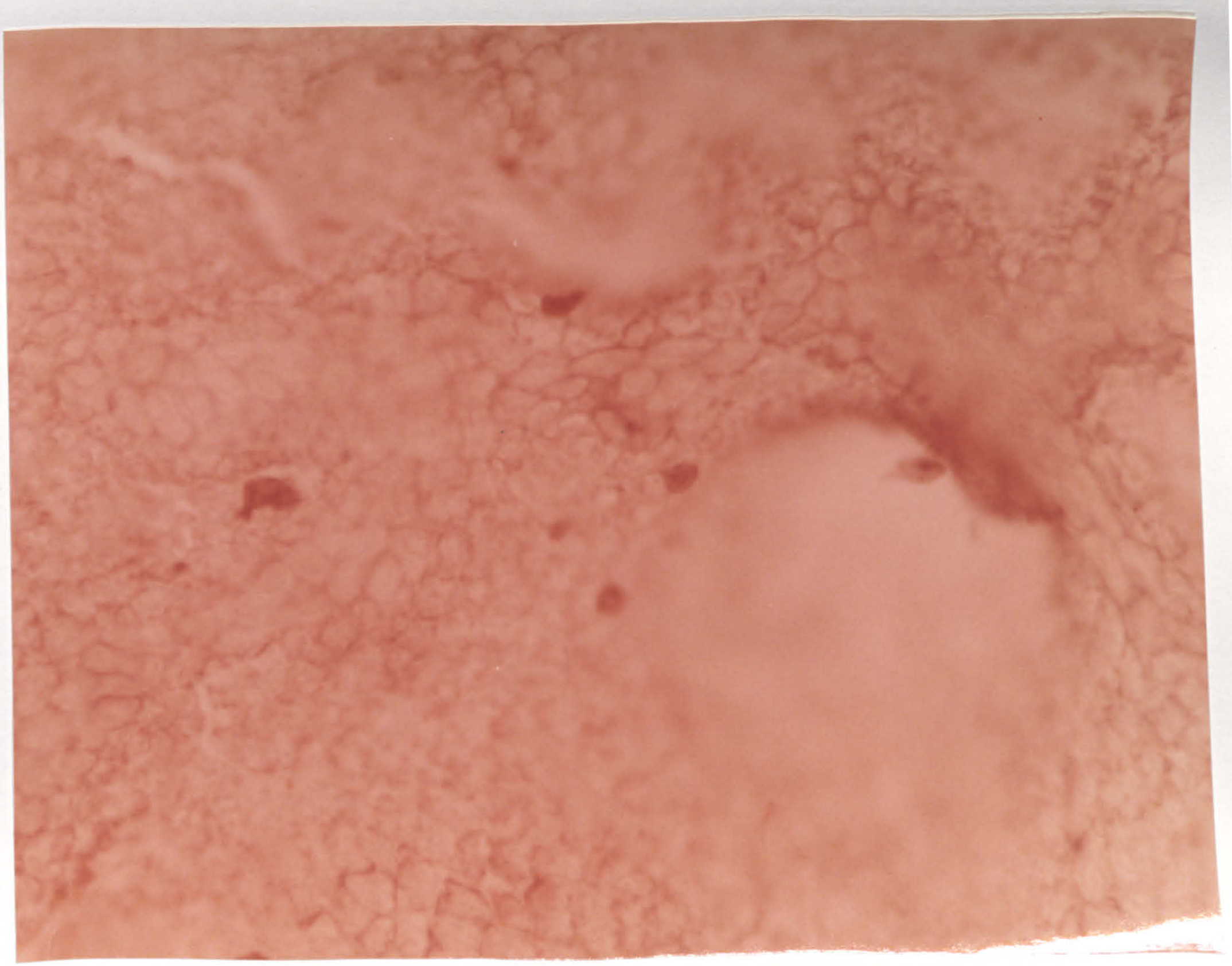


Fig 13. Dopa - incubated epidermis of an involved area from a patient with vitiligo showing a few residual melanocytes. The cells have lost their dendrites and changed into small rounded bodies. (X 128)

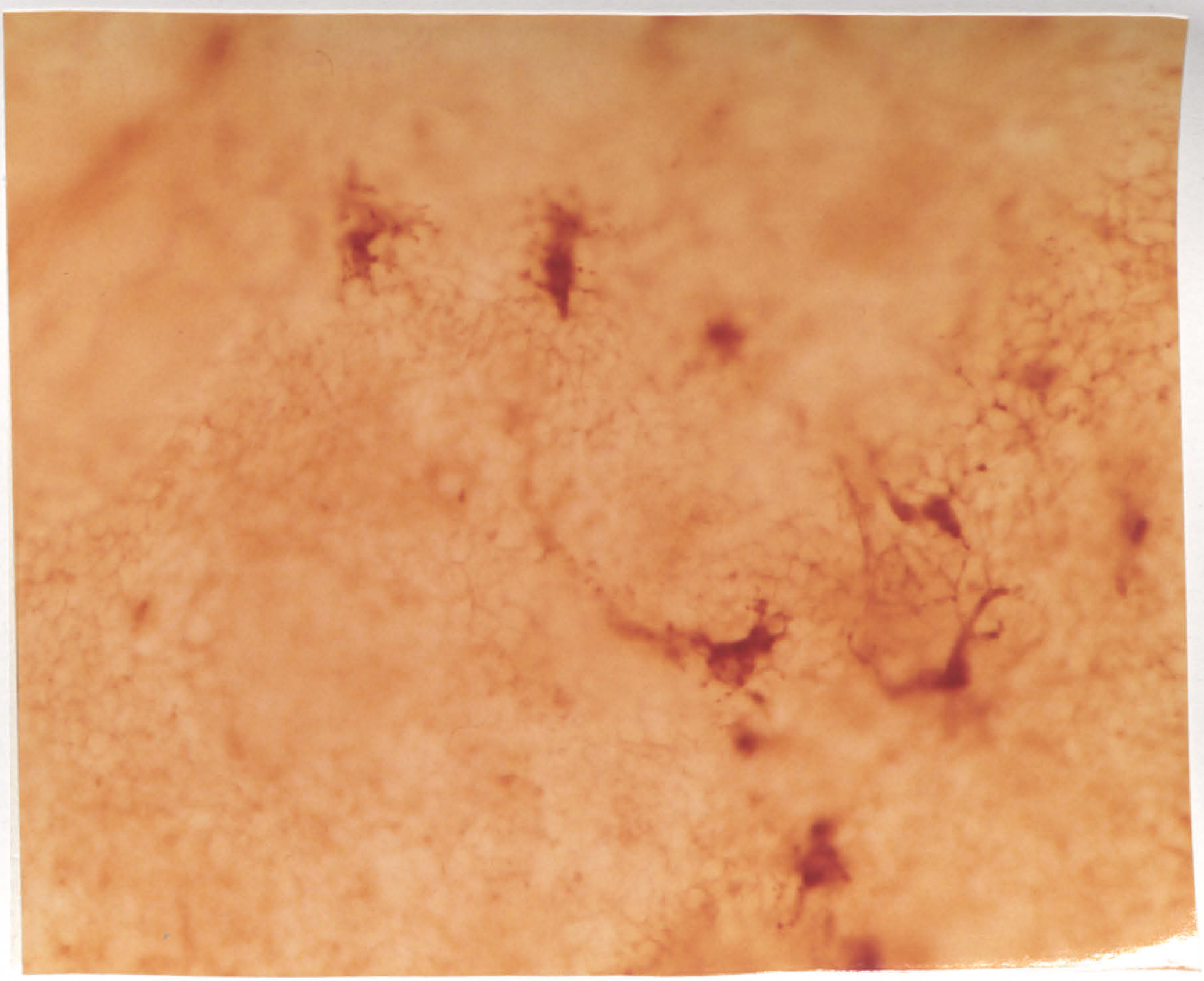


Fig 14. Dopa - incubated epidermis of an involved area from a patient with vitiligo showing a few residual functioning melanocytes. Some of the cells have truncated dendrites. (X 80)

V. EPON EMBEDDED TISSUE

A. NORMAL CONTROLS

Skin biopsies from thirteen normal individuals were studied. The ages of these individuals ranged from 25-35 years, with a mean of 30 years. There were three females and ten males. The shave biopsies were taken in ten patients from the upperarms and in three from the flexor surface of the forearms.

1. Epidermis

(a) General Appearance

The epidermis (fig 15) was composed of a number of layers termed horny, granular, malpighian and basal. The most superficial horny layer consisted of flattened keratinised cells. The next, the granular layer was composed of tightly opposed cells containing keratohyalin granules and about two to three cells deep. The malpighian layer, which constituted the main portion of the epidermis, consisted of polyhedral cells that were attached to each other by intercellular bridges. These cells had large prominent nuclei with the heterochromatin arranged mainly at the periphery. The nucleus usually contained one distinct nucleolus but sometimes two were present. The cytoplasm of these keratinocytes was packed with tonofilaments, leaving a clear perinuclear area. The basal layer consisted of cells arranged in a perpendicular position to dermo-

epidermal junction. The cells in the basal layer of the epidermis contained a variable amount of melanin granules which were principally located at the upper pole of the basal keratinocytes.

(b) Clear Cells (table XIX)

In addition to keratinocytes, the epidermis contained clear-looking cells that appeared clear because of a lack of tonofilaments. These cells did not have intercellular bridges. The clear cells in the epidermis of the normal control skin were melanocytes and Langerhans cells.

Melanocytes

These clear-looking cells were located mainly in the basal layer of the epidermis (fig 15) and surrounded by the basal keratinocytes. The frequency of these cells in the basal layer of the epidermis was variable and there was usually one melanocyte per four to six keratinocytes in the basal layer. The melanocytes made contact at the dermo-epidermal junction without an intervening keratinocyte. In many samples they were seen to be hanging down into the dermis. The bulky cytoplasm of these cells was clear and melanosomes could be seen. Portions of dendrites from these cells were to be found among keratinocytes and occasionally could be seen budding off from the main cell body of the melanocyte. The nucleus was large, rounded, or oval in shape and

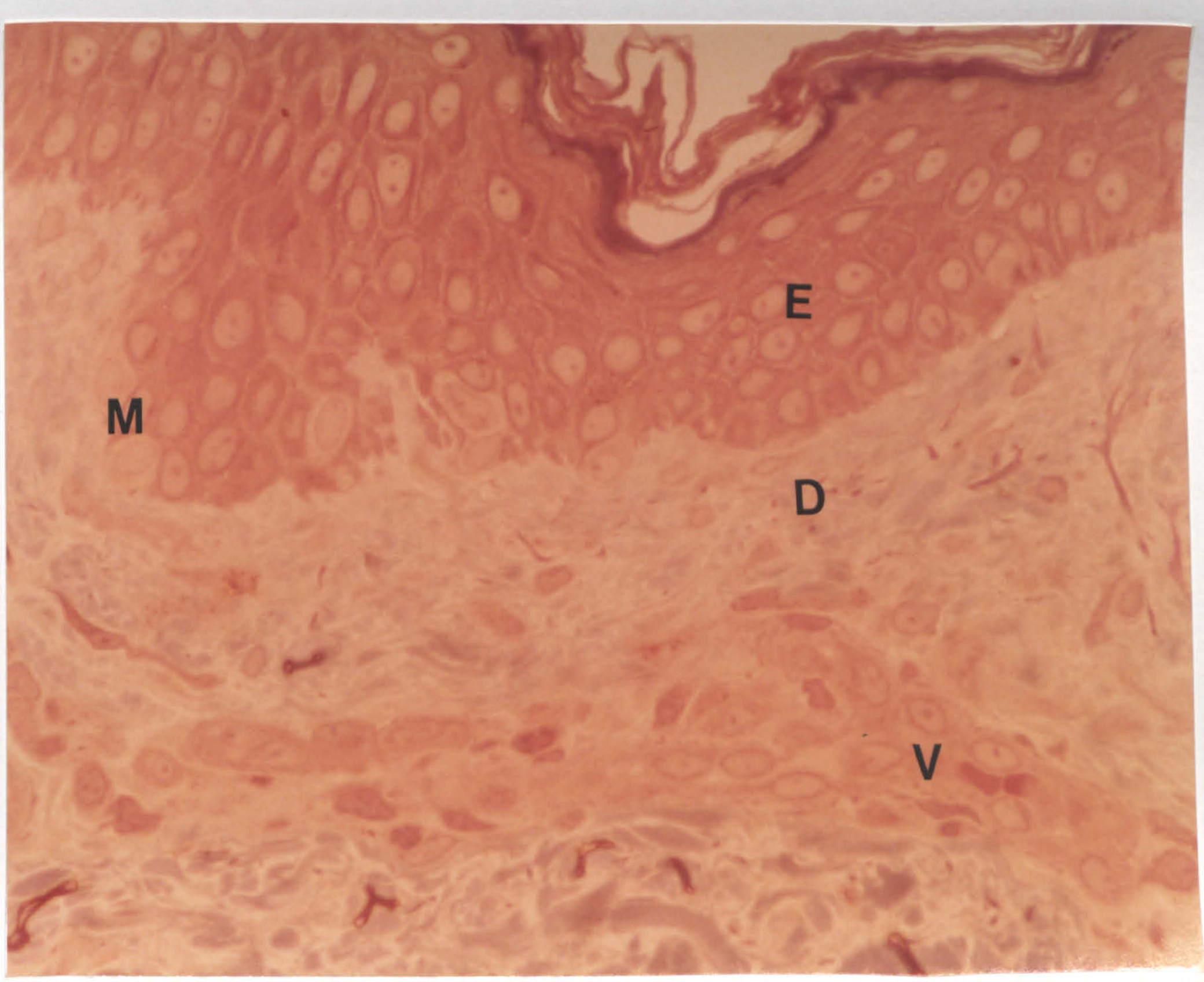


Fig 15. Light micrograph of a normal skin showing the epidermis (E), basal melanocytes (M), dermis (D) and blood vessel (V). Epon-embedded tissue, stained with basic fuschsin and methylene blue. (X 128)

No.	Total clear cells (TCC)	Basal clear cells(BCC)	Langerhans cells (suprabasal clear cells)	Dermal cells	Mast cells	Cytoid bodies
1	15.3	13.6	1.7	1269	59	positive
2	17.3	15.3	2	1065	10	negative
3	16.9	15.3	1.6	1347	0	negative
4	23	21.9	1.1	1072	51	negative
5	36.7	34.1	2.6	1262	66	positive
6	33.6	28.8	4.8	1715	46	positive
7	32.8	32.8	0	1525	36	negative
8	15.3	15.3	0	1452	19	negative
9	23.8	23	0.8	939	0	negative
10	15.3	14.4	0.9	857	0	negative
11	28.2	27.3	0.8	894	22	negative
12	14.4	14.4	0	1178	48	positive
13	28.6	27.2	1.4	532	6	positive
Total mean $\pm$ SEM	23.1$\pm$2.2	21.8$\pm$2	1.36$\pm$0.36	1162$\pm$87.9	27.9$\pm$6.6	

Table XIX Showing the population density in normal control skin of epidermal clear cells/mm of epidermis, total dermal cells/mm^2 of dermis and mast cells/mm^2 of dermis. The presence of cytoid bodies is also indicated.

sometimes was convoluted. The heterochromatin pattern was arranged around the periphery of the nucleus. One or sometimes two nucleoli were seen.

Langerhans Cells

These clear-looking cells were usually seen suprabasal and were high up in the epidermis. They had large amounts of cytoplasm and were sometimes seen to be dendritic. The nucleus was big and convoluted.

2. Dermis

(a) General Appearance

In the biopsies the papillary and upper reticular dermis was present. In addition to the collagen bundles, blood vessels and nerves were also present. Mast cells, fibroblasts and histiocytes could be recognised.

(b) Blood Vessels

They consisted of endothelial cells that lined the lumen and were surrounded by pericytes. Some of the blood vessels contained erythrocytes.

(c) Histiocytes

Histiocytes were identified by their large lobate nucleus and clear bulky cytoplasm and occasionally the cells contained melanosomes and were melanophages (fig 16). Sometimes it was difficult to distinguish these cells from other dermal cells.

(d) Mast Cells

The mast cells were readily identified as fusiform, round or oval cells with round or oval nuclei (fig 16). The cells had one nucleus but portions of mast cell without nuclear material could be seen. The cytoplasm sometimes was large, but sometimes there was a narrow amount ringing around the nucleus. The granules were mostly round and regular in shape and size. They stained bright red with the basic fuchsin and in poorly stained sections blue from the intensity of methylene blue. They were mainly seen in the papillary dermis around blood vessels, although they were also present in the deeper dermis. They were readily distinguished from melanophages by the characteristic granules that also stained differently. The mast cell number in the dermis was very variable, but the mean in papillary region in the thirteen normal individuals was 27.9 $\pm$ 6.6 SEM/mm^2 (table XIX).

(e) Cutaneous Nerves

Polyaxonal nerves were seen mainly in the reticular dermis usually associated with blood vessels. These nerves were composed of many axons that lightly stained with basic fuchsin. These axons were collectively enveloped by bluish staining collagenous endoneurium and perineurium. Some of these axons looked medullated.

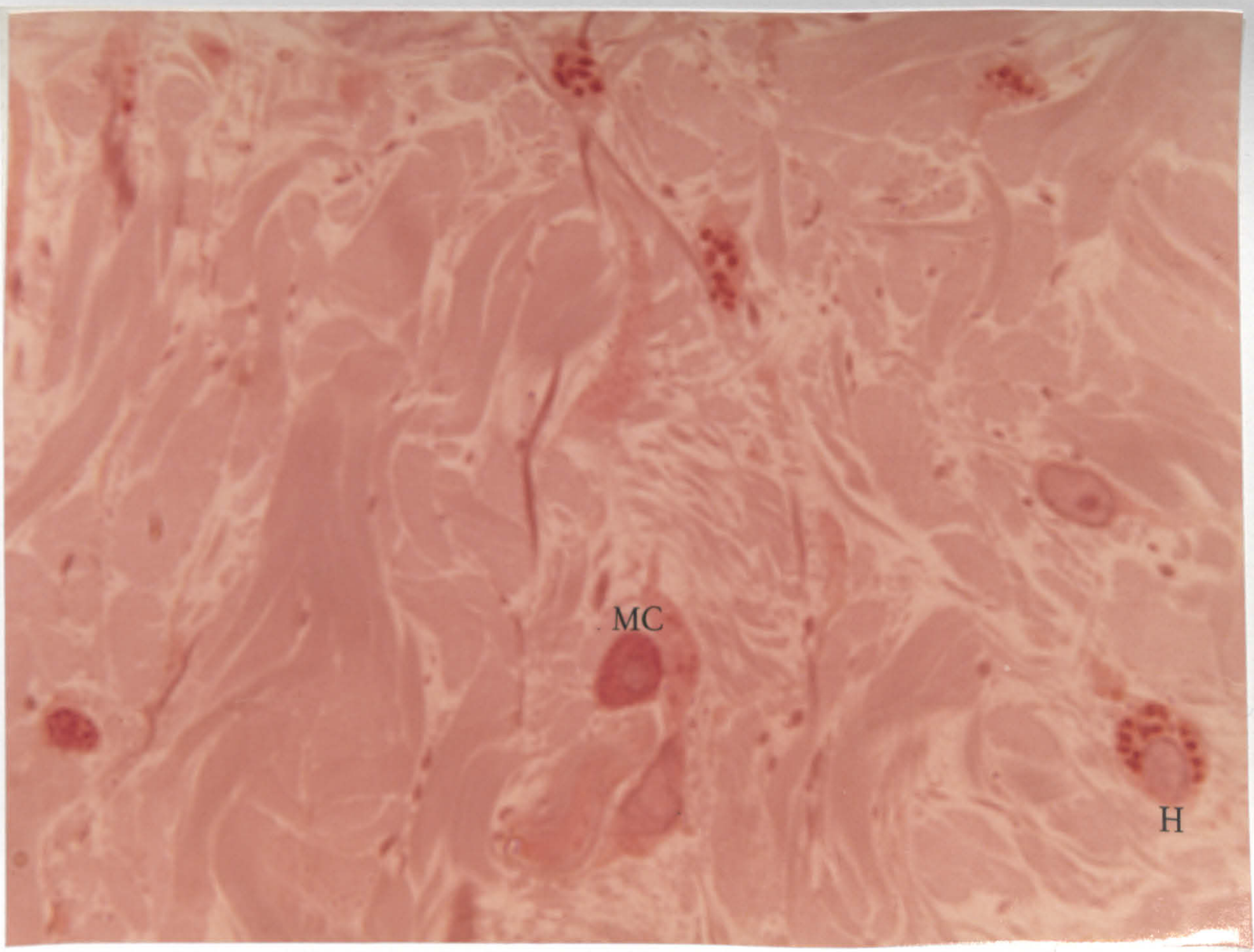


Fig 16. Light micrograph of a dermis showing many macrophage-histiocytes (H), containing melanosomes and mast cells (MC). Epon-embedded tissue stained with basic fuchsin and methylene blue. (X 128)

(f) Cytoid (Colloid/Amyloid) Bodies

These were globular bodies that varied in size, but were usually large. When stained with basic fuchsin they appeared pinky/red, similar in shade to the staining of the basal epidermal cells. They were seen only in the papillary dermis and usually near to the dermo-epidermal junction. Sometimes, they appeared in the basal layer of the epidermis. They were seen in five of the normal controls (table XIX). The frequency of these colloid/amyloid bodies in the normal controls was very low.

B. COMMON VITILIGO

1. Epidermis

(a) General Appearance

Changes in the epidermis were noted mainly in the marginal and involved areas of skin. There was hyperkeratosis seen in the involved skin. This was observed in many of the patients when compared with the uninvolved areas in the same patients and with normal controls. Some spongiosis of the epidermis was seen in many of the biopsies, particularly those from the marginal areas. This was not observed in the controls. The number of melanosomes in the keratinocytes was markedly reduced from the uninvolved to involved areas. Only a few melanosomes were seen in the basal keratinocytes in the involved areas. This was apparent in only a few of the patients biopsied (fig 17). The total number of clear cells seemed to be diminished as one went from uninvolved to involved skin (fig 18, table XX).

(b) Melanocytes

The number of basal clear cells in the uninvolved areas of vitiligo were diminished (forearm mean 19.9 $\pm$ 1.8 SEM) as compared to that of the control group (mean 21.8 $\pm$ 2 SEM). However, this difference was not of statistical significance ($P > 0.05$). The melanocytes were markedly diminished in number, as one proceeded from the uninvolved to

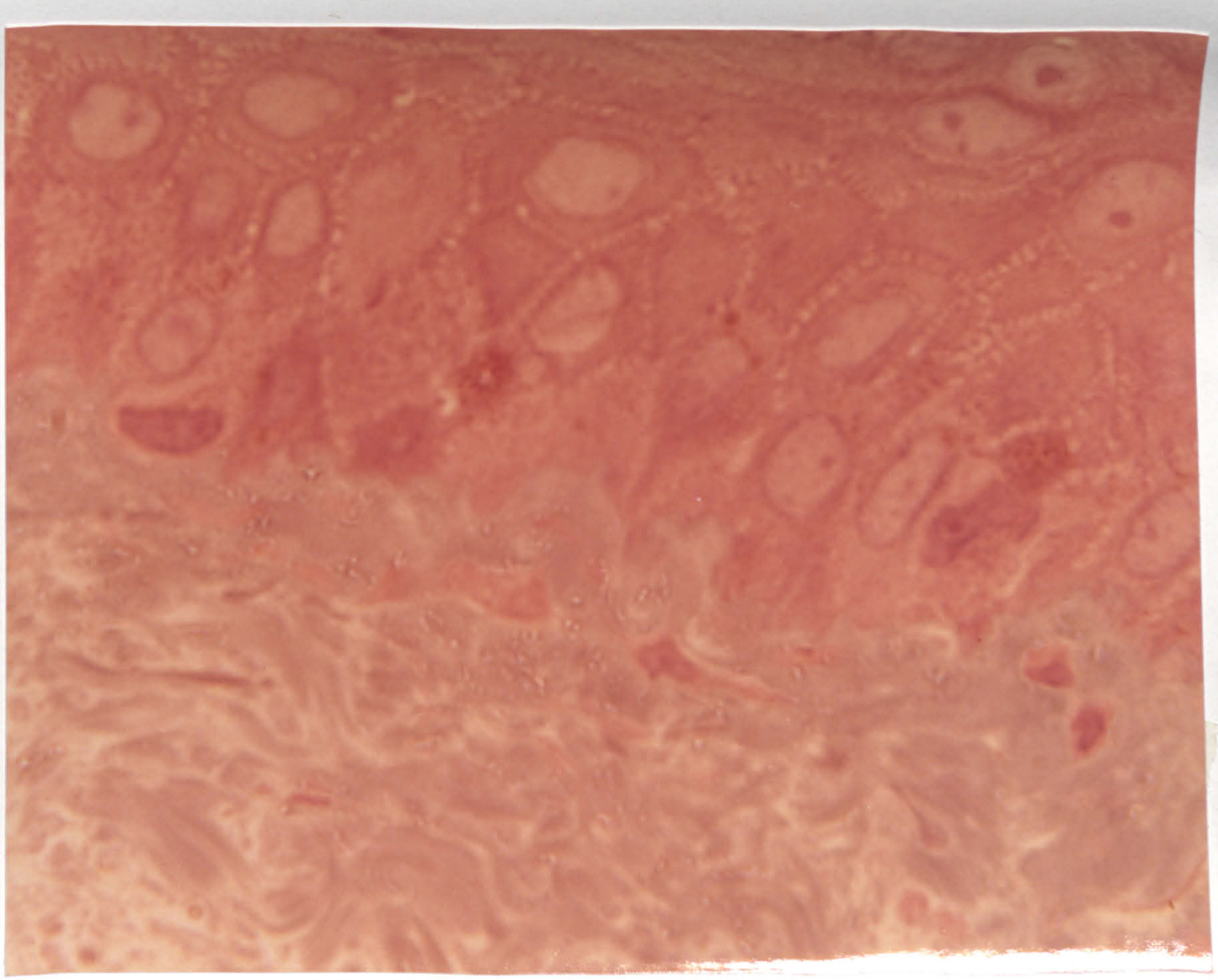


Fig 17. Light micrograph of an involved area from a patient with vitiligo showing basal keratinocytes containing melanosomes and some basal clear cells. The dermis contains a few mononuclear cells. Epon-embedded tissue stained with basic fuchsin and methylene blue. (X 320)

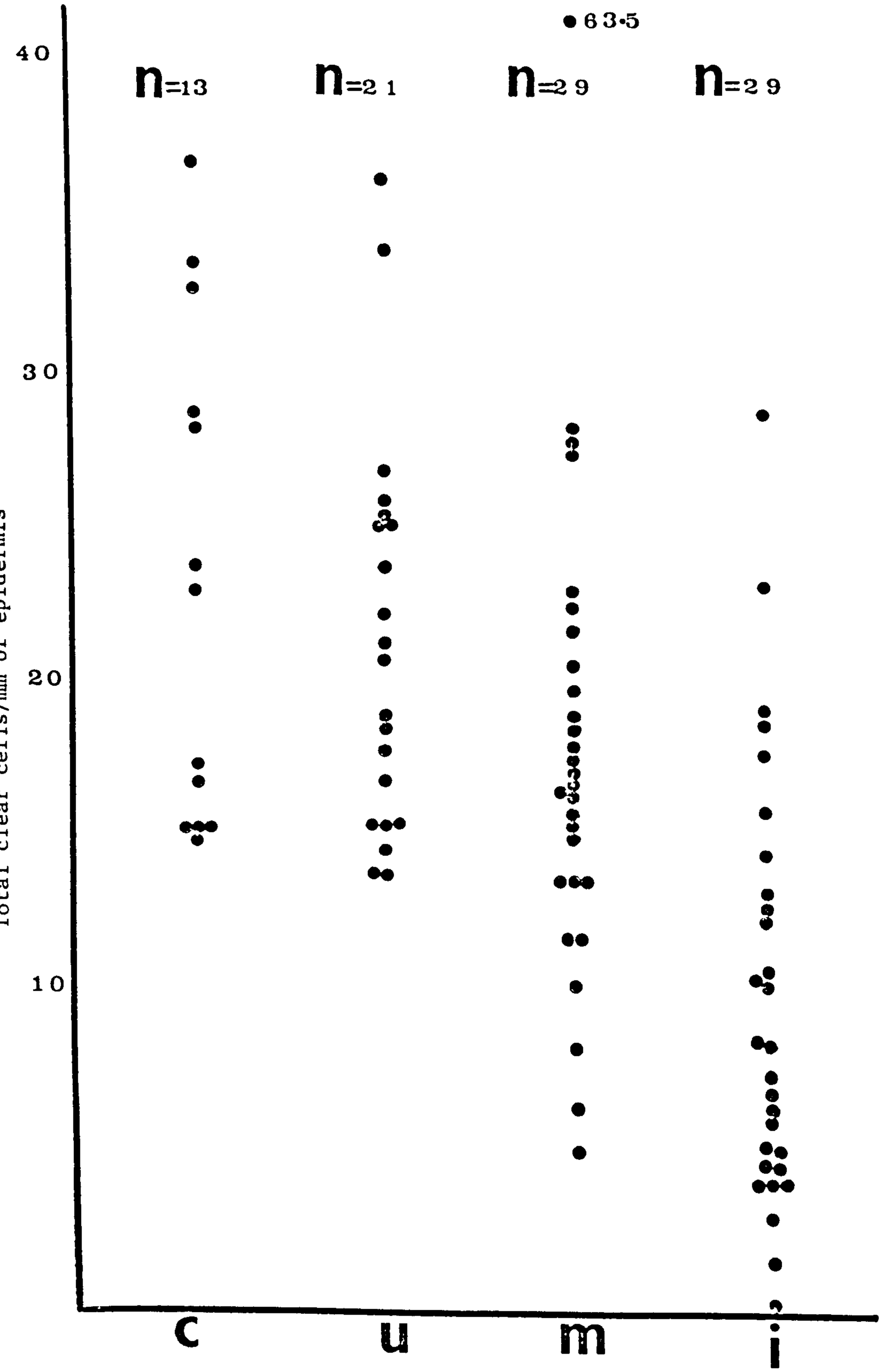


Fig 18 Comparison of epidermal total clear cells in normal controls (c) and uninvolved (u), marginal (m) and involved (i) areas of patients with vitiligo.

		Uninvolved n=21			Marginal n=29		Involved n=29	
No.	Name	BCC	TCC	LC	BCC	TCC	BCC	TCC (LC)
1	DB	ND	ND	ND	5.1	6.2	3	4
2	RY	ND	ND	ND	25.7	28	3.2	12
3	SD	ND	ND	ND	10.5	11.5	3.6	7.2
4	MO	14.2	15.3	1.1	12.7	18.4	6.2	8.2
5	JTR	12.6	14.4	0.8	12.1	15.6	0	4.5
6	CH	23.7	25.6	1.1	17.5	17.5	12.7	12.7
7	JC	21.7	25	3.1	8.9	13.2	2.6	8.3
8	JA	ND	ND	ND	15.1	21.1	10.8	28.2
9	JT	ND	ND	ND	11.2	14.8	19.9	22.7
10	TM	ND	ND	ND	22.7	27.3	10	12.8
11	EM	17	22.1	5.1	11.5	16.6	4.4	10.2
12	JW	14.1	18.5	4.4	11.5	13.2	7.8	10.6
13	LG	11.5	15.3	3.8	8.5	10	14.2	18.3
14	BR	15.3	18.8	3.5	16.2	16.2	4.4	6.6
15	MF	11.5	13.8	2.3	18.1	22	12.7	17.3
16	DH	30.7	36.2	5.5	26	27.7	16.2	18.6
17	AB	18.4	25.3	6.9	22.5	22.5	1.9	1.9
18	EMA	20.1	21.1	0	5	5	0	0
19	JR	ND	ND	ND	8.1	8.1	6	6
20	PL	11.1	13.9	2.8	9.7	13.2	9.4	10
21	GG	23.9	23.9	0	14	15	13.2	15.7
22	SE	32.9	34	1.1	18.9	20.3	3	4
23	CL	16.6	17.9	1.3	16.1	19.7	2.3	4
24	TB	18.8	20.2	1.4	16.8	18.7	3	3
25	CE	16.9	16.9	0	36.8	63.5	4.6	4.6
26	JM	23	26.2	3.2	17	17.3	4.4	5.2
27	JH	ND	ND	ND	13.1	16	3.4	5.1
28	MB	11.1	15.3	4.2	7	11.5	11.5	14.1
29	MT	20.1	25	4.9	16.4	16.4	6.4	6.4
Mean±SEM		18.3±1.3	21.1 ±1.3	2.72 ±0.4	14.9 ±1.2	18.6 ±1.9	6.9 ±.94	9.7 ±1.2

Table XX Showing the population density of epidermal clear cells/mm in areas of patients with vitiligo. BCC basal clear cells, TCC total clear cells, LC Langerhans cells which represent the suprabasal clear cells in the uninvolved areas and total clear cells in the involved areas. ND not done.

involved areas. In the involved areas only a few residual functioning melanocytes were seen.

(c) Langerhans Cells

In the uninvolved skin the Langerhans cells were represented by the clear looking cells that were lying suprabasally and high up in the epidermis. In the involved areas of skin, the Langerhans cells were represented by the total clear cells in the epidermis, as confirmed by electron microscopy. The number of suprabasal clear cells in the uninvolved epidermis in the biopsies taken from patients with vitiligo was significantly increased when compared with those found in the biopsies taken from the normal controls ($P<0.05$).

In the biopsies taken from involved areas, the Langerhans cells were to be found both in the basal layer and also high up in the epidermis (fig 19). In many of the specimens examined the Langerhans cells were only in the basal layer and in only a few of the samples were the Langerhans cells to be found in the upper portion of the epidermis. Langerhans cells were absent in the involved skin from one of the patients.

The number of clear cells (Langerhans cells) in the involved areas of skin was significantly raised when compared with that of those suprabasal clear cells (Langerhans cells) of the uninvolved

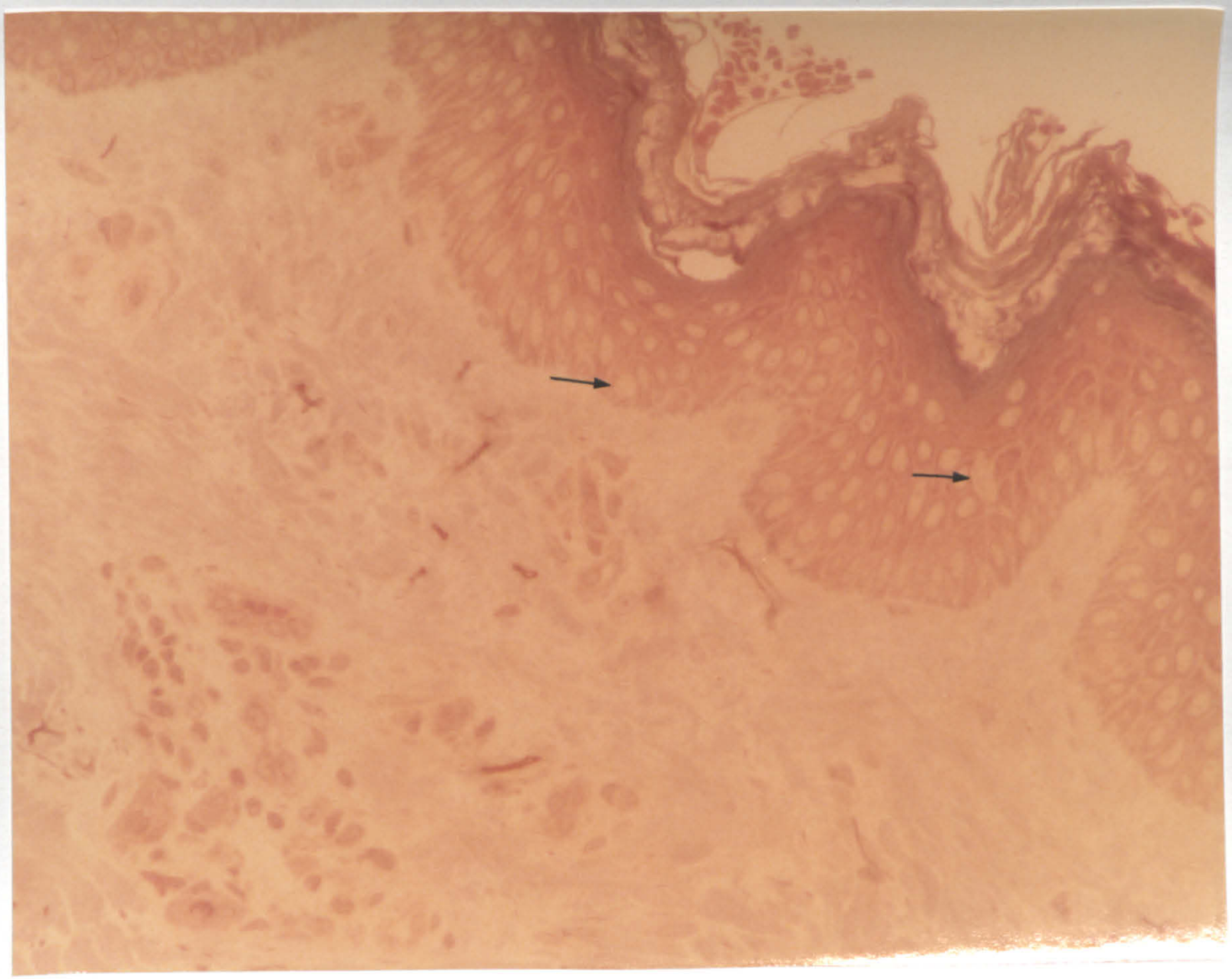


Fig 19. Light micrograph of an involved area from a patient with vitiligo showing a few basal and suprabasal clear cells (arrows). Dermis showing mononuclear cells around blood vessels. Epon-embedded tissue, stained with basic fuchsin and methylene blue. (X 80)

skin in the patients with vitiligo ($P<0.01$), (fig 20, table XX).

It was not uncommon to see in the specimens from patients with vitiligo, two adjacent clear cells that were apparently in contact with each other in the basal layer of the epidermis. This finding was most unusual in the biopsies taken from the normal controls.

(d) Mononuclear Cell Infiltrate of Epidermis

A mononuclear cell infiltrate in the epidermis was present in many of the biopsy specimens. It was mainly observed in the portions from the marginal areas (fig 21,22) and less frequently and less marked in those samples from the involved areas of skin (fig 23). The infiltrate was usually in small foci, the cells being grouped together and lying mainly in the basal part of the epidermis. Sometimes, the cells had a tendency to form Pautrier-like micro-abscesses (fig 24,25).

The main type of cell in this infiltrate was a large lymphocyte (fig 26). This cell had a hyper-chromatic nucleus with many deep invaginations and was easily distinguishable from adjacent histiocytes or Langerhans cells. Some of these lymphocytes had an elongated nuclei. The lymphocytes within the epidermis were similar to those in the dermis and some were seen to be traversing the dermo-epidermal

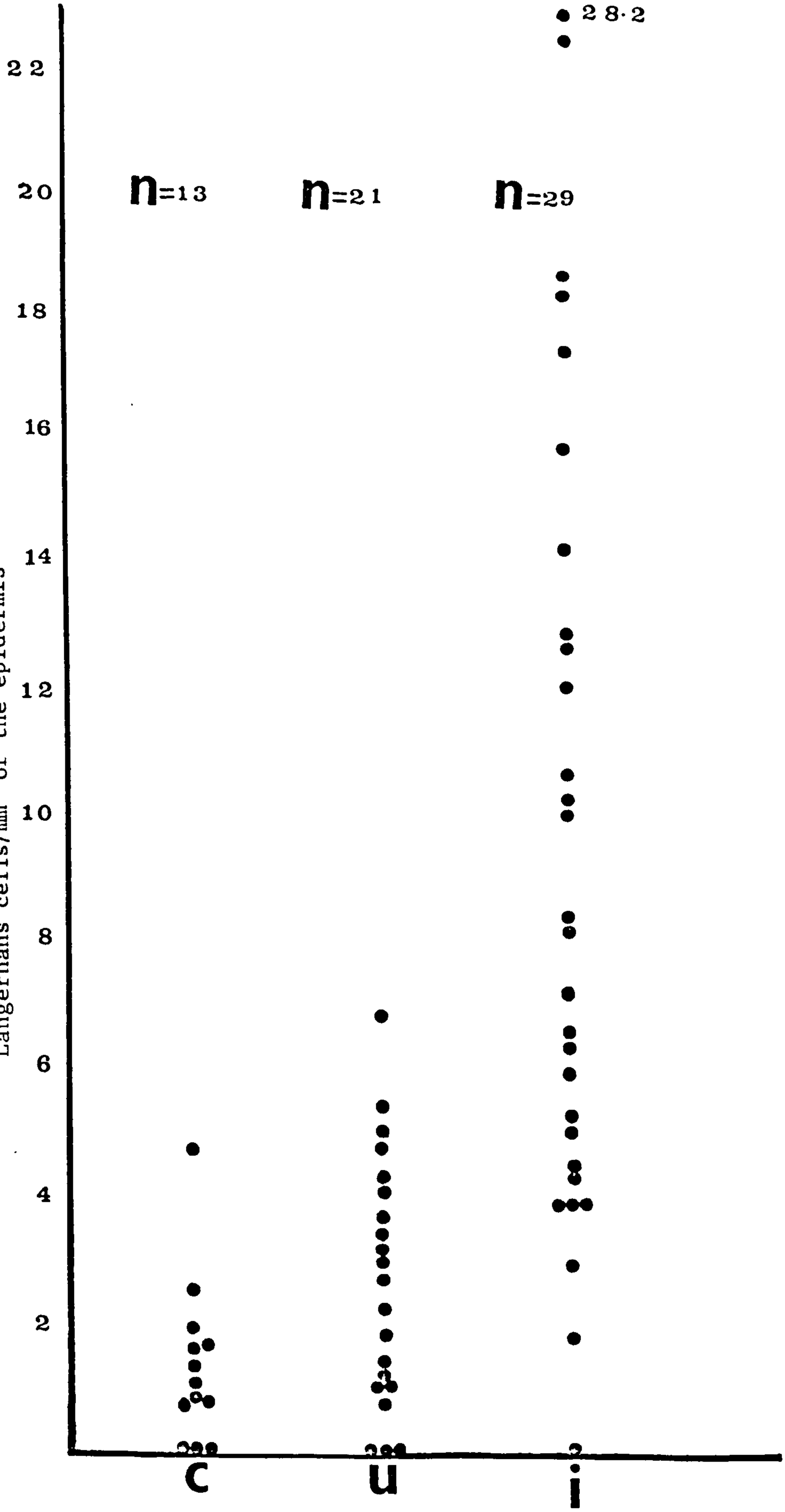


Fig 20 Comparison of epidermal Langerhans cells in normal controls (c, suprabasal clear cells) and uninvolved (u, suprabasal clear cells) and involved (i, total clear cells) areas of patients with vitiligo.

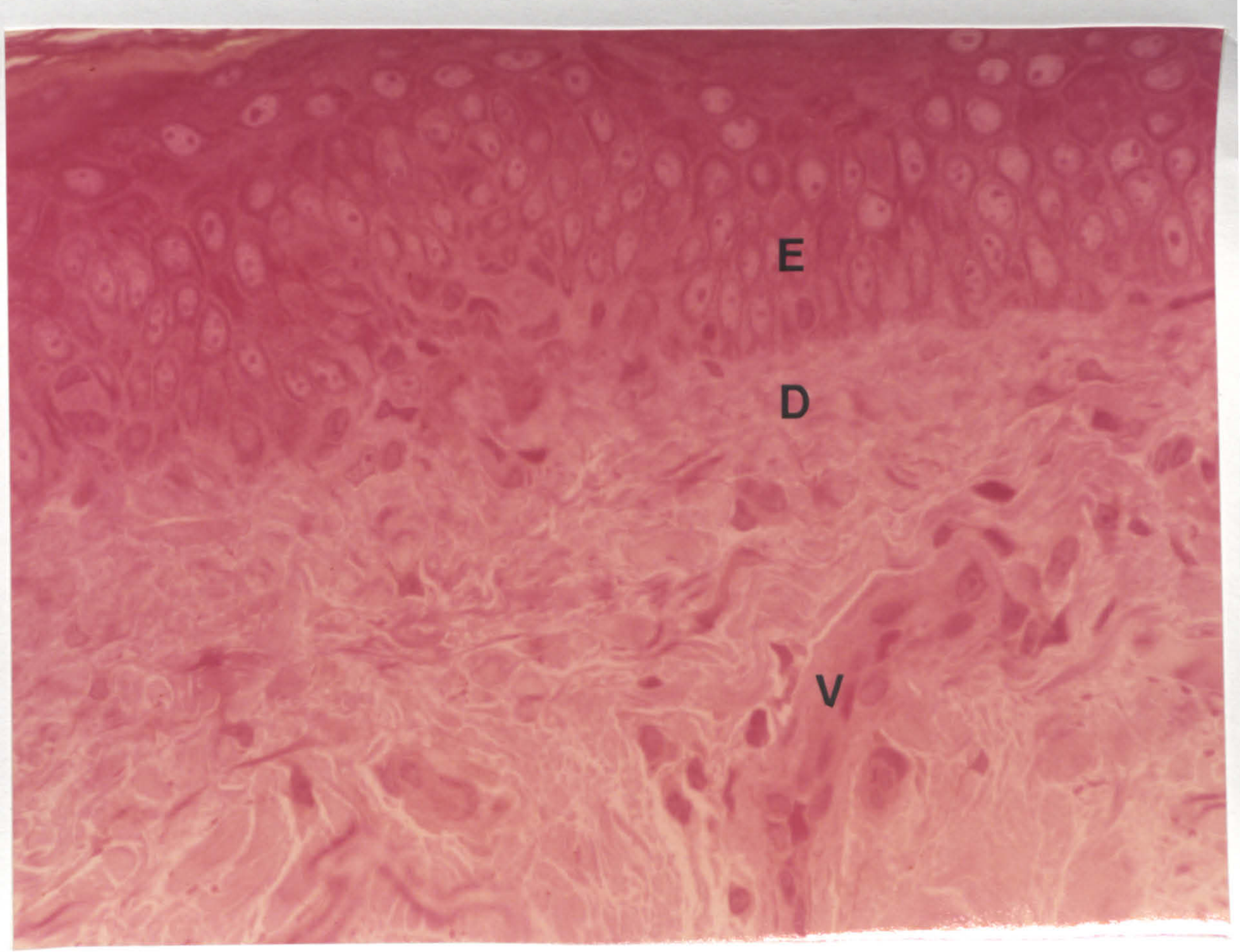


Fig 21. A. Light micrograph of a marginal area from a patient with vitiligo showing a focus of lymphocytes in the epidermis (E) and similar cells in the dermis (D). Blood vessel (V). Epon-embedded tissue, stained with basic fuchsin and methylene blue. (X 128)

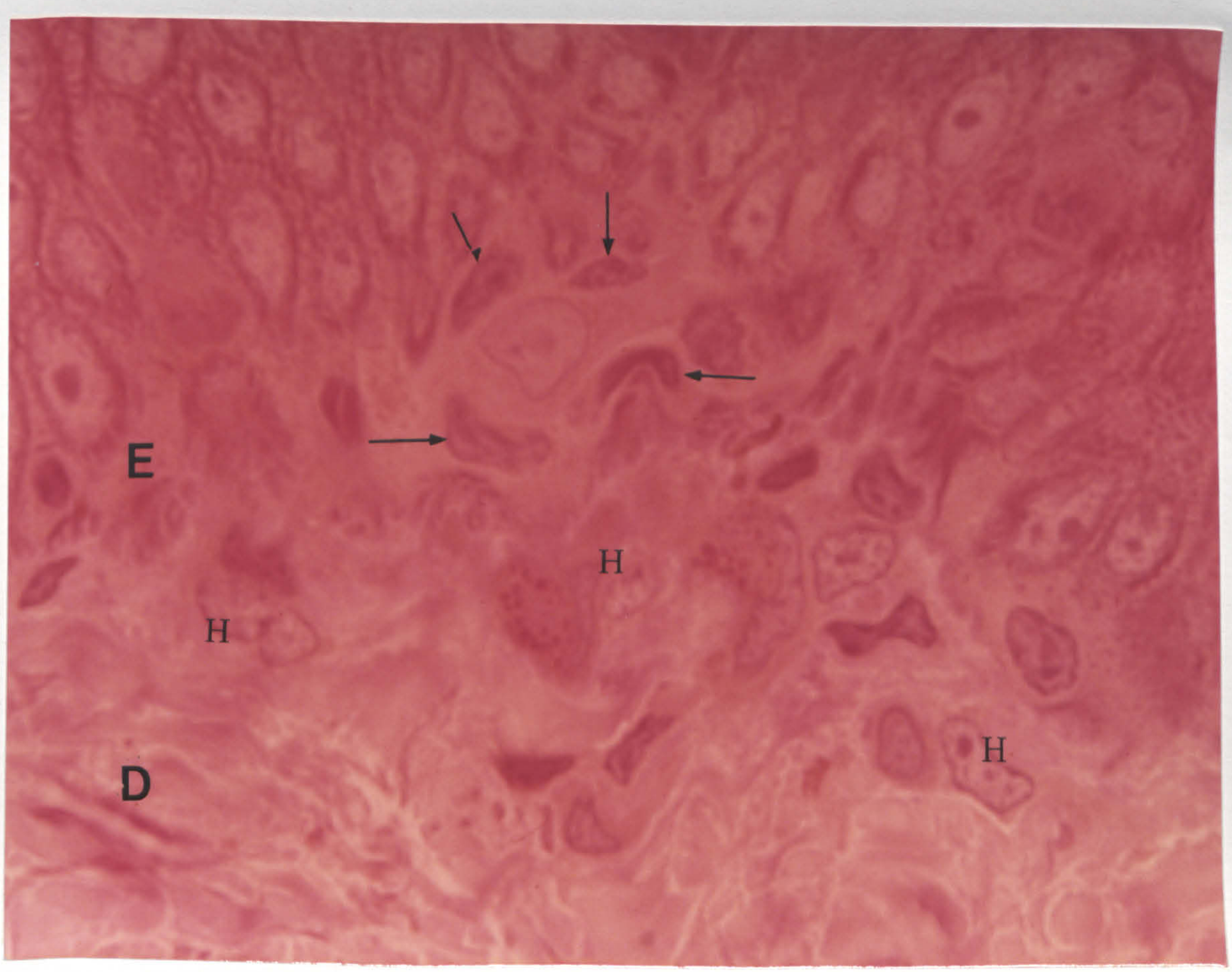


Fig 21. B. Higher magnification showing a group of lymphocytes (arrows) surrounding a clear cell, probably a melanocyte. H Histiocytes, E Epidermis, D Dermis. (X 320)

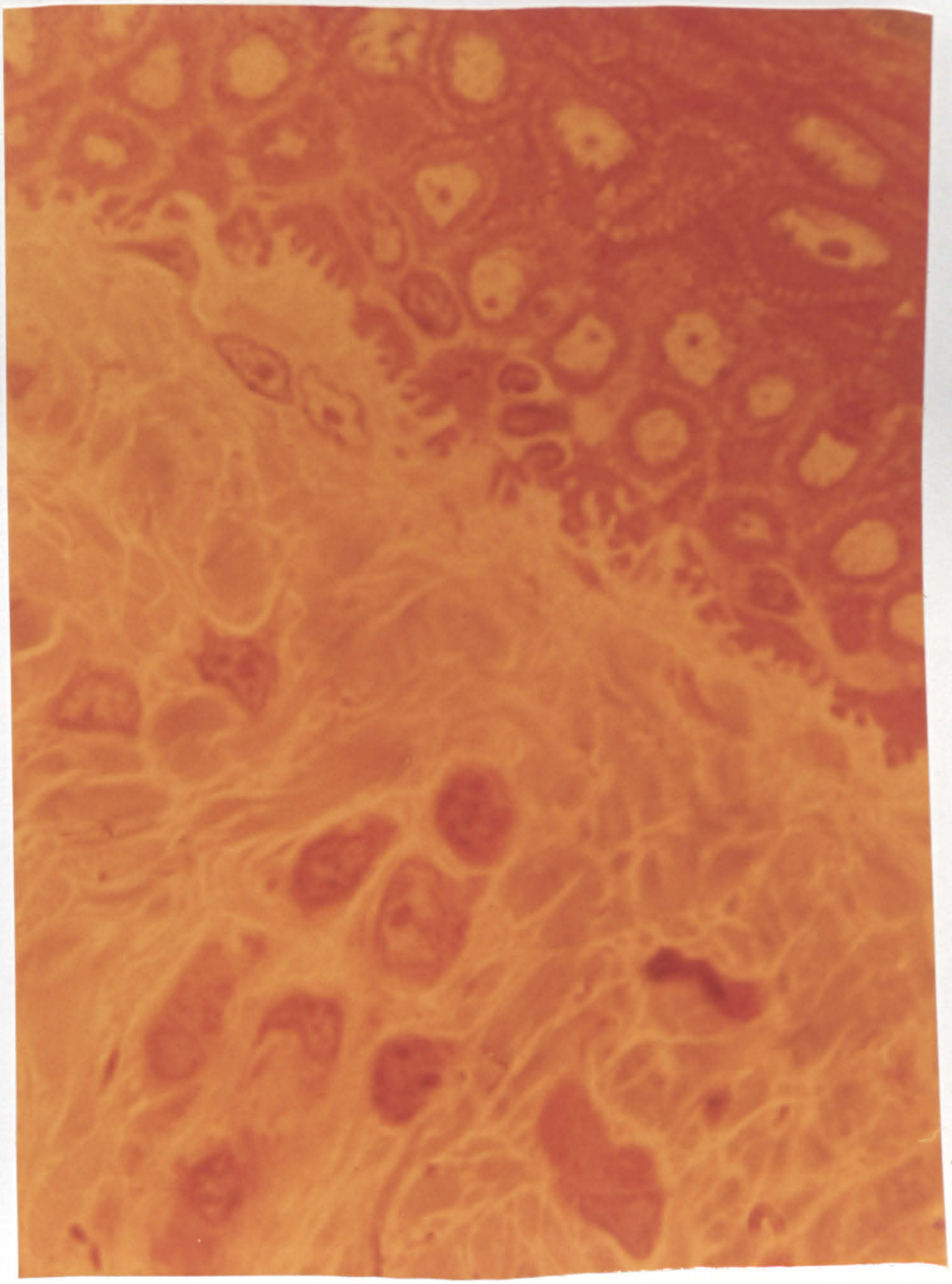


Fig 22. Light micrograph of a marginal area from a patient with vitiligo showing invasion of the epidermis by lymphocytes, similar cells being seen in the dermis. Epon-embedded tissue stained with basic fuchsin and methylene blue. (X 320)

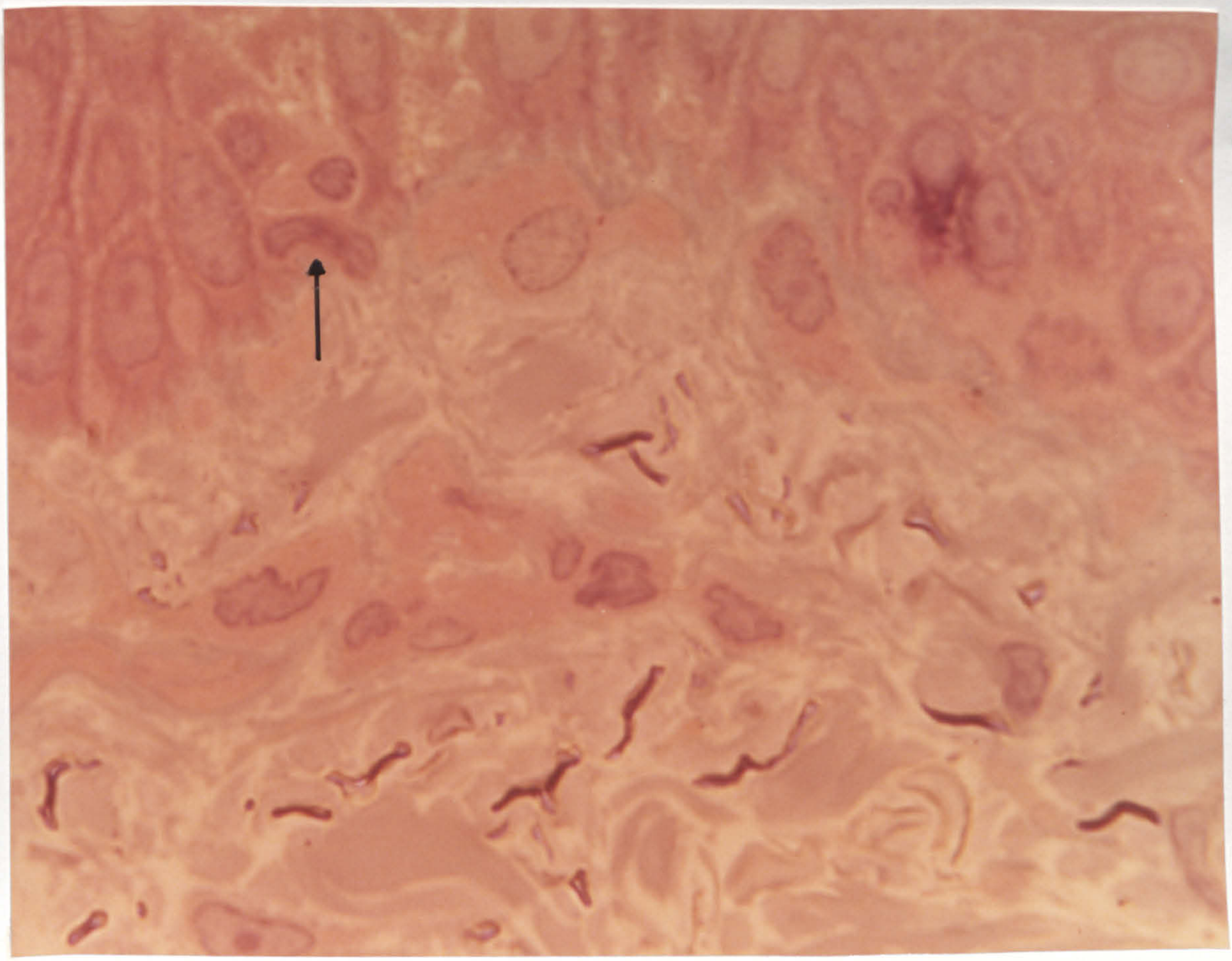


Fig 23. Light micrograph of an involved area from a patient with vitiligo showing a few lymphocytes in the epidermis. Similar cells are seen in the dermis. One of the lymphocytes with an elongated nucleus (arrow) is passing into the basal layer. Epon-embedded tissue, stained with basic fuchsin and methylene blue. (X 320)

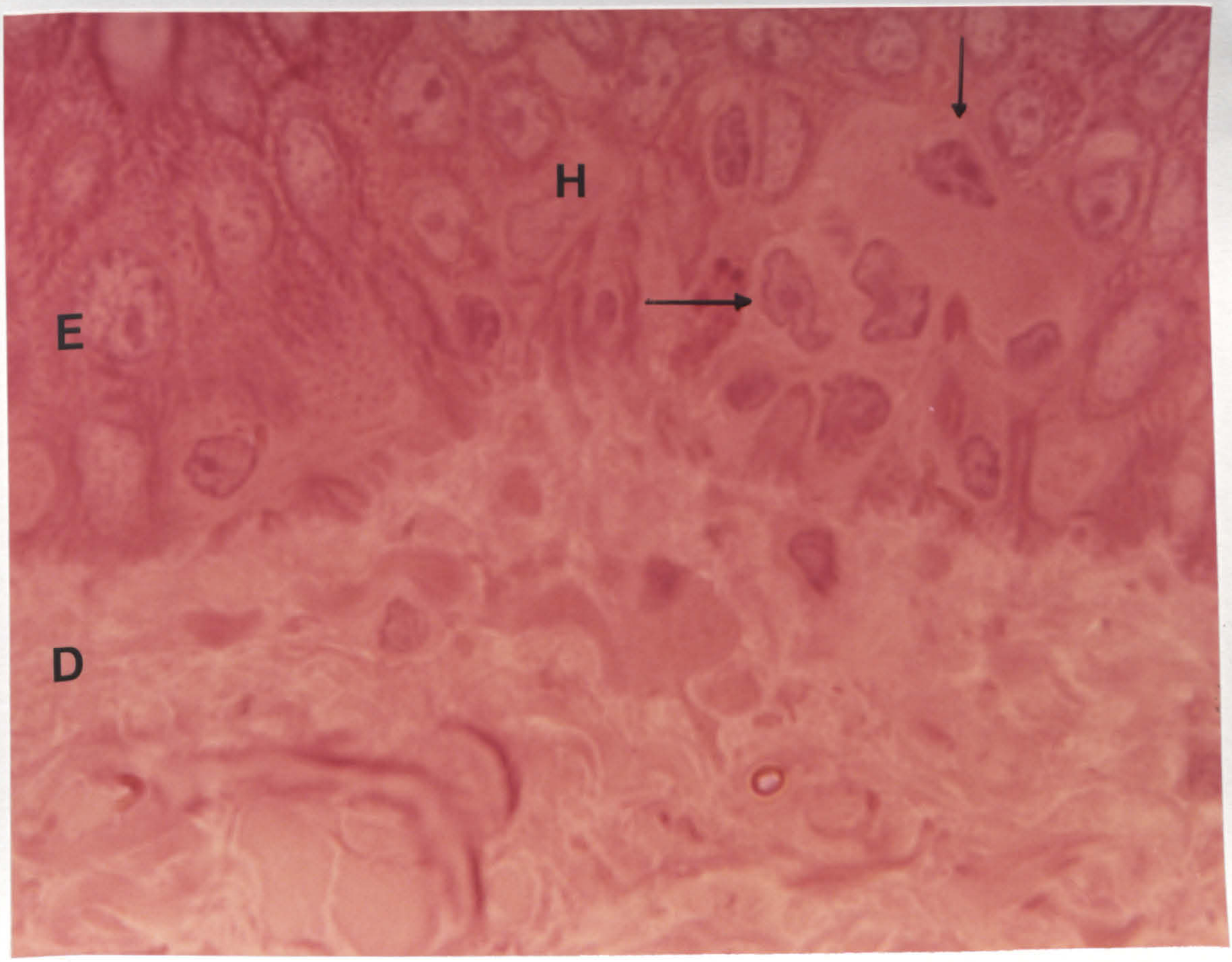


Fig 24. Light micrograph of a marginal area from a patient with vitiligo showing a focus of intra-epidermal lymphocytes (arrows) with a tendency to form Pautrier like micro-abscess. E Epidermis, D Dermis, H Histiocyte. Epon-embedded tissue stained with basic fuchsin and methylene blue. (X 320)

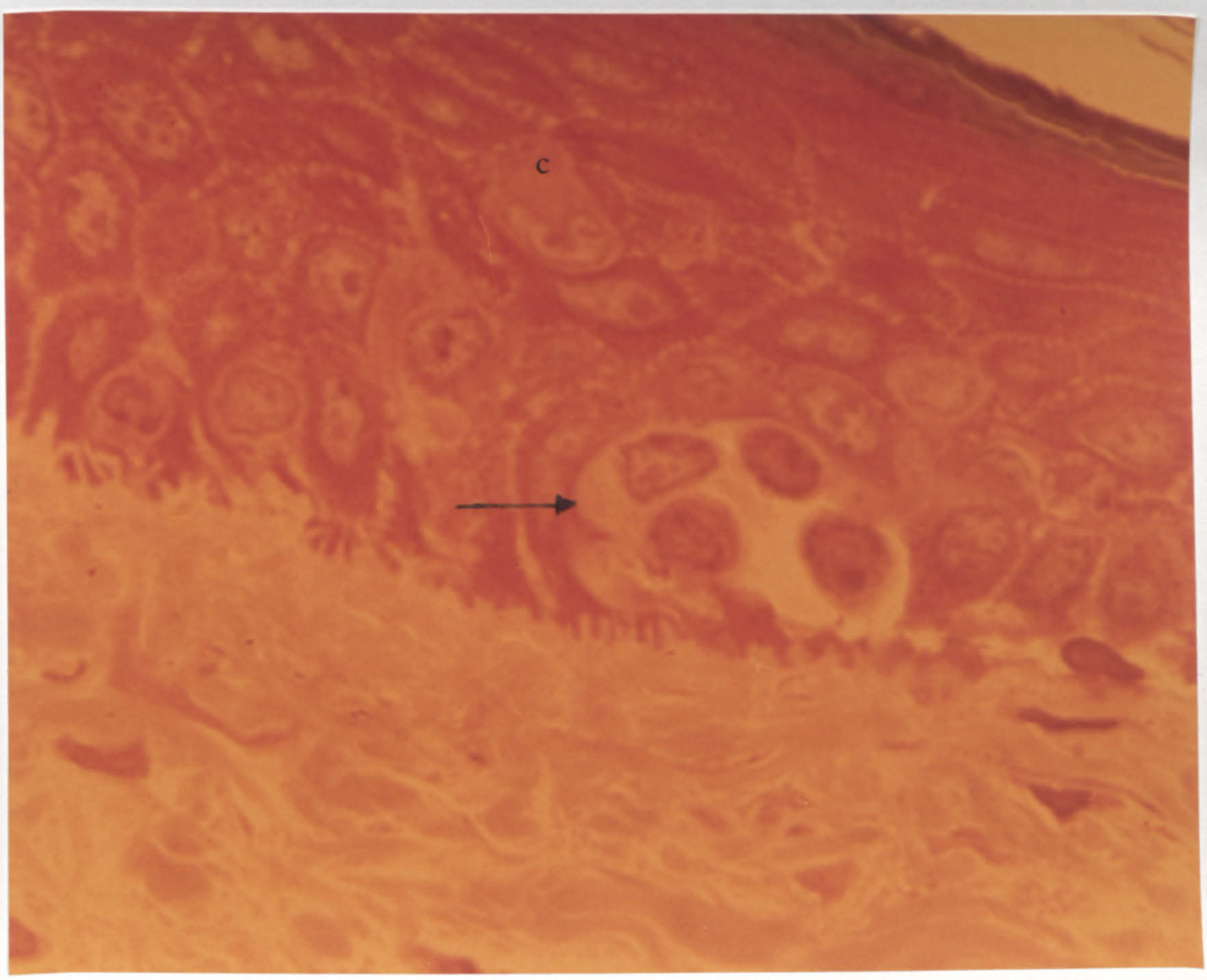


Fig 25. Light micrograph of a marginal area from a patient with vitiligo showing intra-epidermal focus of lymphocytes (arrows) forming a Pautrier-like micro-abscess. C Clear cells, which are either Langerhans cells or histiocytes. Epon-embedded tissue, stained with basic fuchsin and methylene blue. (X 320)

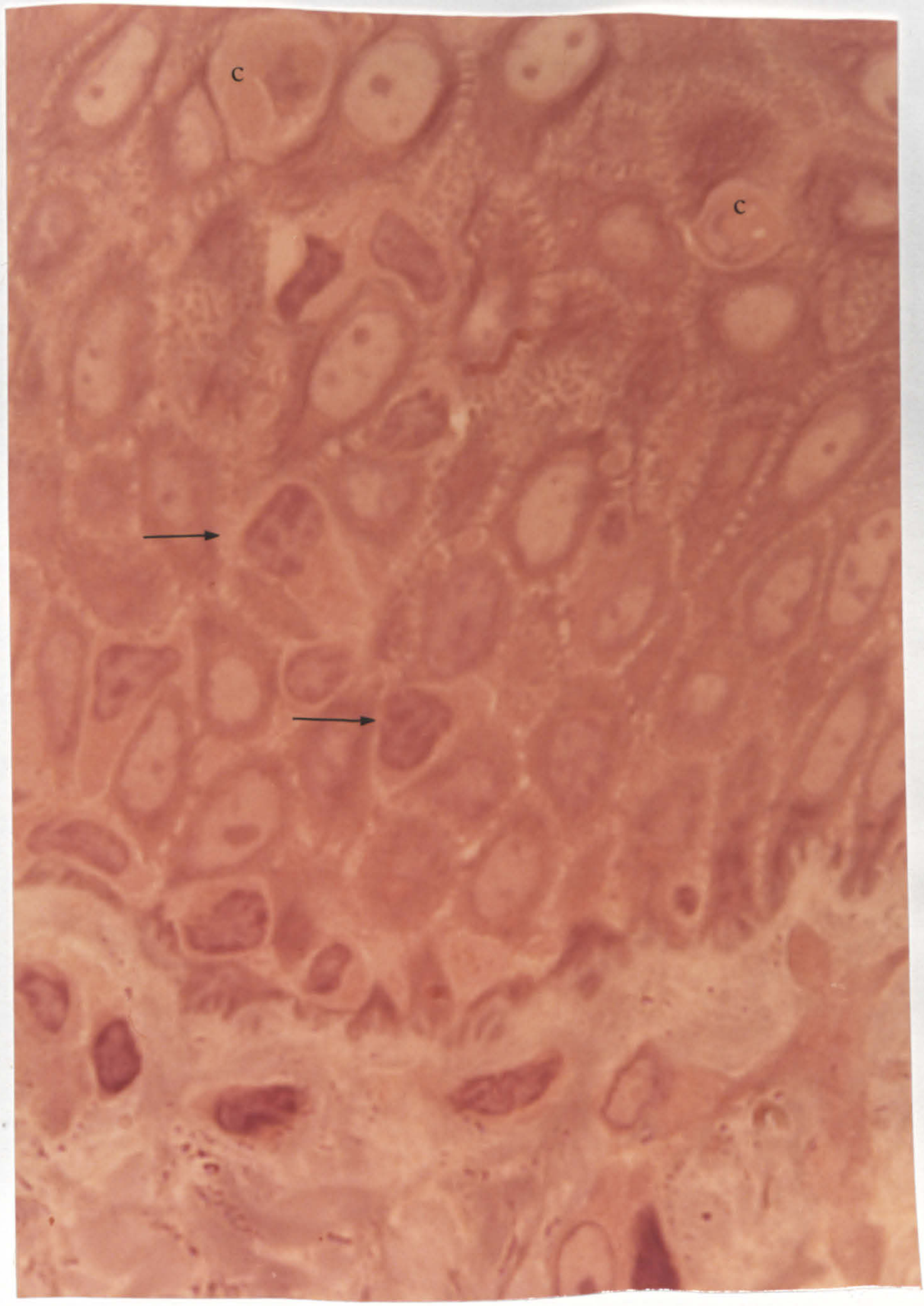


Fig 26. Light micrograph of a marginal area from a patient with vitiligo showing invasion of epidermis with lymphocytes (arrows). Similar cells are seen in the dermis. C clear cells which are either Langerhans cells or histiocytes. Epon-embedded tissue stained with basic fuchsin and methylene blue. (X 320)

junction zone and entering the epidermis.

The degree of the lymphocytic infiltration of the epidermis in many of the biopsies was not parallel with that seen in the dermis. In some specimens there were many lymphocytes in the epidermis, yet the papillary dermis contained only a few cells.

The total number of clear cells in the epidermis of the marginal areas that was expected to be reduced when compared with that from the uninvolved skin due to a reduced number of melanocytes, was in some specimens actually increased. This was attributed to the presence of a mononuclear cell infiltrate in the epidermis in these marginal areas (table XX, fig 18).

2. Dermis

(a) General Appearance

There was an increased cellularity of the dermis in many of the patients biopsies, particularly from the marginal and involved areas, and when compared with those from uninvolved skin and from the normal controls (table XXI). In many of the uninvolved areas in the patients with vitiligo, there was also an increased cellularity of the dermis. The differences between the mean of the total number of dermal cells for an area in the upper dermis in

No.	Name	Uninvolved n=22	Marginal n=29	Involved n=28	Cytoid bodies
1	DB	ND	1071.8	2410.7	Pos
2	RY	ND	1180.6	1437.4	Neg
3	SD	ND	768	1281	Neg
4	MO	1667	1804	1834	Neg
5	JTR	459	1084	924	Neg
6	CH	1413	1841	1343	Neg
7	JC	1271	1798	1504	Pos,U+I
8	JA	ND	1413	606	Neg
9	JT	ND	1782	1612	Pos,M
10	TM	ND	1182	940	Pos,M+I
11	EM	1733	985	524	Pos,M
12	JW	584	1146	401	Pos,U+I
13	LG	1456	2017	1537	Pos,U+I
14	BR	1016	1376	992	Neg
15	MF	1426	579	1478	Neg
16	DH	1104	754	874	Pos,M+I
17	AB	870	1219	780	Neg
18	EMA	1227	361	690	Neg
19	JR	ND	1101	634	Neg
20	PL	1380	584	ND	Neg
21	GG	863	2544	1104	Neg
22	SE	726	1178	1191	Neg
23	CL	1555	1870	1215	Neg
24	TB	790	1216	921	Pos,I
25	CE	1326	1723	906	Neg
26	JM	1251	571	626	Neg
27	JH	458	1225	1841	Neg
28	MB	1387	1706	547	Pos,U+M
29	MT	2003	1368	1873	Neg
Mean±SEM		1180±88.9	1291±93.7	1143±93.4	

Table XXI Showing the population density of dermal cell/mm^2 in areas of patients with vitiligo, also indicating the presence of cytoid bodies in the dermis of these patients.
M Marginal, I Involved, U Uninvolved

various sites was not of statistical significance ($P > 0.05$).

There were no obvious changes of the blood vessels in the skin though in some specimens from the marginal and from the involved areas there was some thickening of their wall (fig 27).

On microscopic examination of the plastic-embedded semi-thin sections stained with Huber method, it was difficult to be certain about the nerves and to comment on any differences.

(b) Histiocytes

These large phagocytic cells were distinguished by the presence of engulfed melanin granules. They were melanophages and were present in the uninvolved and marginal areas. In most of the biopsies from involved areas the histiocytes were devoid of melanosomes.

(c) Lymphocytes

Lymphocytes were frequently found in the dermis, particularly around blood vessels (fig 28). The cells had a big hyperchromatic nucleus with many deep invaginations, sometimes having a cerebriform (Sezary cell). In many, the cells had an elongated indented nucleus. Lymphocytes were seen wandering up into the epidermis, but as mentioned before, the degree of lymphocytic infiltration of the dermis did not parallel that of the epidermis.

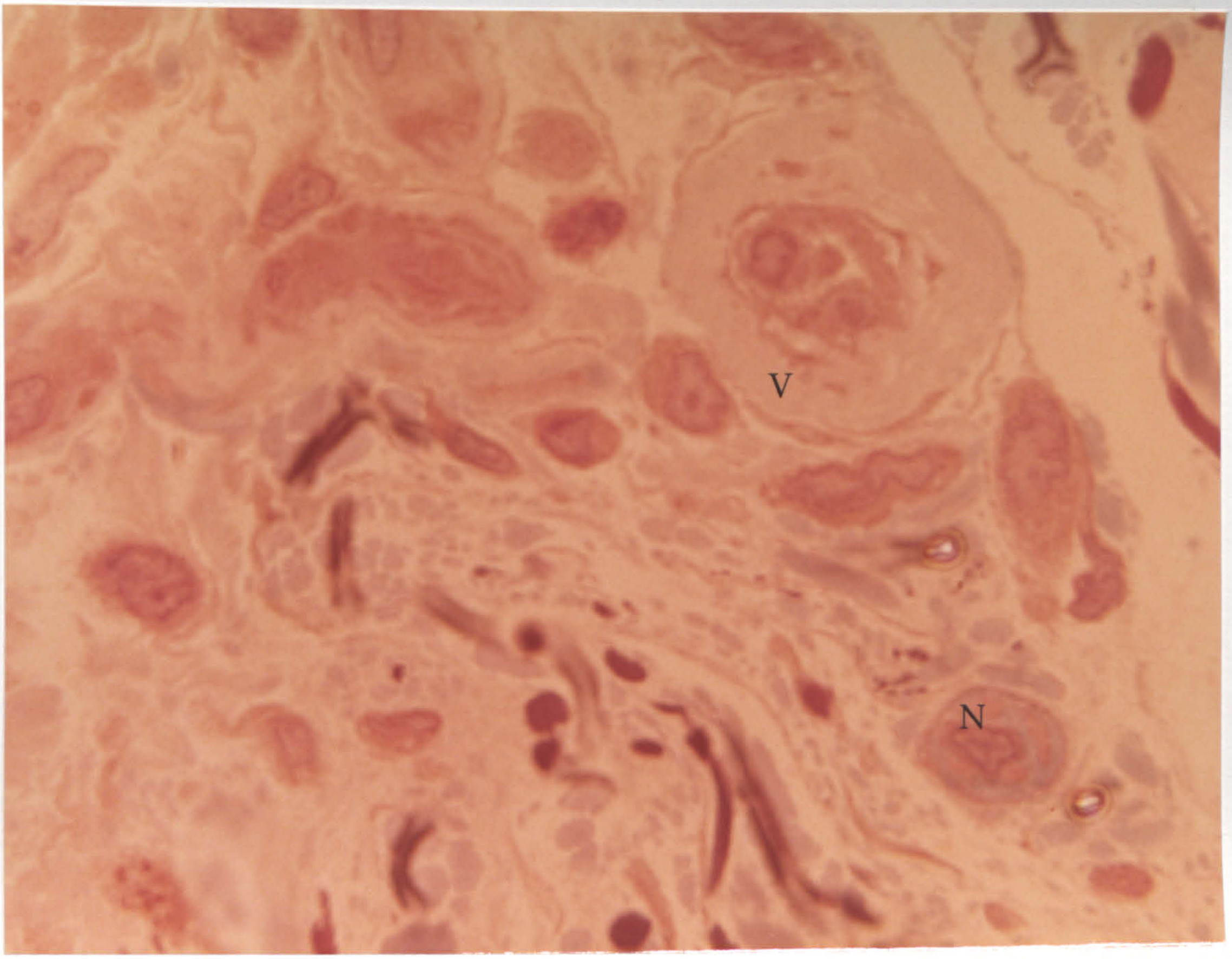


Fig 27. Light micrograph of a marginal area from a patient with vitiligo showing a dermal vessel (V) with thickened wall and mononuclear cells. N Nerve. Epon-embedded tissue, stained with basic fuchsin and methylene blue. (X 320)

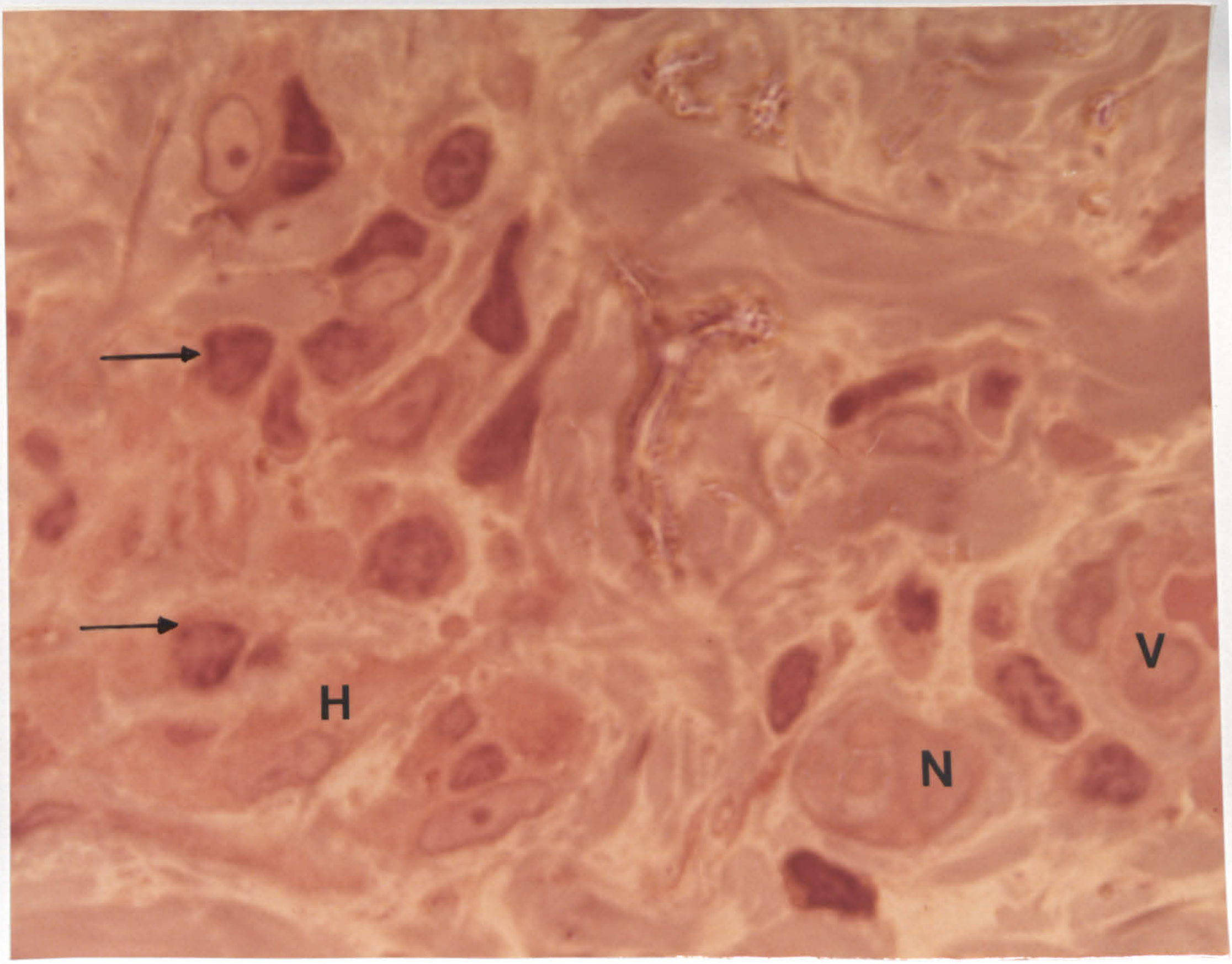


Fig 28. Light micrograph of a marginal area from a patient with vitiligo showing increased dermal lymphocytes (arrows). H histiocytes, N nerve, V blood vessel. Epon-embedded tissue stained with basic fuchsin and methylene blue.

(d) Mast Cells

Mast cells were found to be increased in the marginal and involved areas when compared to uninvolved skin and that of normal controls (table XXII, fig 29). In a few patients mast cells constituted the major number of the cells in the dermis. They were found mainly around the blood vessels and in the upper dermis. However, when the total mean number of mast cells in the different groups were compared with each other there was no statistical significance ($P>0.05$).

(e) Cytoid (Colloid/Amyloid) Bodies

These had been observed in the uninvolved, marginal and involved areas (fig 30, table XXI). They were seen mostly in the papillary dermis, some of them were seen in the basal layer of the epidermis. In some patients they were most frequent, particularly in the marginal and uninvolved skin, while in the involved areas they were very few. Although it was difficult to quantitate these bodies, they were much numerous in the vitiligo skin than in the normal controls.

No.	Name	Uninvolved n=22	Marginal n=28	Involved n=29
1	DB	ND	59.1	86
2	RY	ND	9.8	8.4
3	SD	ND	ND	33.8
4	MO	31.8	14.7	44.3
5	JTR	0	0	0
6	CH	51.8	73.9	0
7	JC	17.7	0	29.5
8	JA	ND	52.5	73.9
9	JT	ND	114.6	59.1
10	TM	ND	46.4	0
11	EM	39.4	26.2	9.8
12	JW	14.7	25.3	17.7
13	LG	44.3	0	16.9
14	BR	36.9	0	22.1
15	MF	0	0	14.7
16	DH	22.1	43	100.5
17	AB	29.5	19.7	0
18	EMA	3.5	0	0
19	JR	ND	23.6	12.6
20	PL	85.4	169	9.8
21	GG	7.3	0	8.4
22	SE	7.8	29.5	122.8
23	CL	14.7	36.9	110.9
24	TB	5.9	133.1	29.5
25	CE	0	0	65.7
26	JM	24.6	0	36.1
27	JH	0	23	22
28	MB	31.8	86.1	0
29	MT	29.5	44.3	29.5
Mean±SEM		22.64±4.4	36.81±8.2	33.24±6.6

Table XXII Showing the population density of dermal mast cells/mm^2 in areas of patients with vitiligo. ND not done.

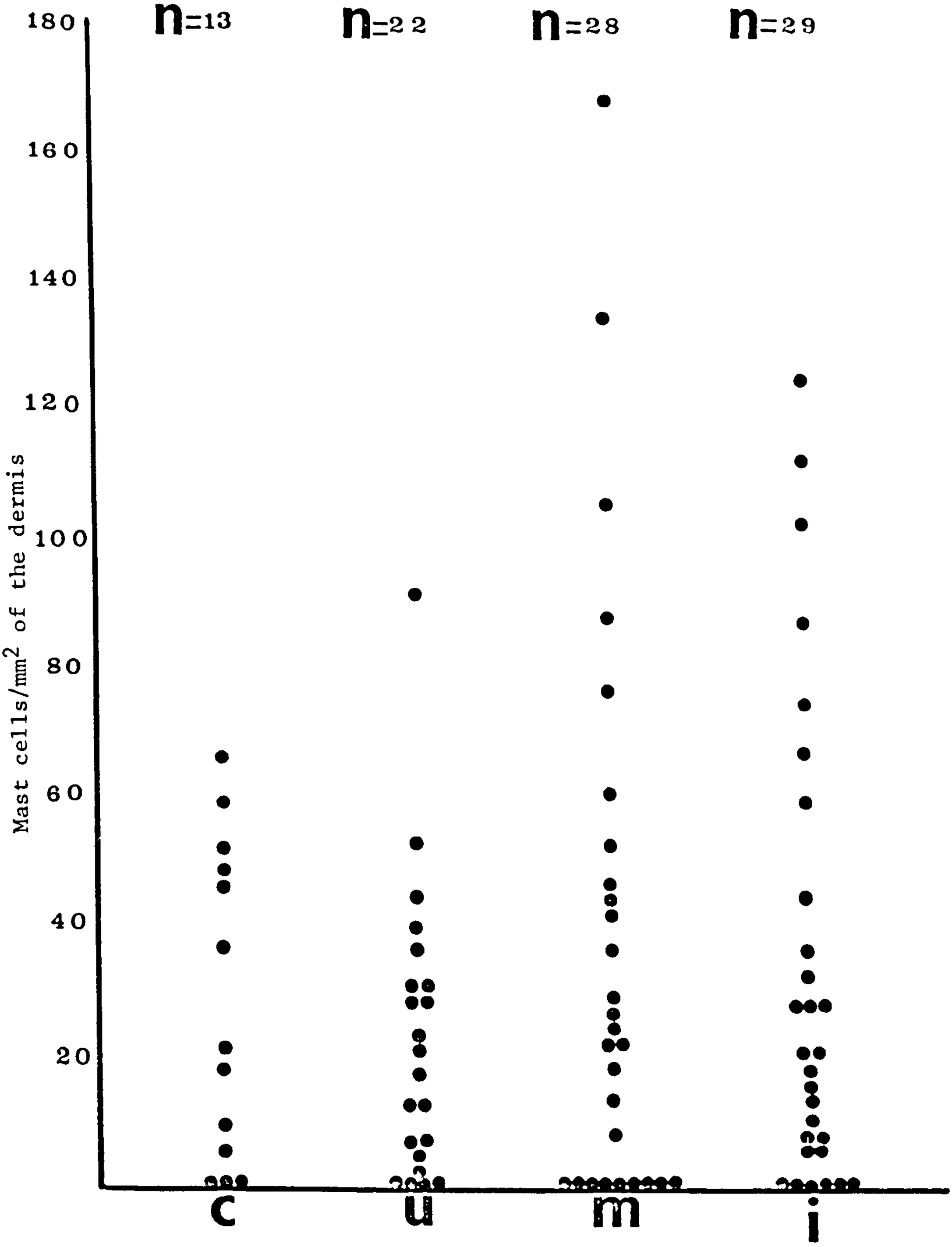


Fig 29 Comparison of dermal mast cells in normal controls (c) and uninvolved (u), marginal (m) and involved (i) areas of patients with vitiligo.

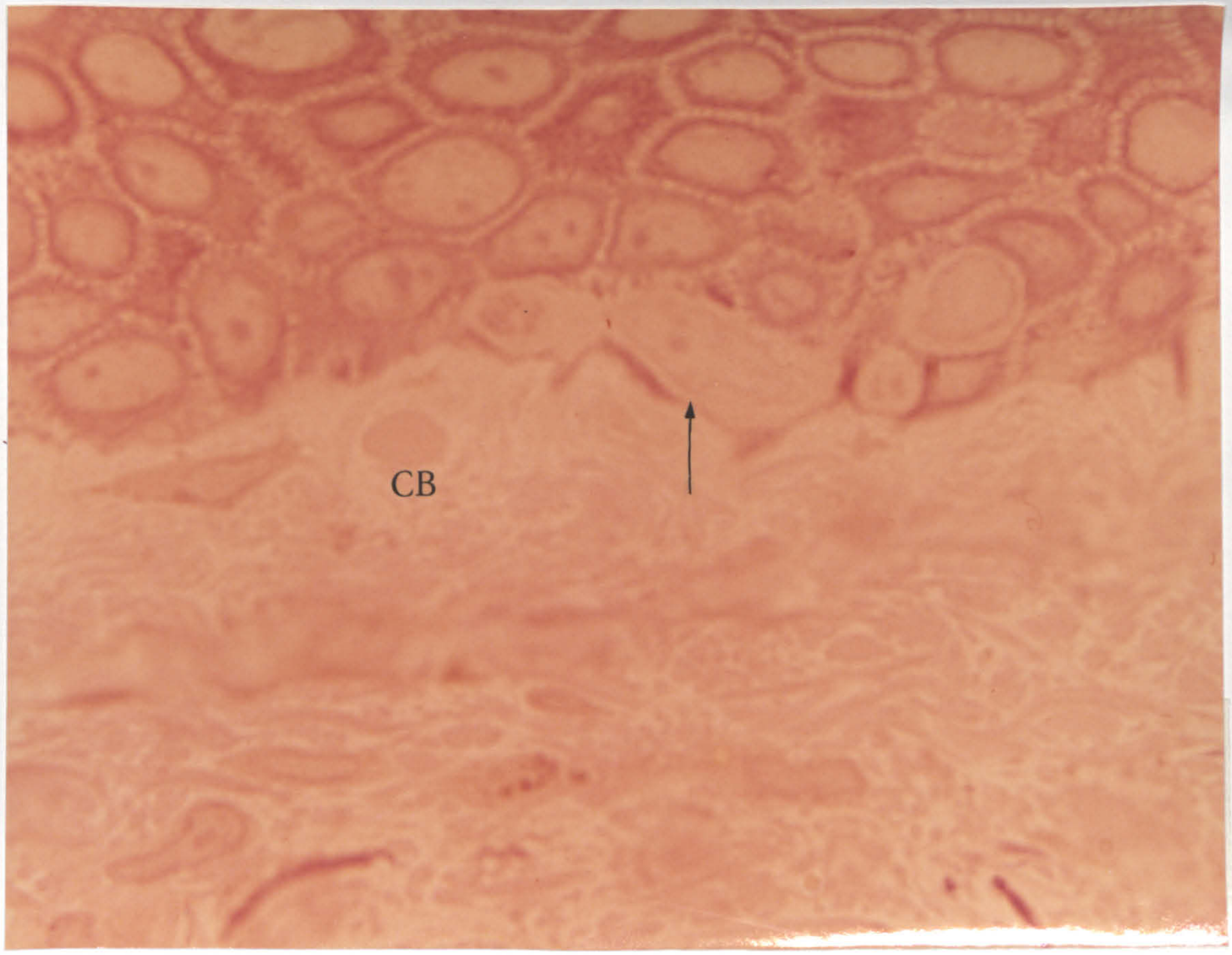


Fig 30. Light micrograph of an involved area from a patient with vitiligo showing a cytoid (colloid/amyloid) body (CB) in the papillary dermis. Many mononuclear cells (arrow) in the basal layer of the epidermis are also seen. Epon-embedded tissue stained with basic fuchsin and methylene blue. (X 320)

C. OCCUPATIONAL VITILIGO

The histopathological features in the biopsies of skin from the two patients with occupational vitiligo are shown (table XXIII, XXIV). The changes were similar to that found in common vitiligo.

There was some hyperkeratosis of the epidermis in the involved skin in both patients. This was very marked in one of them when compared with the epidermis from the uninvolved area. No melanosomes were seen in the keratinocytes from the involved areas of skin. The melanocytes were diminished in number in the marginal areas and appeared to be absent in the involved skin.

In one patient there was a marked increase in the cellularity of the dermis, mainly lymphocytes. Melanophages were also seen in the dermis in the marginal and uninvolved skin, but not in the involved amelanotic area.

Mast cells were also seen to be increased in number in one of the patients, both in the marginal and involved areas. Cytoid (colloid/amyloid) bodies were seen in one patient.

	Uninvolved Dermis		Marginal Dermis		Involved Dermis	
Name	Dermal cells	Mast cells	Dermal cells	Mast cells	Dermal cells	Mast cells
AG	1420	39.4	1400	32.8	ND	ND
AD	1319	23.6	2873	85.4	1408	72.3

Table XXIII Showing the population density of total dermal cells and dermal mast cells/mm^2 in areas of patients with occupational vitiligo. ND not done.

	Uninvolved			Marginal		Involved	
Name	BCC	TCC	LC	BCC	TCC	BCC	TCC(LC)
AG	27.6	36.1	8.5	18.8	27.3	4.7	5.3
AD	24.4	26.5	2.1	7.6	12.8	10	11.5

Table XXIV Showing the population density of epidermal clear cells/mm of epidermis in areas of patients with occupational vitiligo. BCC Basal clear cells, TCC total clear cells, LC Langerhans cells.

VI. ELECTRON MICROSCOPY

A. NORMAL CONTROLS

Biopsies were taken from nine controls. The mean age of these patients was twenty-nine, the age range nineteen to thirty-five. The majority of these biopsies were taken from the upperarm.

1. Epidermis

(a) General Appearance

The epidermis was recognised to be divided into four strata or layers.

Basal Layer

This was composed mainly of keratinocytes (fig 31) and whose principal function is that of division. It can be regarded as the stratum germinativum. Keratinocytes in this layer had an elongated shape, their nuclei were oval and were perpendicular to the plane of the basal surface of the epidermis. The nucleus contained one or more prominent nucleoli. In addition to the normal cytoplasmic organelles the cell also contained bundles of tonofilaments that were arranged mainly at the periphery of cytoplasm, leaving a clear perinuclear zone. The keratinocytes were attached to each other by complete desmosomes and to the basal lamina by hemi-desmosomes. In addition to the keratinocytes, melanocytes, Langerhans cells and occasionally Merkel cells were found in the basal layer. Melanosomes were to be frequently found in the basal keratino-

cytes and were most likely to be present at the upper pole of the nucleus forming what had been described as a supranuclear cap (fig 31). The melanosomes of Caucasoids are small and aggregated in groups of two or more within membrane-bound packages, while in negroes the melanosomes are larger and remain unaggregated in the cytoplasm.

Stratum Spinosum

This consisted of many layers of keratinocytes. They were polyhedral in shape in the deeper part, but become more flattened and elongated near to the granular layer. Desmosomes occurred at intervals along the cell membrane which was very folded with many villous like projections. Tonofilaments were more numerous than in the cells of the basal layer and were grouped together, particularly in relation to the desmosomes. They frequently enveloped the nucleus leaving a clear perinuclear zone. Membrane bounded melanosomes were present in the cytoplasm of these cells. Dendritic Langerhans cells were also found, but they mainly occurred in the upper portion of this layer.

Stratum Granulosum

This was composed of about two layers of cells. The cells were now more flattened than in the stratum spinosum cells and their nuclei were elongated with their long axis horizontal to the surface.

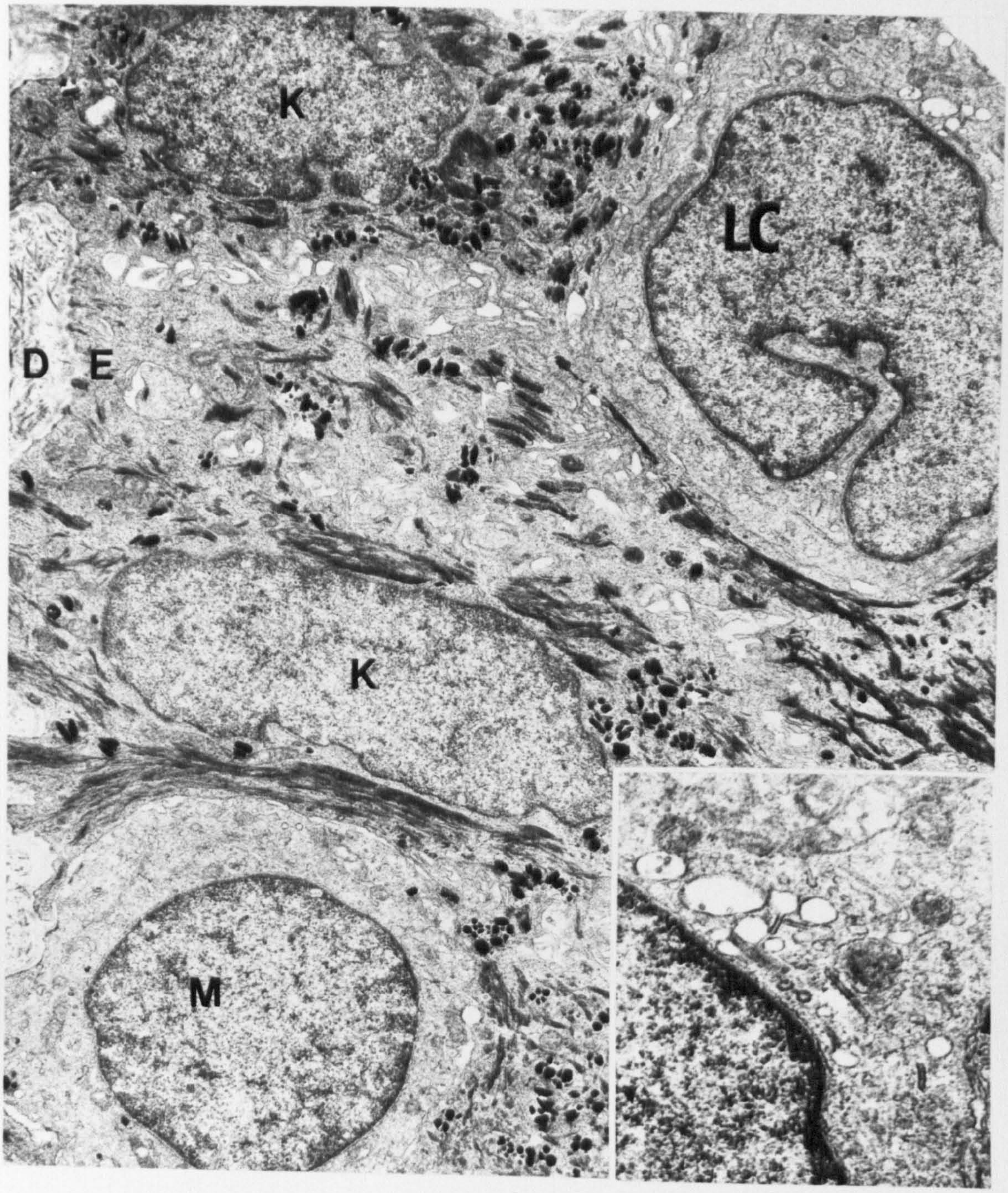


Fig **31** . Electron micrograph of a normal skin showing a relatively inactive melanocyte (M) and suprabasal Langerhans cell (LC). The keratinocytes (K) have a lot of melanosomes. D Dermis, E Epidermis. Inset showing a higher magnification of Langerhans granules. (X 25000)

The cytoplasm contained few organelles. Two characteristic granules were present; keratohyalin granules appearing as amorphous and highly electron dense bodies. They were irregular in shape and vary in size and seem to intermingle with tonofilaments. Not infrequently the tonofilaments seemed to be condensing together to form keratohyalin granules that measure 100-200nm. Lamellar or Odland bodies were much smaller granules that were ovoid or round in shape and were seen in close proximity to infolding of the cell membrane. On higher magnification the lamellar structure of these granules was apparent. The parallel dense lamellae were separated from each other by more translucent zones.

The keratinocytes in this layer were still attached to each other by desmosomes. In Caucasians only a few melanosomes were seen in the keratinocytes, but in more pigmented races well preserved melanosomes were frequently found high up in the epidermis.

Stratum Corneum

This comprised of variable number of flattened keratinised cells that were piled on top of each other. The number of layers of cells varied according to different sites. There was a sudden change from granular cells to fully keratinised cells. The electron density of the cells varied. The intercellular space contained granular or reticular

material. There were intercelluar dense areas which represented the remains of desmosomes. The cytoplasm of these cells was usually free of any organelles apart from dense aggregates of filaments and occasionally a few melanosomes.

Desmosomes

These were specialised connections between the keratinocytes. Desmosomes were primarily composed of the plasma membranes of two adjacent cells. They are made up of a series of dense and lucent zones. On the external surface of the plasma membrane there was a row of electron dense granules. In the middle of the space between the two plasma membranes there was also a row of electron dense granules. There was also an electron dense thickening on the inner surface of each plasma membrane, forming the attachment plaque of the desmosomes. This attachment plaque was separated from plasma membrane by lucent areas.

The size of desmosomes varied, and they were either short or very long depending on the plane that they were cut. The distribution also varied. It was not infrequent to see collections of desmosomes between the keratinocytes and, in particular, near the Langerhans cells. Bundles of tonofilaments frequently appeared to stream onto and terminate upon the attachment plaque of desmosomes.

(b) Clear Cells

The clear cells to be found in the normal

epidermis were melanocytes, Langerhans cells and the occasional Merkel cell.

Melanocytes

These pigment-producing cells were usually confined to the basal layer (fig 31), where the incidence of melanocytes in relation to keratinocytes was variable. Melanocytes were usually basal in their position, but occasionally were just suprabasal as a result of the sectioning. Effete melanocytes were not seen higher up in the epidermis. The majority of the melanocytes had a direct relationship with the dermo-epidermal junction and to the basal lamina. Frequently, melanocytes had a pendulous appearance dipping into the papillary dermis but separated from it by the basal lamina. The dendritic nature of melanocytes was rarely fully apparent due to sectioning, but dendritic processes were present interspersed among the neighbouring keratinocytes.

The melanocytes were readily distinguishable from surrounding keratinocytes by a more translucent cytoplasm and there were no tonofilaments. Fine cytofilaments could be seen within the cytoplasm of most melanocytes. The cytofilaments had a diameter that ranged from 80-120Å, but the majority were about 100Å. These were very well developed in the more inactive cells and formed bundles and

whorls. Desmosomes were not seen between melanocytes and the neighbouring cells. Hemi-desmosomes were not found, though in some cells thickening of their plasma membrane forming attachment like structures may be seen along the dermo-epidermal junction.

The most characteristic feature of the melanocytes was the presence of specific cytoplasmic granules, the melanosomes. These pigmented granules were present in the cytoplasm and four formative stages are recognised. Stage I were vesicles, with no apparent internal structure, that were located usually near the Golgi system. Stage II contained coiled filaments in a smooth-walled vesicle, but with no significant deposition of melanin. Stage III melanosomes were recognised by deposition of melanin which partially obscured their internal structure. The melanofilaments exhibited a periodicity of particles along their length of 60-80Å. Stage IV melanosomes, the internal structure was completely obscured by the deposition of melanin. This fully melanised melanosome had an ellipsoidal shape with a mean transverse diameter of 0.1μm. In the relatively inactive melanocytes, these melanosomes were mainly seen in portions of the dendrites with very few in the cytoplasm of the main cell body. However, in the active cells melanosomes were mainly

seen around the nucleus and in relation to the Golgi system. Collections of melanosomes without an outer limiting membrane were seen in the cytoplasm of several melanocytes in few of the biopsies (fig 37). The distribution of melanosomes in the cytoplasm of keratinocytes was mainly in complexes, where two or more melanosomes were grouped together within a limiting membrane. In few biopsies the melanosomes were distributed both singly and in complexes. In general, the larger the melanosome the more the tendency towards single dispersion.

Langerhans Cells

These dendritic cells were present mainly in the suprabasal layer of the epidermis (fig 31), but were also found in the basal layer. They could be distinguished from the keratinocytes by their clear appearance and the lack of desmosomes and tonofilaments. Specific organelles (Birbeck granules) were present within the cytoplasm of the cell. The nucleus of the cell was usually lobate and indented, sometimes it was highly convoluted. The heterochromatin was condensed at the periphery of the nucleus. In addition the cytoplasm contained many lysosomes. Langerhans cells found high up in the epidermis might contain only a few rod or racquet-shaped Langerhans cell granules. Microfilaments were not

so well developed in these cells although sometimes they could be as numerous as found in the melanocytes.

The Langerhans cell granule was considered to be the specific marker for this cell. These organelles were frequently arranged near the Golgi but also could be found in relation to nuclear and cell membranes. Sometimes there were aggregates of rod-shaped granules that were arranged in stacks parallel to each other. The characteristic morphology of the Langerhans cell granule was that of tennis racquet with an expanded vacuolated end and a lower rod-like handle of variable length. In the central part of this handle, there was a linear electron-density with periodicity of particles along its length of about 90Å. Sometimes a paracrystaline net or lattice again with periodicity of 90Å was also seen. Three dimensional reconstructions of these granules indicated that they were basically discoid bodies with vesicular expansion at the margin of the disc. These granules could sometimes be found budding off from the nuclear membrane and they occasionally were on the outer limiting cell membrane.

Lysosomal structures were frequently found within these cells, many of them being spherical membrane bounded bodies with amorphous or granular

contents. Lamellated lysosomal bodies were also found as well as vacuoles. Rarely membrane bounded melanosomal complexes could be found within these cells.

Indeterminate Cells

These were dendritic non-keratinocytic cells that closely resemble the Langerhans cell and the melanocyte. The cytoplasm of these cells appeared clear and they lack desmosomes and tonofilaments. Their morphology resembled that of melanocytes and Langerhans cells, but they lacked the characteristic organelles for these cells. Occasionally, in serial sections, Langerhans cell granules were to be found and these cells were then identified as Langerhans cells. Most of these cells were to be found either in the basal layer or just above this zone in the epidermis. Not infrequently, these cells had the same 100Å microfilaments as was seen in the melanocytes. However, even on serial sections melanosomes could not be identified within the cells.

Indeterminate cells appeared to be rather infrequent in the epidermis of the skin from normal controls.

Merkel Cells

These were also non-keratinocyte cells and were present in the basal layer of the epidermis (fig 32). Their cytoplasm was clear and they contained characteris-

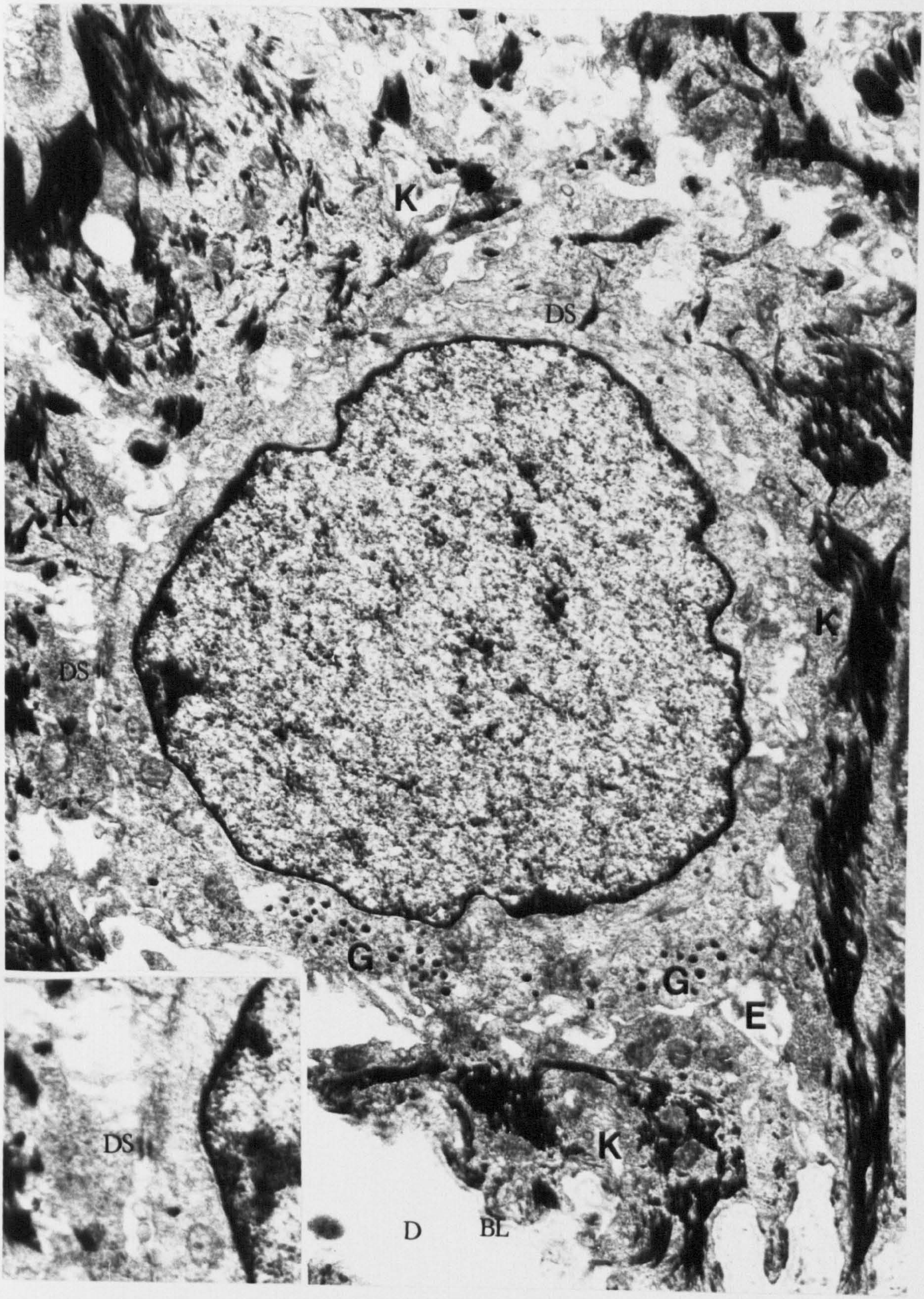


Fig **32** Electron micrograph of an involved area from a patient with vitiligo showing a basal Merkel cell with its two characteristic features; the electron dense granules (G) and the desmosomes (DS). Inset showing desmosome between the keratinocyte and Merkel cell. E epidermis, K keratinocytes, D dermis, BL basal lamina. (X **19971**)

tic round electron-dense granules measuring 60-110nm in transverse diameter. These granules were membrane bounded and were most frequently to be found on the opposite side of the nucleus to the Golgi. A further feature of these cells was the presence of desmosomes on their outer limiting membrane. The cytoplasm also contained micro-filaments. One of these cells was found in the involved skin of a vitiligo patient. However, they were not found in the biopsies taken from normal controls.

2. Dermis

This was divided into papillary and reticular portions and consisted of bundles of collagen fibres and ground substance. In addition to the collagen fibres there were elastic and reticulin fibres. Cells to be found in the dermis were fibroblasts, histio-cytes, occasional lymphocytes, mast cells as well as those of the nerves and blood vessels.

(a) Dermo-epidermal Junction

This was the boundary between the epidermis and dermis and was composed of; the basal cell plasma membrane with hemi-desmosomes; the lamina lucida; the lamina densa (basal lamina); the sub-basal lamina and its fibrous elements. Anchoring fibrils, with an irregular banded pattern, were

present in the papillary dermis and extended to the lamina densa. There were also special fine filaments that transversed the lamina lucida perpendicullarly to the outer surface of the plasma membrane of the basal cells into the lamina densa. These fine filaments were mainly seen in the region of the hemidesmosomes.

(b) The Collagen and Elastic Fibres

Collagen fibres were to be found throughout the dermis and they were mainly arranged in the form of bundles, particularly in the deeper part of the dermis. On higher magnification collagen fibres showed a striation with a repeated banded pattern along the length of the fibre measuring 550-640Å.

Elastic fibres were also seen between the collagen bundles, recognised by being more electron dense. They consist of an amorphous very electron dense part that was admixed with microfibrils.

(c) Dermal Cells

Fibroblasts

These were elongated or spindle-shaped cells that had an oval nucleus. The cytoplasm of these cells was bulky and they had well developed rough endoplasmic reticulum. These cells were found throughout the dermis and were more active in the papillary dermis.

Histiocytes (Macrophages)

These were again large cells that had a rather indented nucleus and sometimes this might be very lobate. In addition to normal organelles, the cytoplasm contained membrane bounded electron dense bodies that were lysosomes. In the inflammatory disorders the macrophages lying in the papillary dermis frequently contained melanosomal complexes. These melanophages were particularly to be seen in those with racially pigmented skins.

Mast Cells

They were to be found in the dermis and the population density of these cells varied considerably. Most of these cells were normally to be found in relation to blood vessels. The size of these cells was very variable and they were easily recognised by the characteristic granules and by the villous , like processes of the outer cell membrane. The mast cell granules were usually spherical in shape and often had very electron dense portion with less dense granular peripheral zone. Some of the granules showed lamellated internal structure, some of these in the form of scrolls. Some of mast cells were packed with granules, others might contain very few. Nuclei of these cells were usually central in location and occasionally binucleate cells could be seen. Mast cells were more common in the papillary dermis

but could be found in the deeper portion of the dermis and also in the subcutaneous tissue.

Lymphocytes and Plasma Cells

Also to be found, particularly around blood vessels, were lymphocytes and occasionally plasma cells. Plasma cells were recognised by highly developed rough endoplasmic reticulum and also very prominent Golgi.

(d) Cutaneous Nerves

The nerves most commonly found in the papillary and reticular dermis were unmyelinated, but one also finds myelinated ones occasionally. The nerves were composed of a number of axons that were enveloped by process of the Schwann cell. The Schwann cell and processes were surrounded by a well defined basal lamina. The axons seen in the reticular dermis were embedded in the endoneurium collagen. The endoneurium was enclosed by perineurium that consisted of concentric layers of flattened cells, similar to fibroblasts, but surrounded by basal lamina. The axons seen in the superficial dermis and in particular in the papillary part were lacking in endoneurium and perineurium. These nerves in the papillary dermis usually run parallel with dermo-epidermal junction and were only very rarely vertical in their arrangement.

The nucleus of the Schwann cell was usually indented and the cytoplasm of the cell, in addition

to having the normal organelles, had as well many microfilaments, lysosomes and occasionally melanosomes were to be found. The cells could be distinguished from other dermal cells by the presence of axons and surrounding basal lamina. The axons associated with the cell and its processes were usually completely infolded, although occasionally might be partly exposed. Each axon was composed of axoplasm that was surrounded by its plasma membrane. It contained mitochondria and well developed neurotubules 23nm in diameter. The neurotubules were usually running along the long axis of the axon and only a few obliquely. The axons also contained few neurofilaments. At the nerve terminals near the dermo-epidermal junction zone the axon was occasionally seen swollen like a button that was full of mitochondria and a few electron dense cored granules. Unmyelinated nerves were seen containing either large (105nm in diameter) and small (60nm in diameter) dense core vesicles of adrenergic type or small agranular vesicles of cholinergic nature. Also larger electron dense bodies could be seen in the axons, some of them resembling dense mitochondria. A number of myelinated axons that occasionally were present in the dermis, each being surrounded by a myelin sheath that consisted of concentric lamellae. It was a regular sequence of electron dense and less

dense lines in these lamallae.

(e) Blood Vessels

Most of the vessels seen in the papillary dermis were small capillaries. These were cut in various cross-sections and consisted of a small lumen with large endothelial cells that formed the lining of the vessel. Surrounding the capillaries were peri-endothelial cells. The endothelial cells had large prominent nuclei and in addition to having well developed cytofilaments also had electron dense bodies (Weibel - Palade bodies). A type of junction was present, points of contact between the adjacent outer limiting plasma membranes of the endothelial cells. The peri-endothelial cells (pericytes) were closely related to the capillaries and were surrounded by the basal lamina that enveloped the capillary. Replication of the basal lamina surrounding the blood vessels in the superficial dermis was not infrequently seen, particularly in the sun exposed area of the skin.

(f) Filamentous Colloid/Amyloid Bodies

Sometimes these were seen in the normal skin and were to be found in the papillary dermis near the dermo-epidermal junction zones. These globular bodies consisted of whorls and rays of unbranched filaments of varying cross-sectional diameter. They were only found in one of the normal controls.

B. COMMON VITILIGO

1. Epidermis

a) General Appearance

Spongiosis of the epidermis was noted in almost 50% of biopsies from marginal and involved areas of skin and was usually more common and apparent in the marginal sites (fig 33). This spongiosis was mainly in the lower part of the epidermis with oedema between keratinocytes and adjacent clear cells. There was widening of the intercellular spaces with preservation of cellular attachments. Villous-like processes were found between the keratinocytes and some of the nuclei of the cells appeared pyknotic with a prominent heterochromatin pattern. The tonofilaments were not infrequently aggregated together forming clumps. Occasionally, there was complete disruption of the basal part of the epidermis with degeneration of keratinocytes leaving debris between the cells (fig 55). The spongiosis was not infrequently associated with a mononuclear cell infiltrate of epidermis.

(b) Melanocytes

Uninvolved areas: These were present usually in the basal layer of the epidermis, but also occasionally just suprabasally. The nucleus was usually round or oval in shape (fig 34), but not infrequently lobate with a convoluted nucleus and

dense chromatin around its margin. Ellipsoidal melanosomes, mostly stage III and IV, were found with few premelanosomes, stages I and II. Inactive melanocytes were present containing very few melanosomes, but with prominent cytofilaments 100Å in diameter. In many instances the melanocytes were pendulous, dipping into the dermis, but always lying above and in relation to the basal lamina. Occasionally melanocytes were found apparently in the dermis but still enclosed within a sheath of basal lamina. In some areas melanocytes were found that showed degenerative changes. The nuclei of these cells were pyknotic with a prominent heterochromatin pattern (fig 36). The cytoplasm was vacuolated and contained auto-phagosomes. This was particularly observed in a spongiotic epidermis. In some sections there was marked reduction or even complete absence of melanocytes and melanosomes, but on further sectioning of the same block of tissue, melanocytes were found. This could represent minor foci of vitiligo in the 'uninvolved' skin. In one of these areas an intra-epidermal adrenergic polyaxonal nerve was seen in direct contact with a secretory melanocyte (fig 72).

Marginal: There was a reduction in the population density of melanocytes. The melanocytes were larger than those found in the uninvolved skin, often

with many dendrites that were full of melanosomes and were to be seen between the keratinocytes. Inactive melanocytes (fig 35) were also seen containing only a few melanosomes and with prominent arrays of cytofilaments. In many areas there was an apparent complete loss of melanocytes. However, portions of dendrites that could be seen contained melanised melanosomes. It was in these areas that the keratinocytes still contained melanosomes. Large collections of melanosomes, without a definite limiting membrane, had been seen in the cytoplasm of melanocytes in some biopsies (fig 37).

In areas where there were spongiosis of the epidermis the melanocytes were involved and appeared to be separated from the adjacent keratinocytes by the intercellular oedema. Even in the absence of spongiosis the melanocytes showed evidence of degeneration with pyknotic-like changes of the nucleus with a prominent heterochromatin pattern and vacuolation of the cytoplasm (fig 36). In the epidermis of these marginal areas, lymphocytes were seen to be in contact with secretory melanocytes (fig 38,39,40).

Involved areas: A complete absence of melanocytes was almost universally found in the biopsies from these areas. Only a few residual melanocytes were found though some of these cells were highly active and contained many melanised melanosomes.

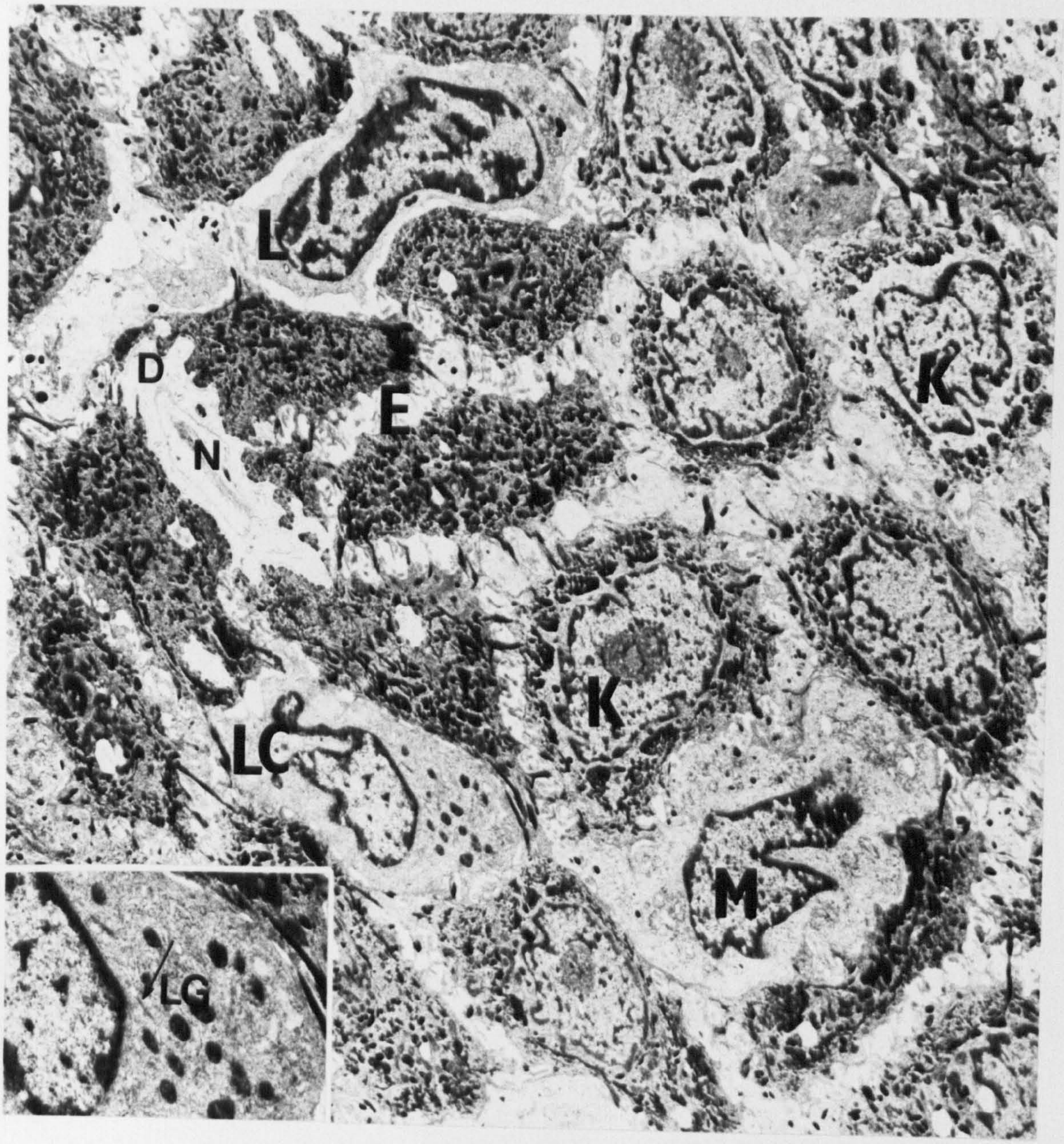


Fig **33** . Electron micrograph of a marginal area showing spongiosis of the epidermis (E) with oedema of the dermis (D). N nerve affected by the oedema (see Fig. **71**), M Melanocyte, L Lymphocyte, LC Langerhans cell, with its granule (LG) shown in the inset, K Keratinocyte. (X 6250)

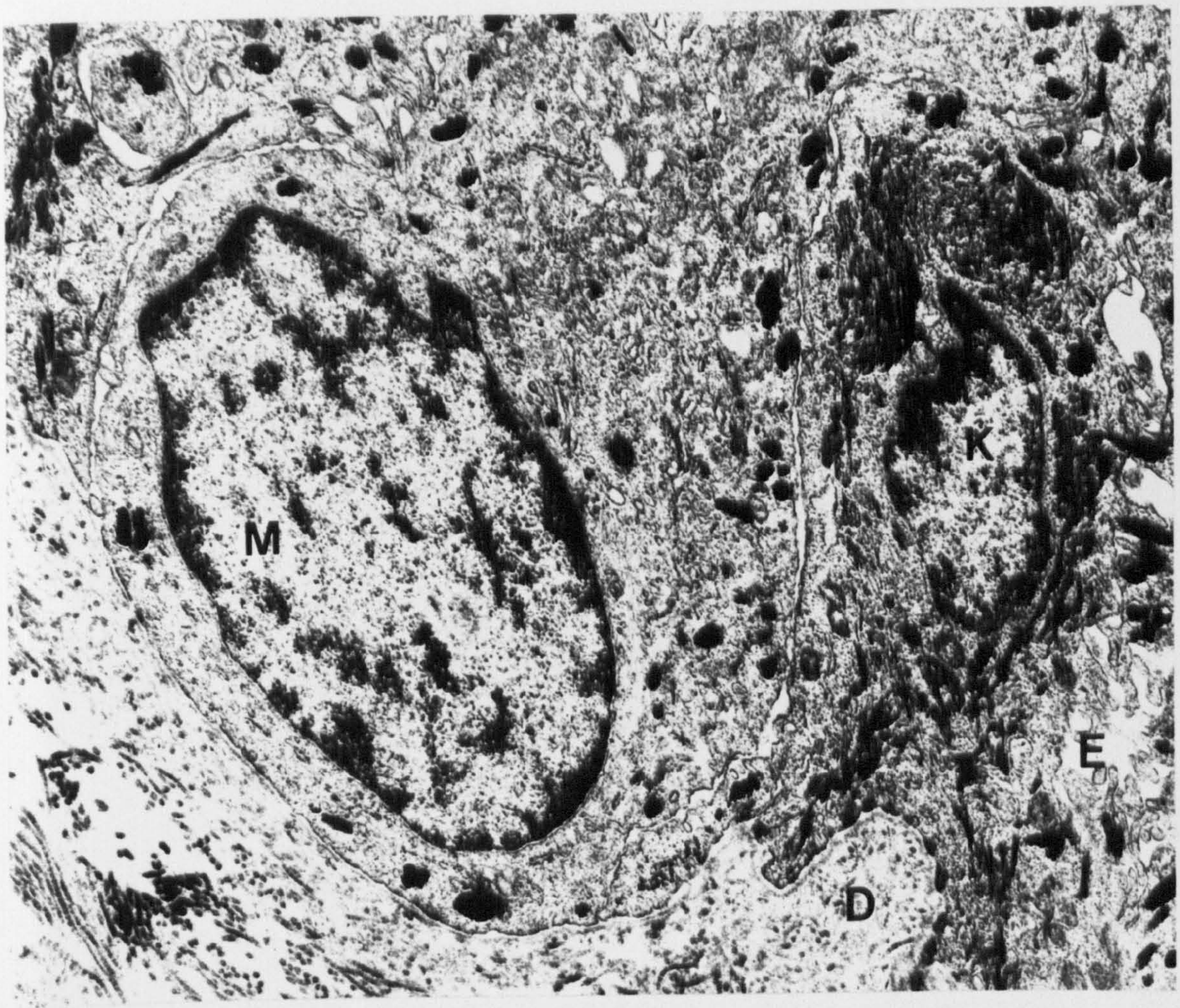


Fig **34** . Electron micrograph of a normal functioning basal melanocyte (M). D Dermis, E Epidermis, K Keratinocyte. (X 14175)

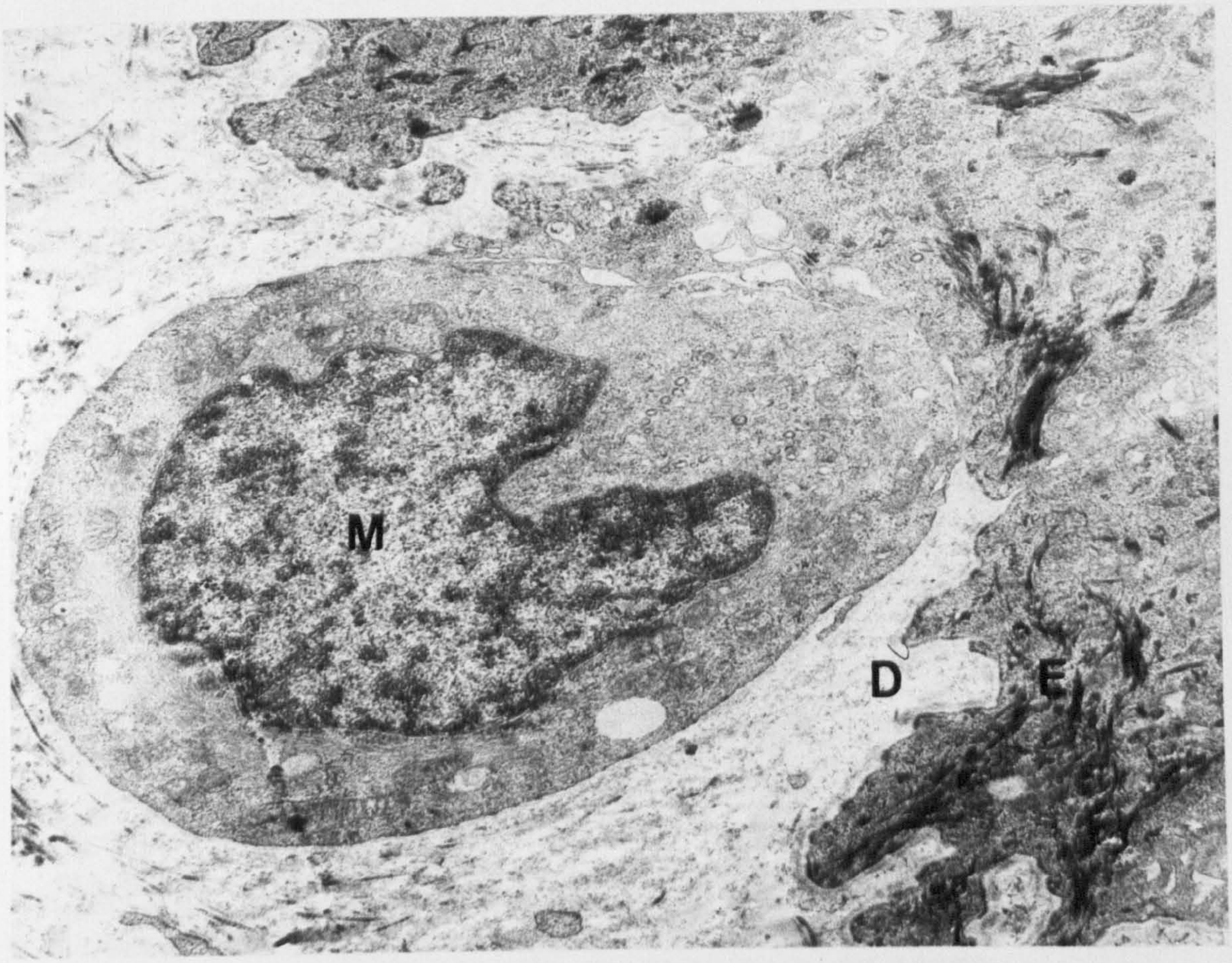


Fig **35** . Electron micrograph from a marginal area showing inactive pendulous melanocyte (M). D Dermis, E Epidermis. (X 15120)

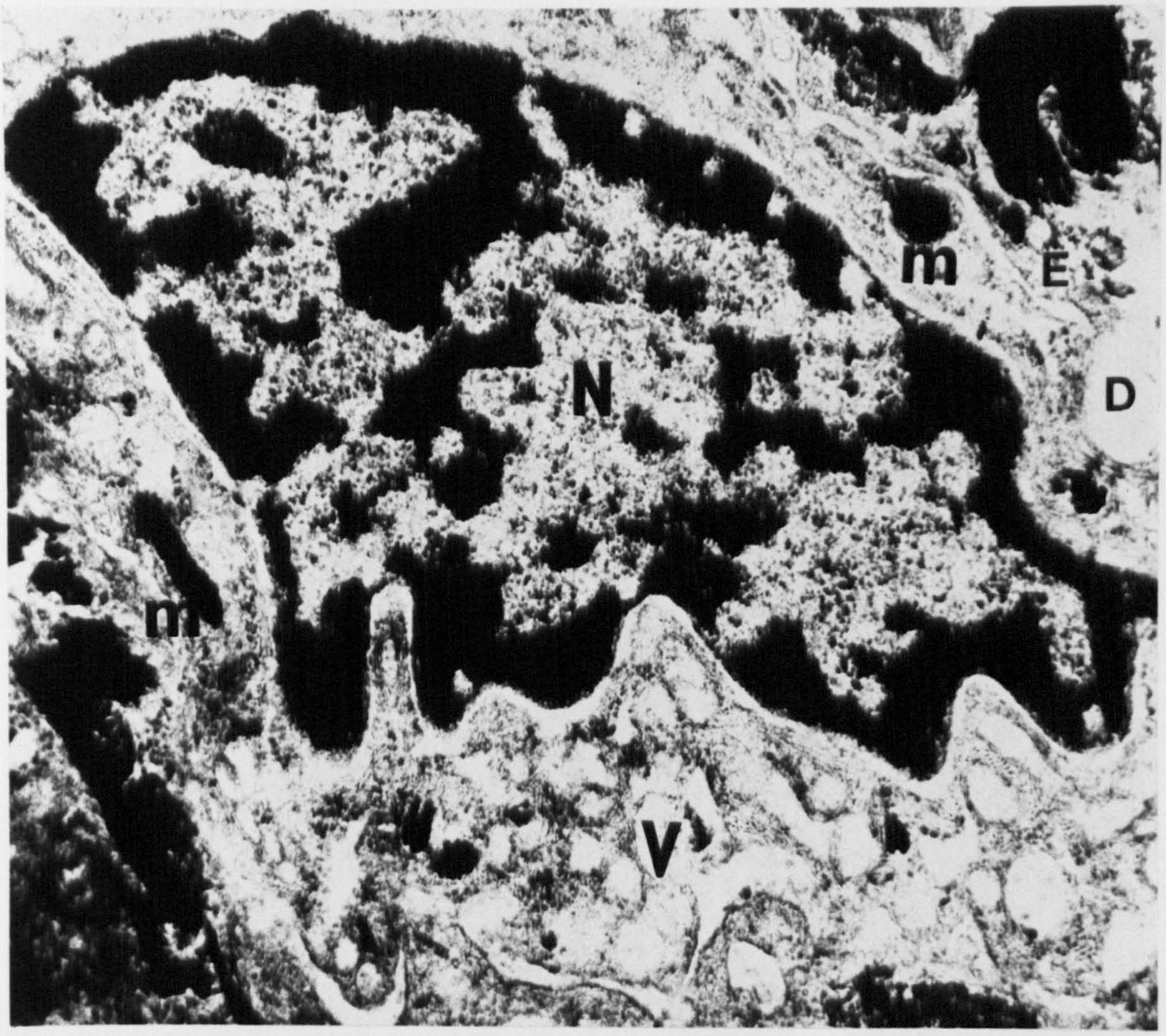


Fig **36** . Electron micrograph of degenerating melanocyte showing pyknotic nucleus (N) and vacuolated cytoplasm (V). m melanosomes, D Dermis, E Epidermis. (X 38400)

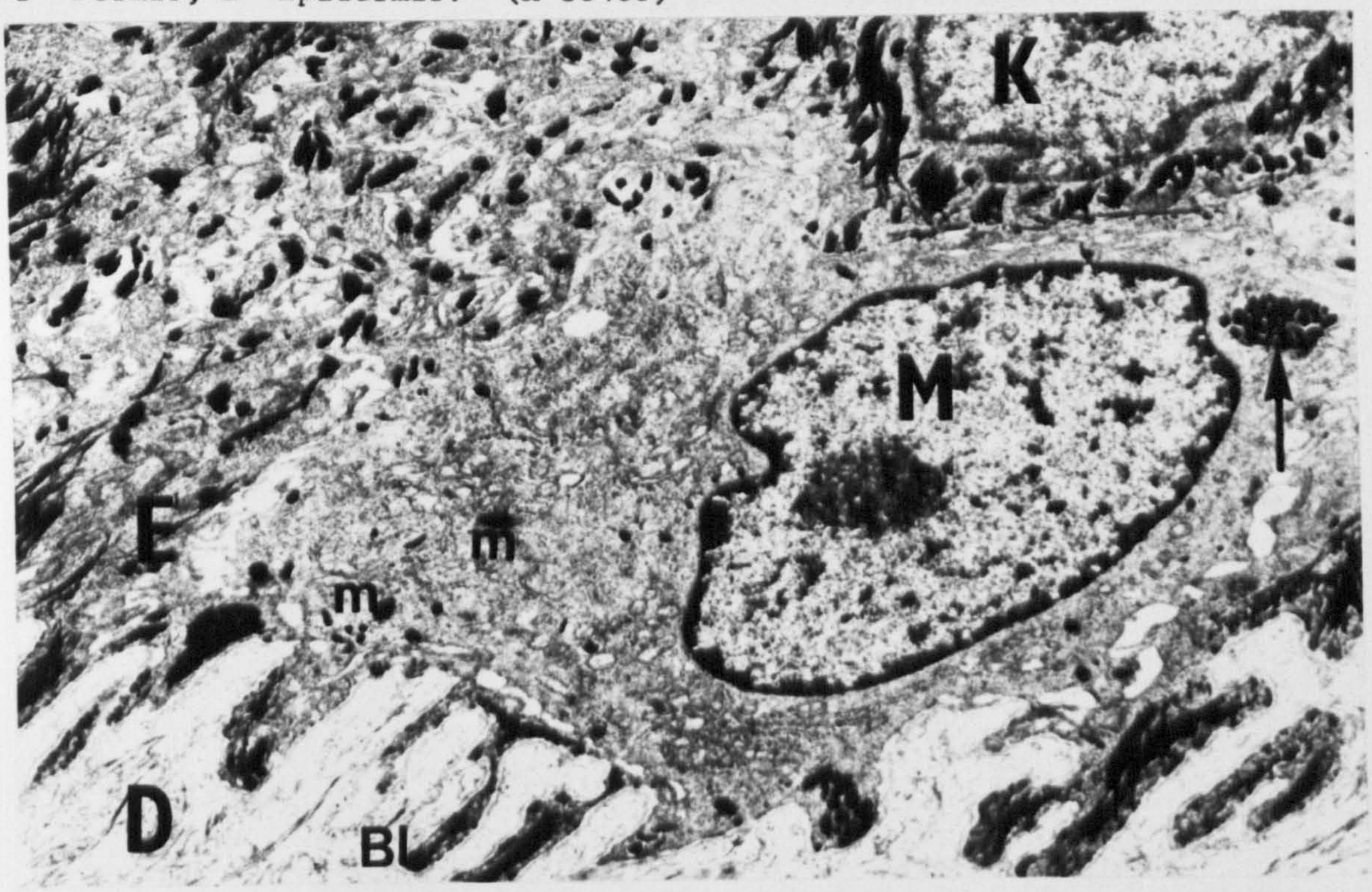


Fig **37** . Electron micrograph of melanocyte (M) showing melanosomes collection (arrow) inside the cytoplasm without a limiting membrane. m melanosome in different stages of development, K Keratinocytes with melanosome, D Dermis, BL Basal lamina. (X 10000)

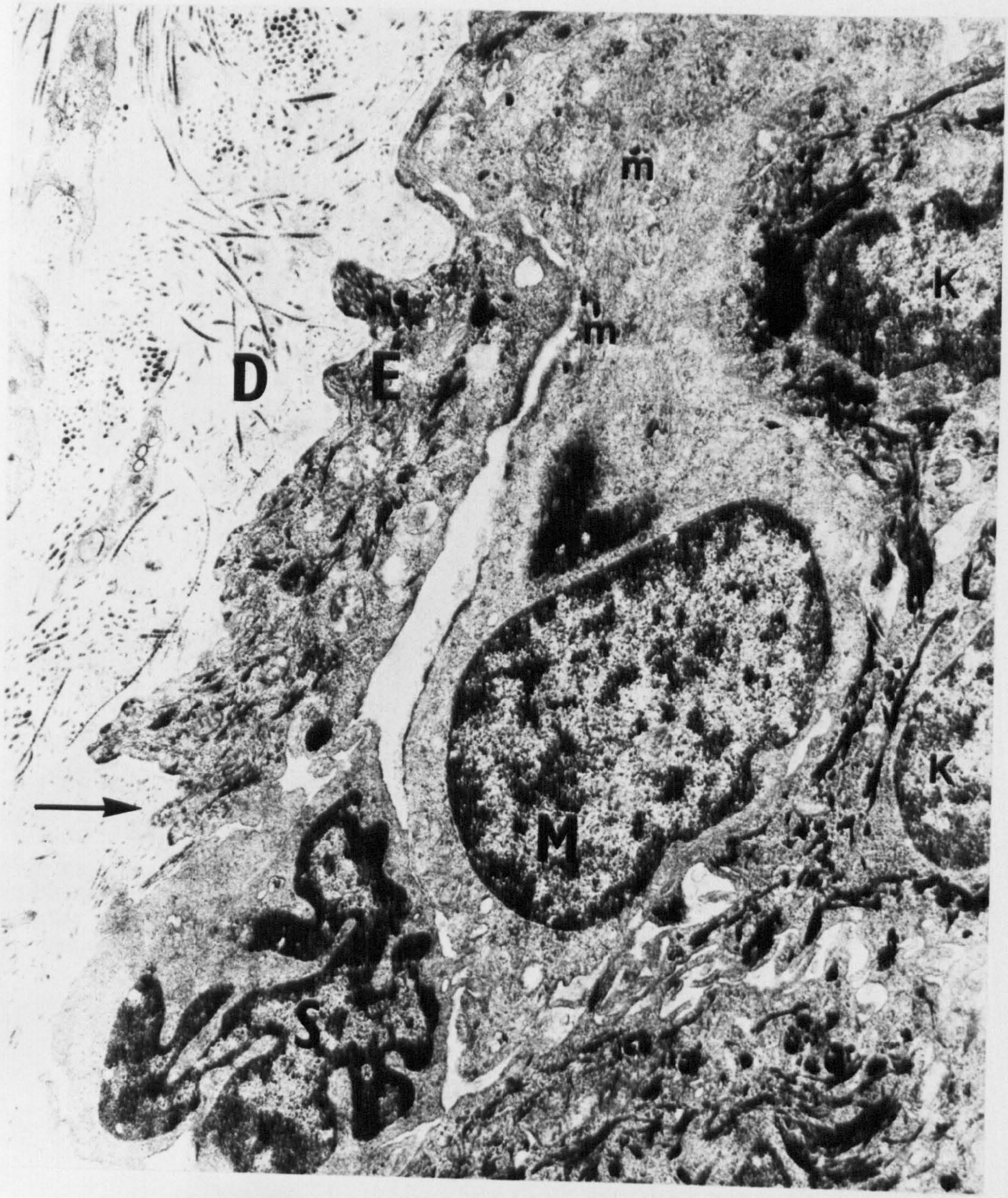


Fig **38** . Electron micrograph of a marginal area showing **S**ézary like lymphocyte (S) passing through the dermo-epidermal junction and coming in direct contact with a melanocyte (M) and even making interdigitations with it. Arrow: showing a break in the dermo-epidermal junction. D Dermis, E Epidermis, m melanosomes. (X 17325)

Although there was almost complete absence of melanocytes in these areas, melanosomes were often found in the keratinocytes and this occured in more than a third of the patients biopsied. It is presumed that the presence of a few residual functioning melanocytes in these involved areas were responsible for the production and transfer of melanosomes to these keratinocytes.

Melanosomes: Most of the patients in this study were Caucasians and it was observed that in the sections from uninvolved and marginal areas of the skin the melanosomes, instead of being arranged in membrane bound complexes of three or more, tended to be arranged singly. The size of the melanosome was variable, but the mean transverse diameter of uninvolved areas was 0.13μ.

(c) Langerhans Cells

Uninvolved areas: These dendritic cells were usually seen high up in the epidermis. They had an indented nucleus and only a few Langerhans cell granules were to be found in the cytoplasm of the cell.of the Langerhans cells found in the basal layer, their nucleus was more convoluted and granules were more frequent. In some of the uninvolved areas the Langerhans cells were only found in the basal layer. Langerhans cells were not found in those areas where there were many mononuclear cells in

the epidermis. Some of the Langerhans cells had vacuolated cytoplasm with a shrunken nucleus. Also occasionally melanosomal complexes were seen in these cells. In one section Langerhans cell granules were found in a membrane bounded body that was a lysosome (fig 41). In general the number of Langerhans cells appeared to be increased when compared with those biopsies from normal controls. This was only an impression, but could not be proven statistically.

Marginal areas: The Langerhans cells were mainly basal in location (fig 42) with a few being suprabasal. In many patients the cells were few or were absent, in particular, when there was a mononuclear cell infiltrate in the epidermis. The Langerhans cells sometimes appeared more 'active' than those in the uninvolved areas and had more convoluted nucleus with many characteristic granules and more lysosomal structures, and a well developed Golgi. Some of the Langerhans cells had vacuolated cytoplasm with a shrunken nucleus (fig 47A). Frequently, the Langerhans cells were seen in direct relation to lymphocytes, particularly in the basal layer of the epidermis. Melanosomal complexes were also found in the cytoplasm of these cells. A desmosome-like structure was noticed in the cytoplasm of one of these cells which was similar to the Langerhans granule.

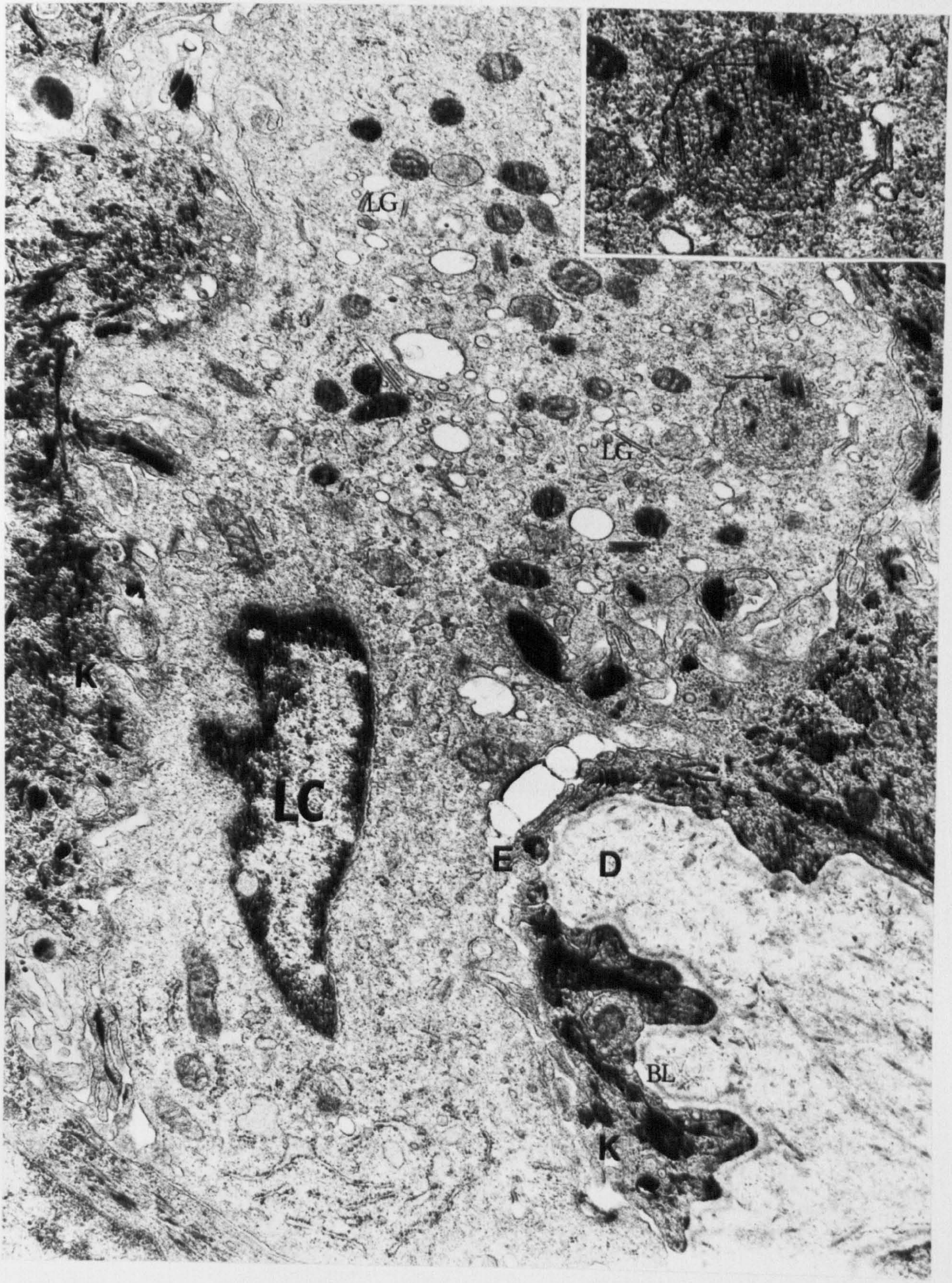


Fig **41** . Electron micrograph of an uninvolved area showing a basal Langerhans cell (LC) with intralysosomal Langerhans granules (arrow), as shown in the inset. LG Langerhans granules, D Dermis, BL Basal lamina, K Keratinocytes. (X 22050)

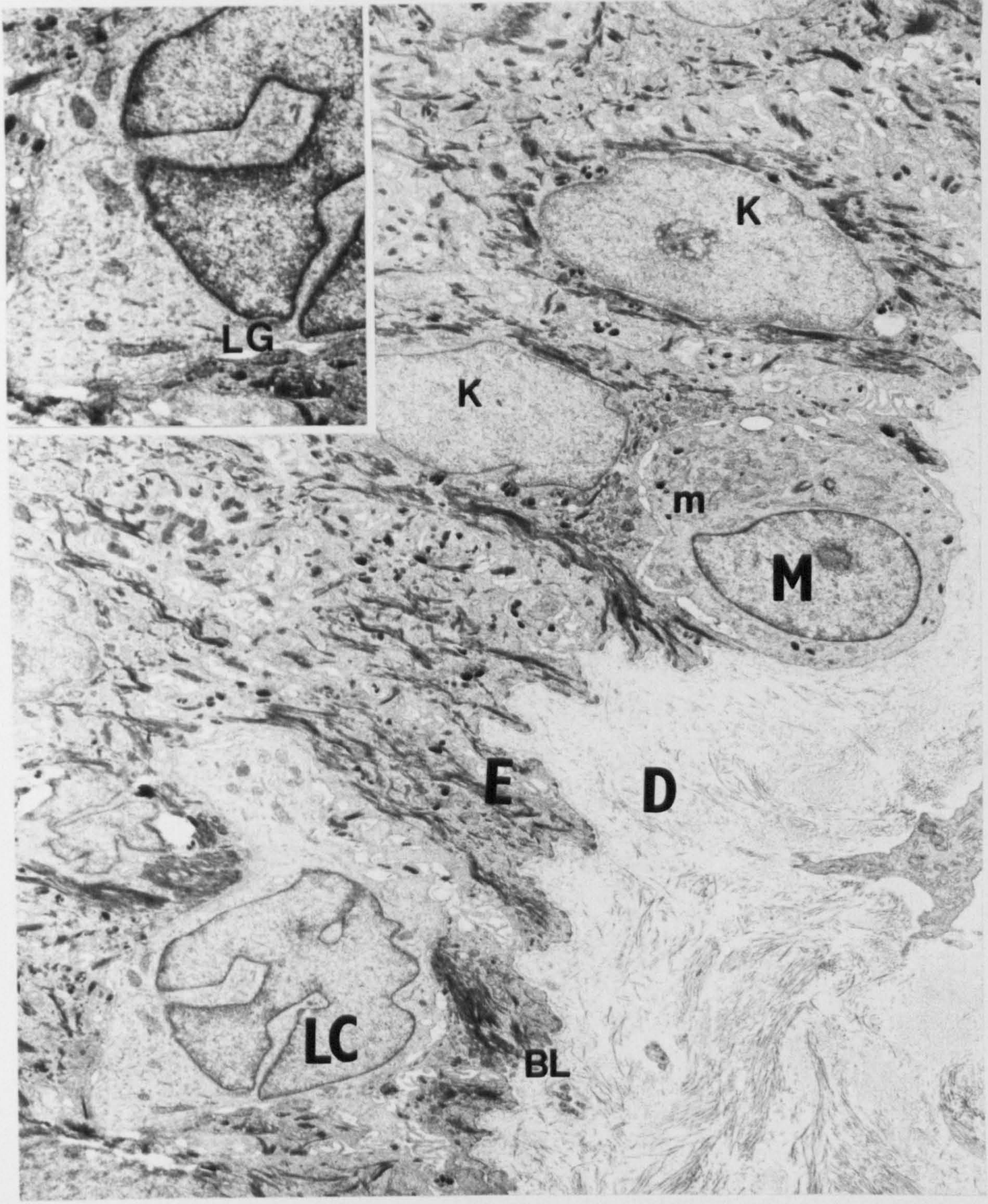


Fig 42 . Electron micrograph of a marginal area showing a basal melanocyte (M) with different formative stages of melanosomes (m). Also a basal Langerhans cell (LC) with its granules (LG) shown in the inset. D Dermis, BL Basal lamina, E Epidermis. (X 7000)

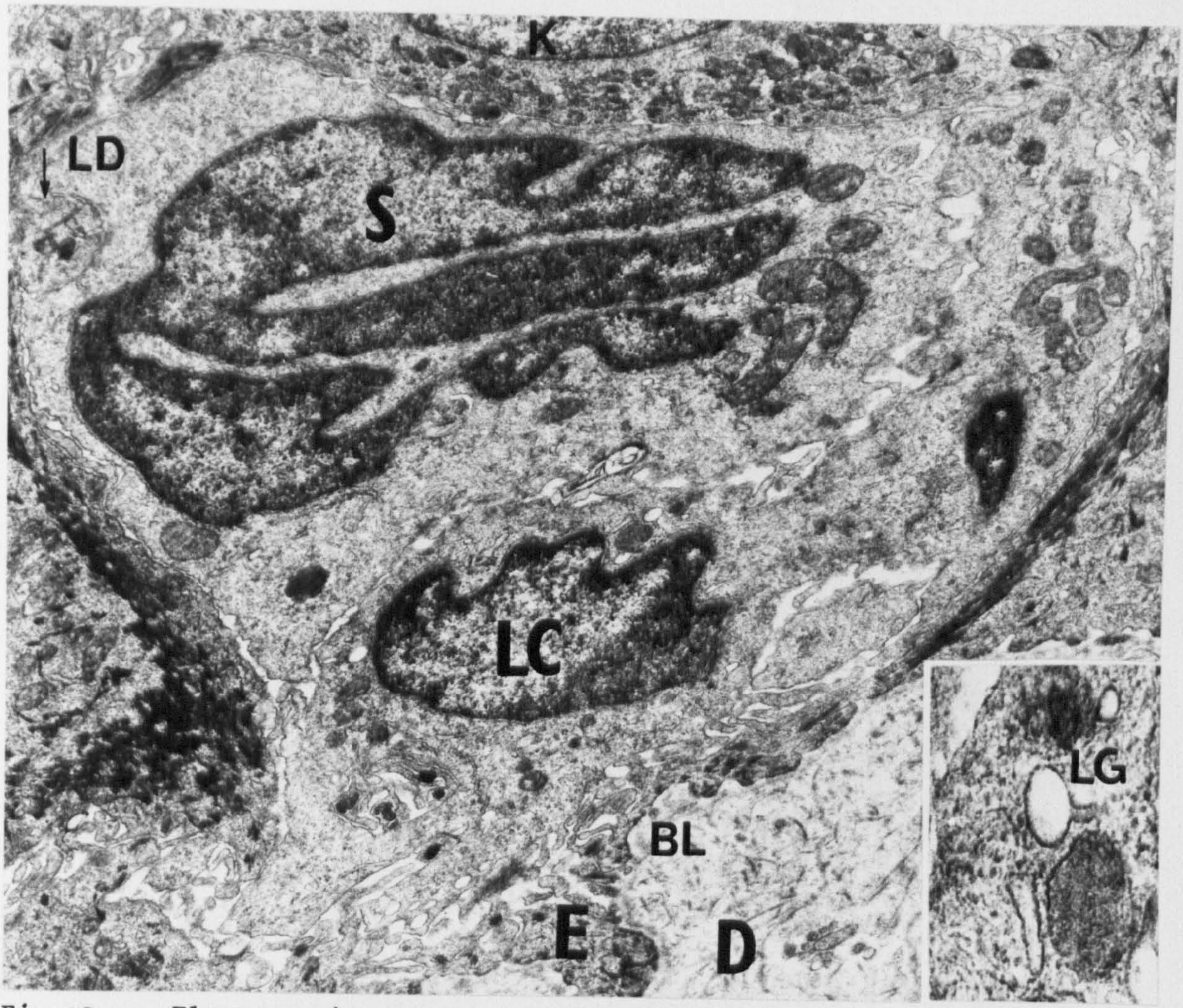


Fig **43** . Electron micrograph of an involved area showing Sezary like lymphocyte (S) in contact with Langerhans cell (LC). Inset showing a higher magnification of Langerhans granules (LG). LD Langerhans dendrite, D Dermis, BL Basal lamina, E Epidermis. (X 14490)

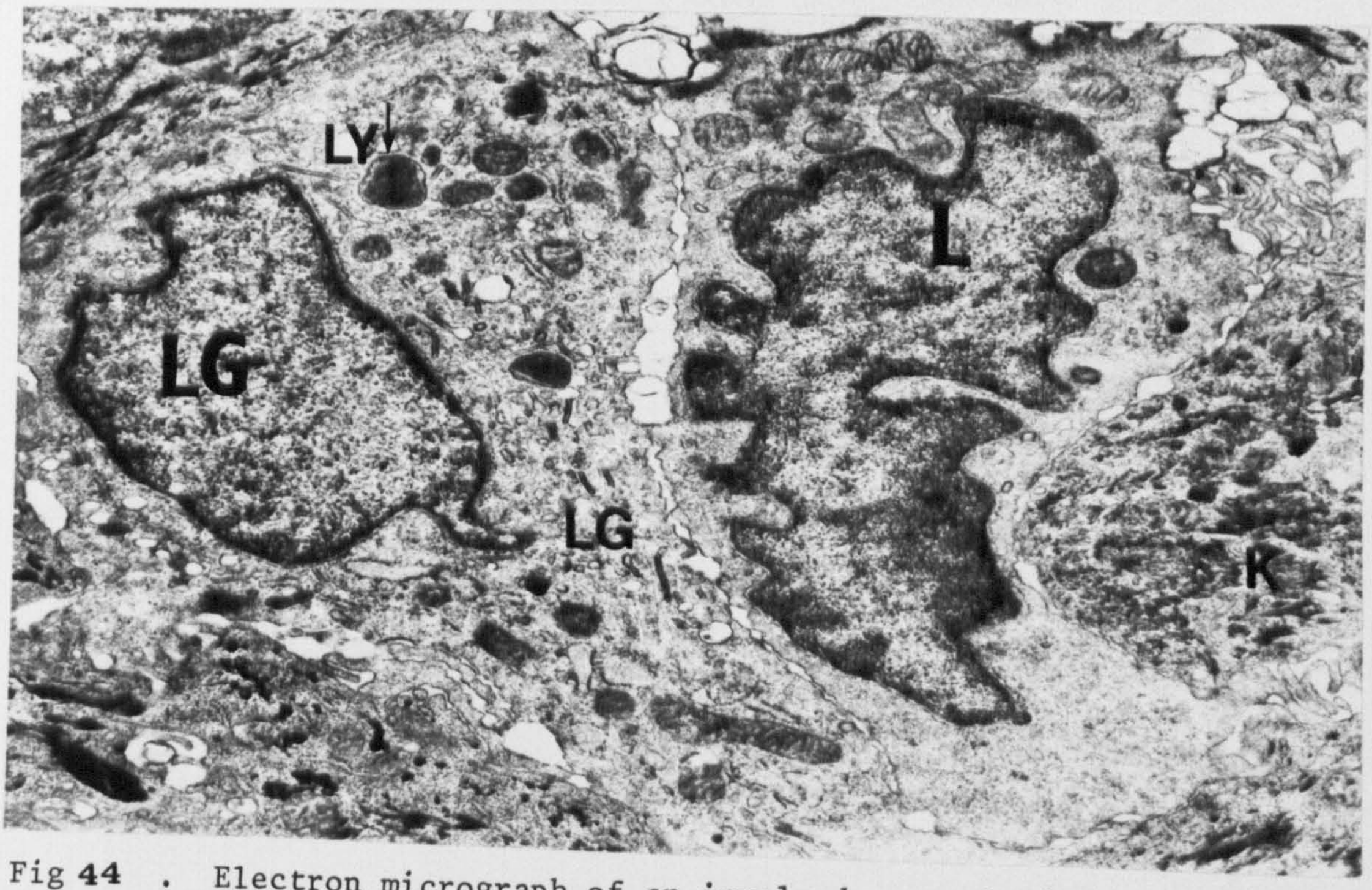


Fig **44** . Electron micrograph of an involved area showing lymphocyte (L) in contact with Langerhans cell (LC). LG Langerhans granules, LY Lysosome, K Keratinocyte. (X 17325)

Involved areas: In the involved areas of the skin the Langerhans cells were mainly basal, but were also found above this layer. In some patients only suprabasal Langerhans cells were identified. In a number of patients in serial sections of the biopsies from involved skin, no Langerhans cells could be positively recognised. However, the morphology of the clear cells in the basal layer of the epidermis were very suggestive of Langerhans cells (see indeterminate cells). Langerhans cells were also found to be in close contact with lymphocytes, particularly in the basal layer of the epidermis (fig 43,44).

The number of Langerhans cells in the basal and suprabasal layer appeared to be increased as compared with the numbers found in the epidermis of uninvolved in patients with vitiligo and in the normal skin from controls.

(d) Langerhans Cell Granules and Desmosomes

It was not infrequent to see a group of desmosomes aggregated near a Langerhans cell and often they were partially enclosed by the Langerhans cell cytoplasm (fig 45). In one section a desmosome was seen inside the cytoplasm of a Langerhans cell and this was similar to the characteristic granule, but it had an attachment plaque (fig 46). Also on many occasions desmosomes were seen that are similar to

Langerhans cell granules even, possessed vesicular portions. These were seen between the keratinocytes and near the Langerhans cells (fig 47).

(e) Indeterminate Cells

These cells were found in all areas, but appeared to be more frequent in the marginal (fig 49) and involved (fig 48) areas of skin in patients with vitiligo. Morphology of these cells was identical to that of Langerhans cells, but no Langerhans cell granules could be recognised even in serial sections. In the marginal areas of the skin the cells appeared to be more frequent and were related with mononuclear cells. One Langerhans cell/indeterminate cell was found in the marginal area lying in the upper dermis. In the normal and uninvolved skin, in vitiligo, these indeterminate cells were only occasionally seen and were much less frequent than melanocytes and Langerhans cells.

(f) Mononuclear Cell Infiltrate of the Epidermis

A mononuclear cell infiltrate was found within the epidermis in about a third of the patients biopsied. Most invariably, this was in the marginal areas of skin (fig 50,51,52), but in a few of the biopsies taken from uninvolved (fig 53) and involved areas of skin showed the presence of mononuclear cells within the epidermis. Associated with this mononuclear cell infiltrate within the epidermis

there was also frequently some degree of spongiosis. In some biopsies there was considerable disruption of the epidermis with degeneration of keratinocytes and cell debris (fig 54,55). A number of breaks were present at the dermo-epidermal junction zone involving also the basal lamina. Mononuclear cells were to be found passing through these breaks into the epidermis (fig 38,56,57). Most frequently, the mononuclear cell infiltrates were arranged in foci, particularly in the basal layer of the epidermis. These cells were to be found aggregated and in loose contact with each other. In some specimens, particularly from the marginal areas there were many of these mononuclear cells, sometimes aggregated to form Pautrier micro-abscesses. However, in the majority of the biopsy specimens these cells were arranged singly and were to be found in the epidermis, but particularly in the basal layer. Sometimes there were foci of these mononuclear cells in a particular area, but further sections through the same block showed relatively normal epidermis with only occasional mononuclear cells.

Mononuclear cells seen were usually atypical (stimulated) lymphocytes (fig 51,52) but also histiocytes were clearly recognised. The lymphocytes usually had large convoluted nuclei with a prominent heterochromatin pattern that was particularly con-

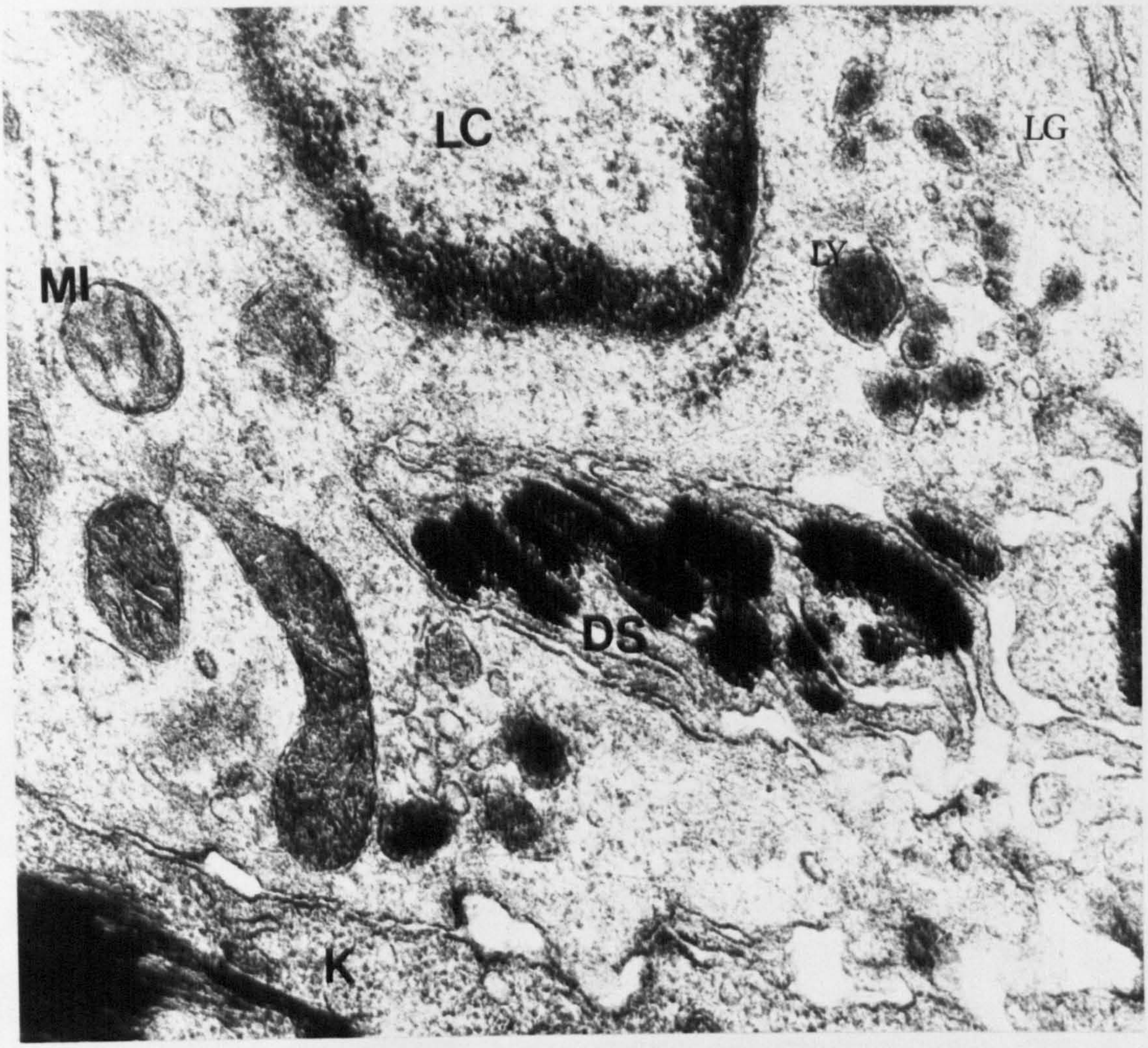


Fig **45** . Electron micrograph showing Langerhans cell (LC) partially phagocytosing a collection of desmosomes (DS). LG Langerhans granules, MI Mitochondria, LY lysosome, K Keratinocyte. (X 48000)

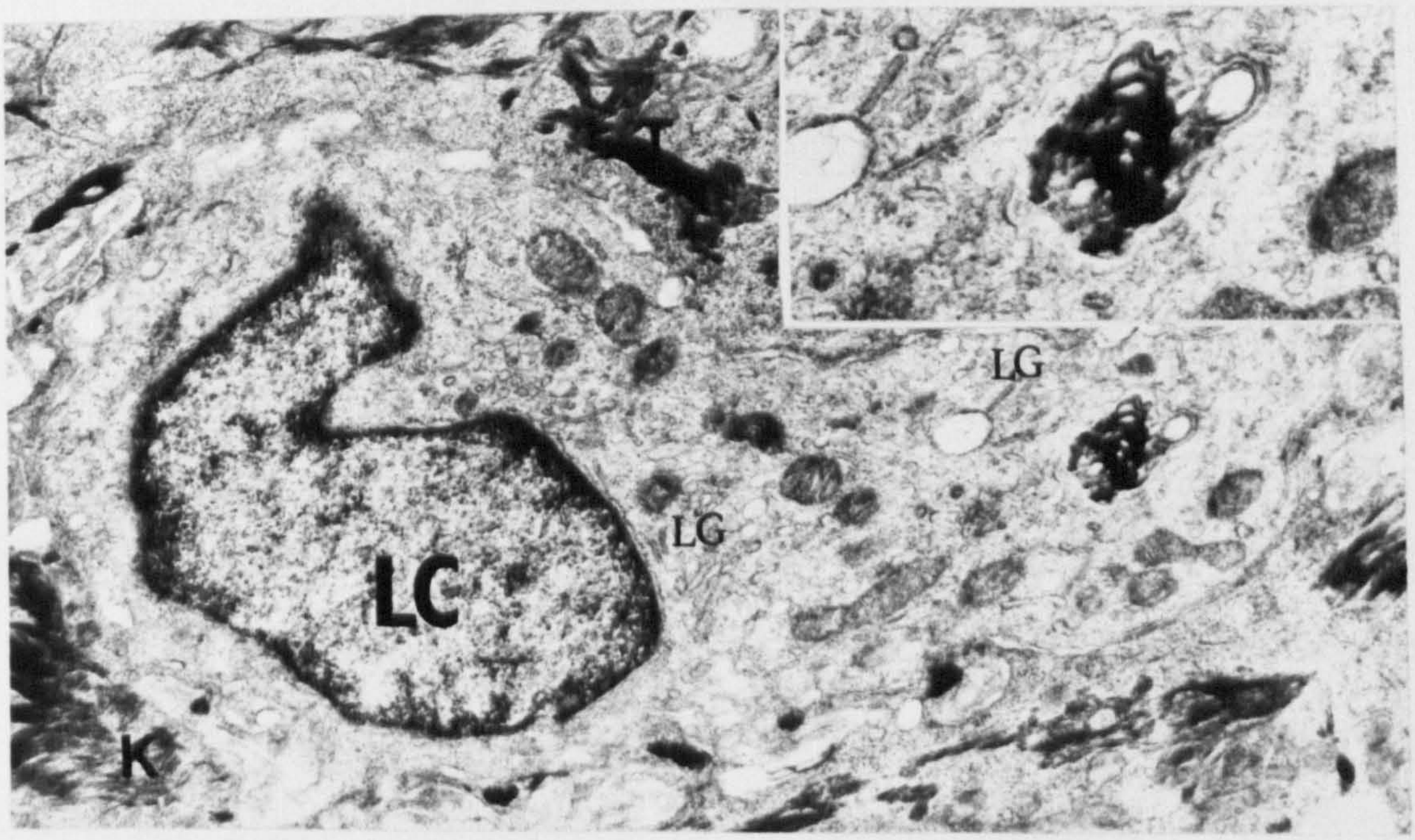
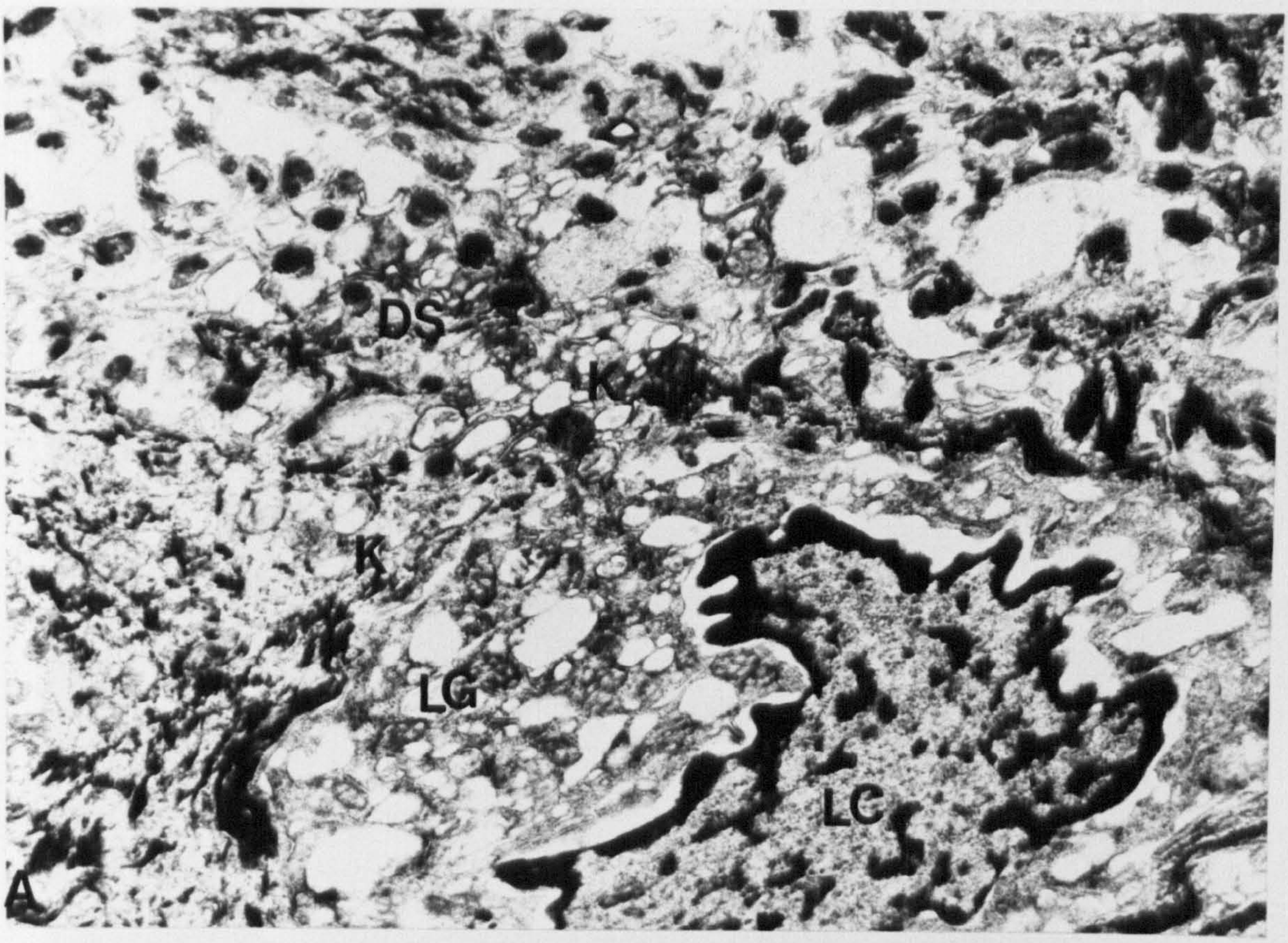
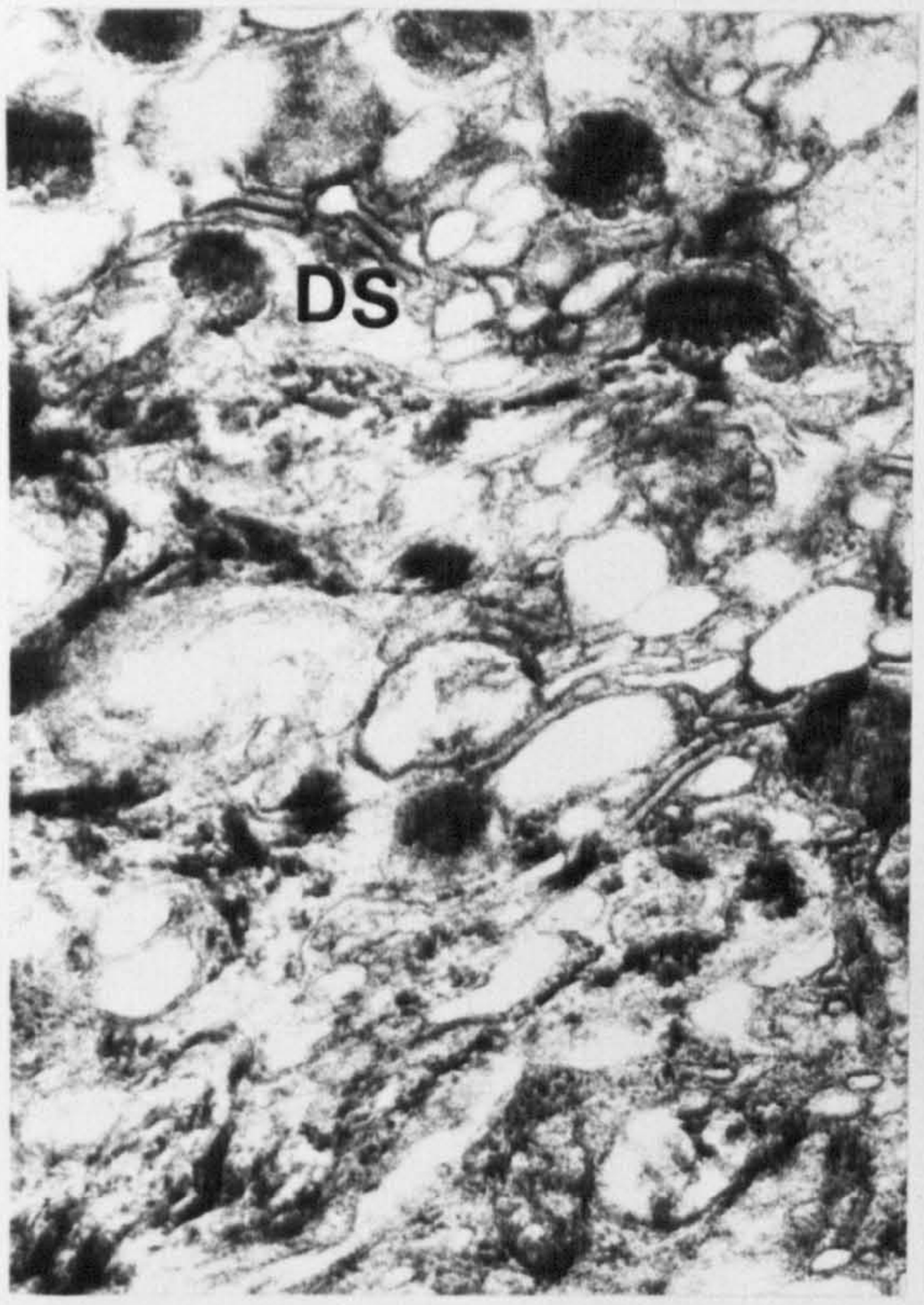


Fig **46** . Electron micrograph showing Langerhans cell (LC) with phagocytosed desmosomes. Inset is higher magnification of desmosomes. K Keratinocyte, T Tonofilaments, LG Langerhans granules. (X 18199)





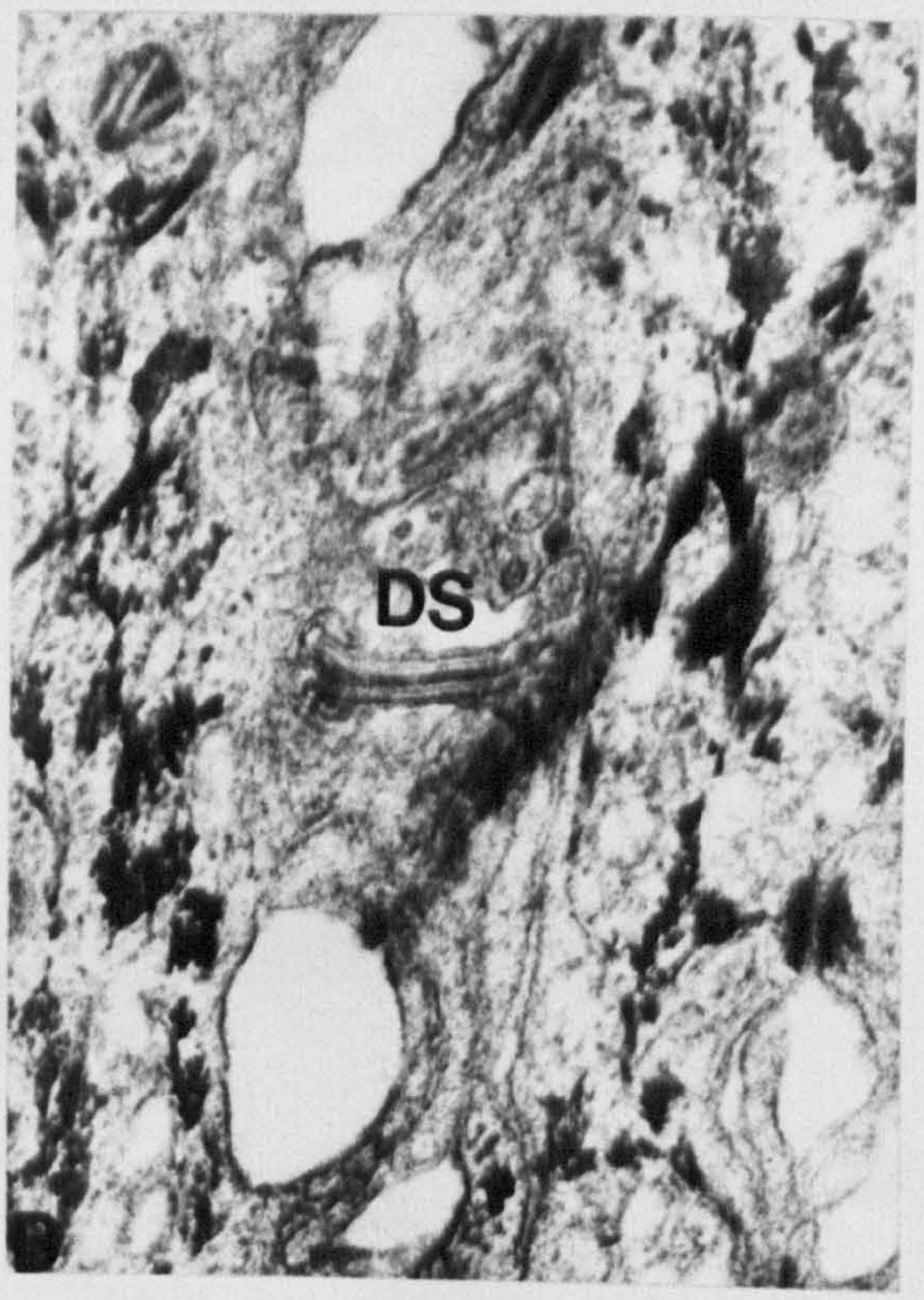


Fig 47 . A - Electron micrograph of a marginal area showing Langerhans cell (LC) with marked degenerative changes. On the top of this cell some of the intercellular desmosomes (DS) had changed into Langerhans granule like structures (X 16380). Inset in the left lower corner showing a higher magnification of these structures. LG Langerhans granules, K Keratinocytes.

B - showing intercellular desmosomes (DS) changed into Langerhans granule like structures. (X 40000)

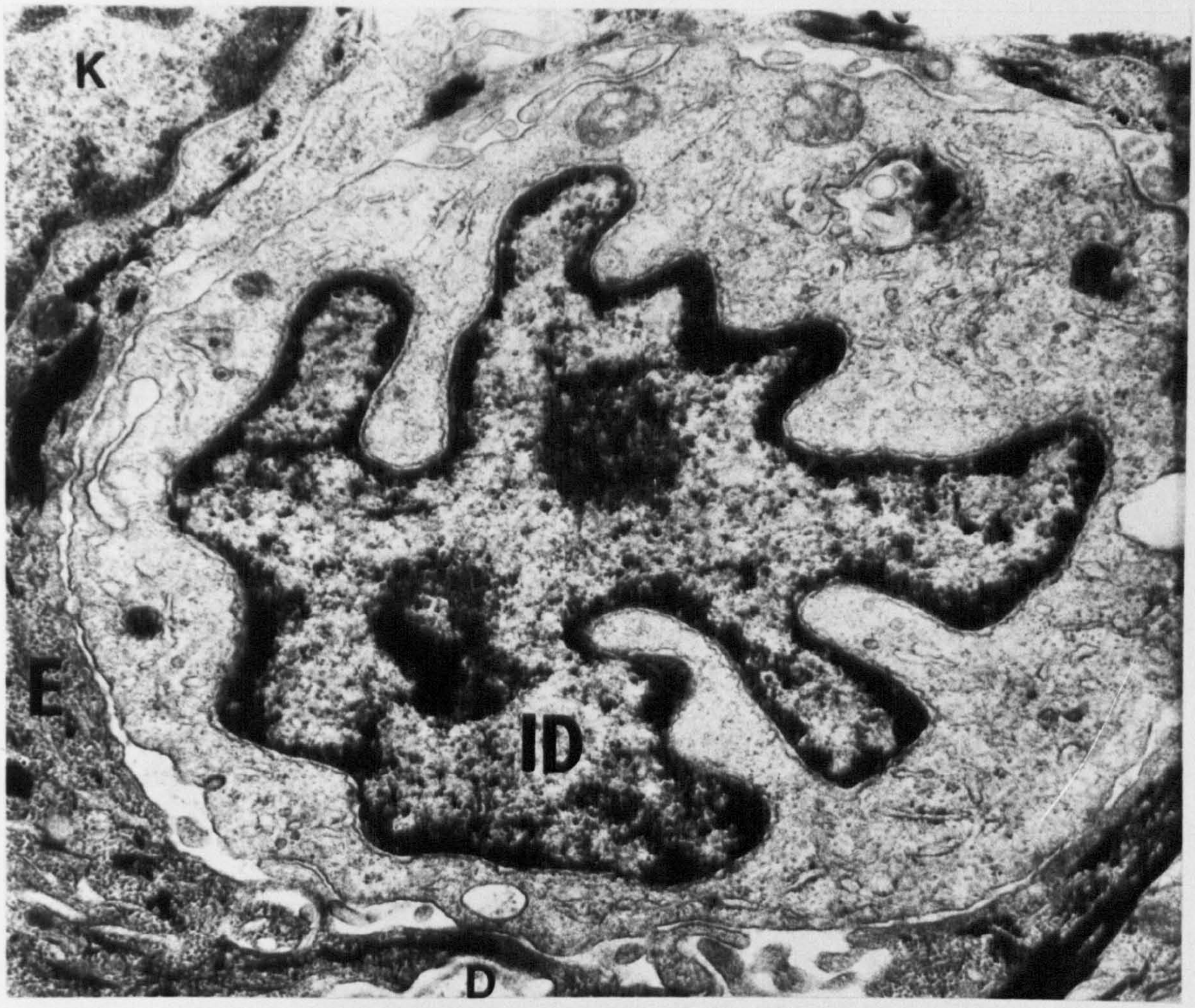


Fig 48 . Electron micrograph of an involved area showing a basal indeterminate cell (ID). D Dermis, E Epidermis. (X 2400)

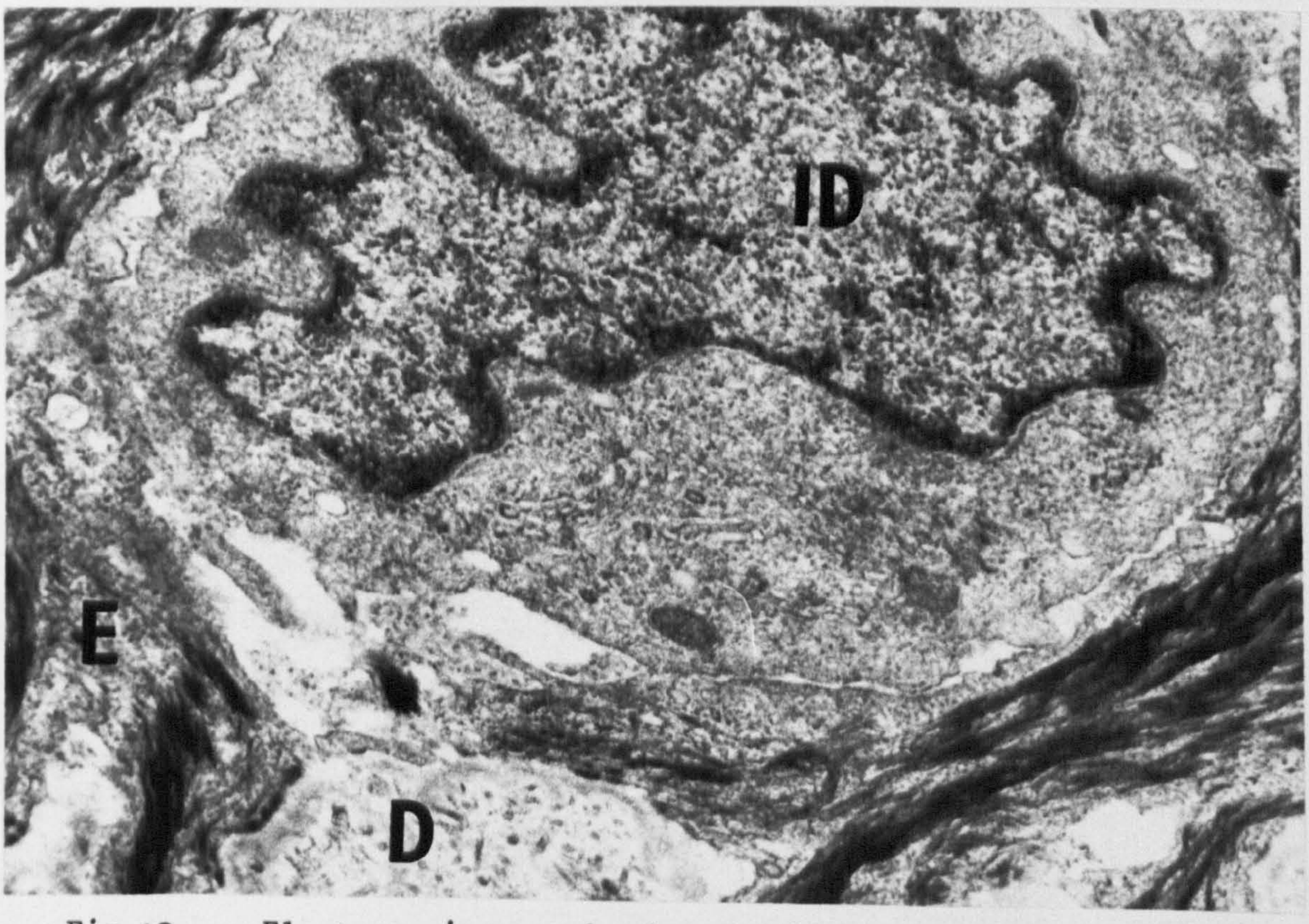


Fig 49 . Electron micrograph of a marginal area showing a basal indeterminate cell (ID). D Dermis, E Epidermis. (X 2400)

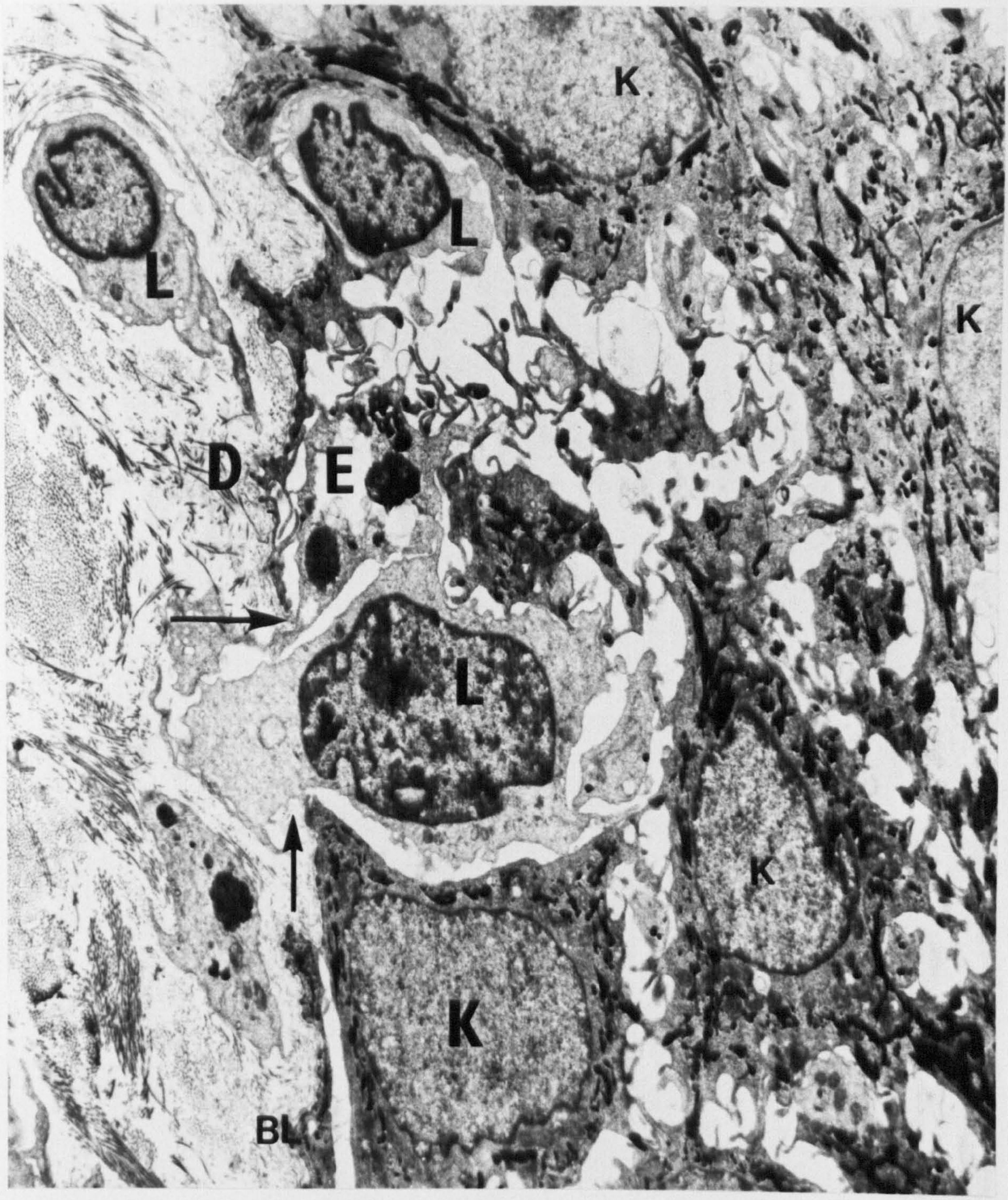


Fig 50 . Electron micrograph of a marginal area showing marked inflammatory changes, mainly spongiosis with lymphocytic cell infiltrate (L) of the epidermis (E). One of the lymphocytes is just passing into the epidermis through a break in the dermo-epidermal junction (arrows). D Dermis, BL Basal lamina, K Keratinocyte. (X 7500)

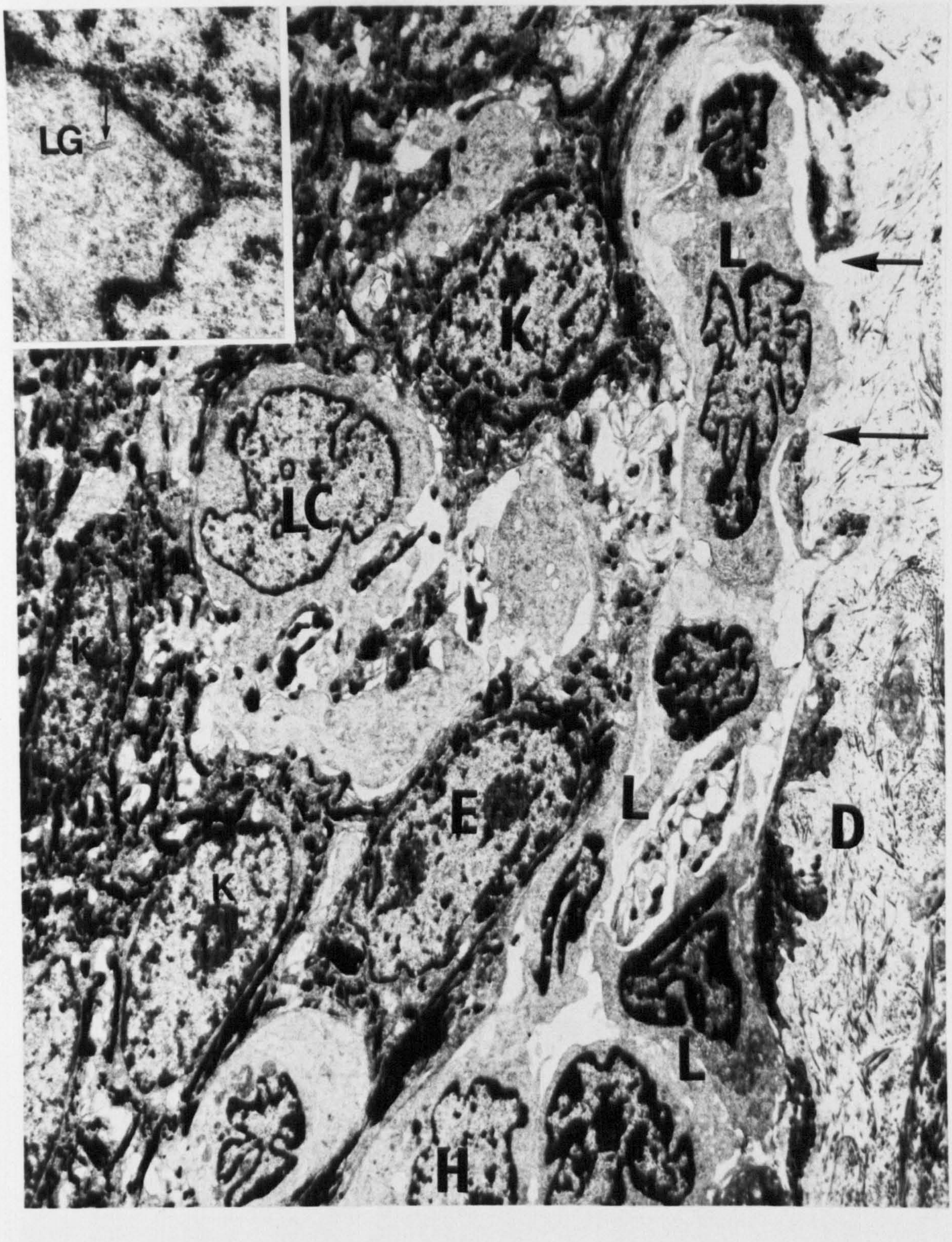


Fig **51** . Electron micrograph of a marginal area showing atypical lymphocytes (L) infiltrating the epidermis (E). LC Langerhans cell with its Langerhans granule shown in the inset. H Histiocyte, D Dermis, K Keratinocytes. Arrows showing a break in the dermo-epidermal junction . (X 13275)

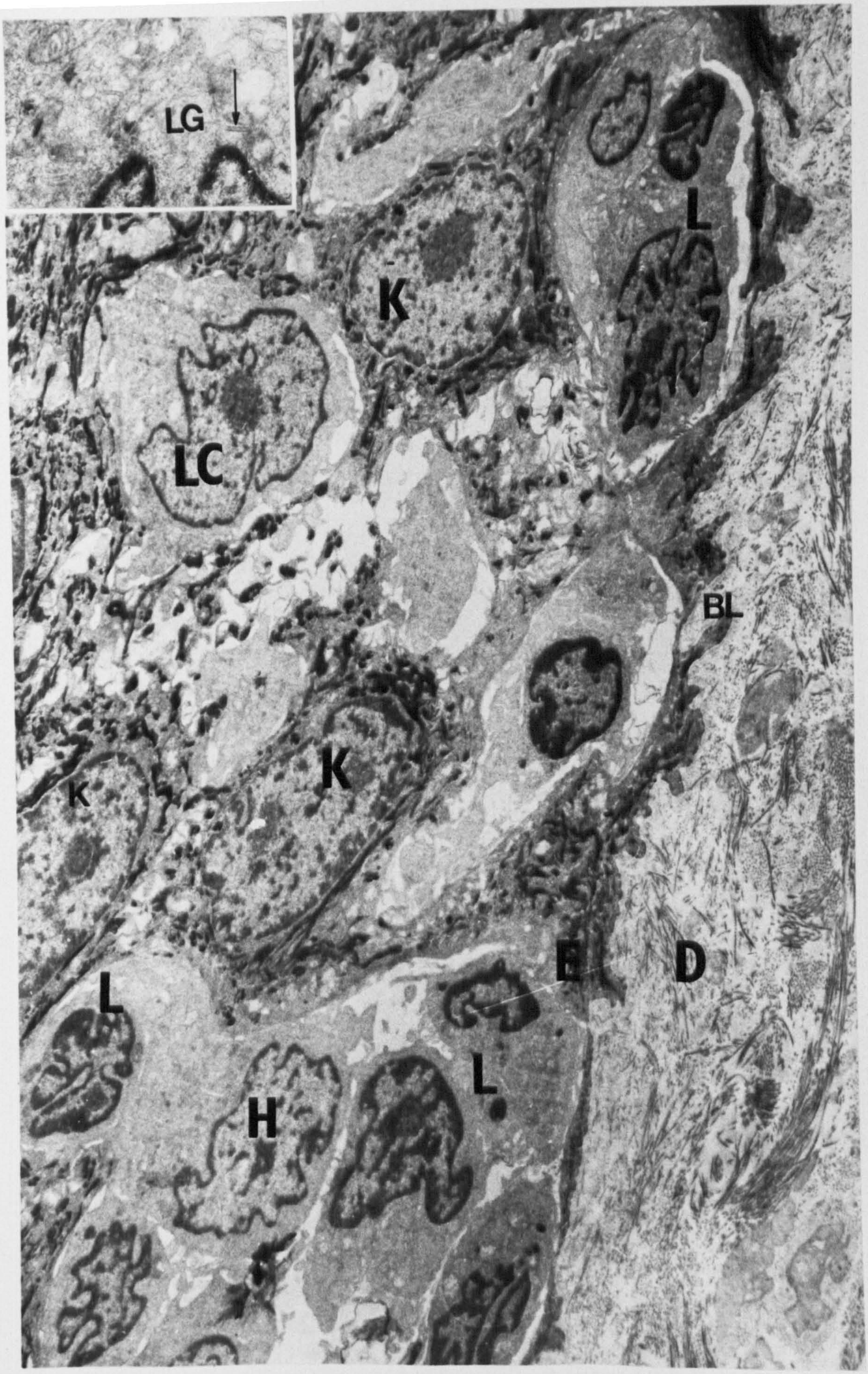


Fig **52** . Electron micrograph of a marginal area showing atypical lymphocytes (L) in the epidermis (E). LC Langerhans cell with its granules (LG) shown in the inset. H Histiocytes, D Dermis, BL Basal lamina. (X 6000)

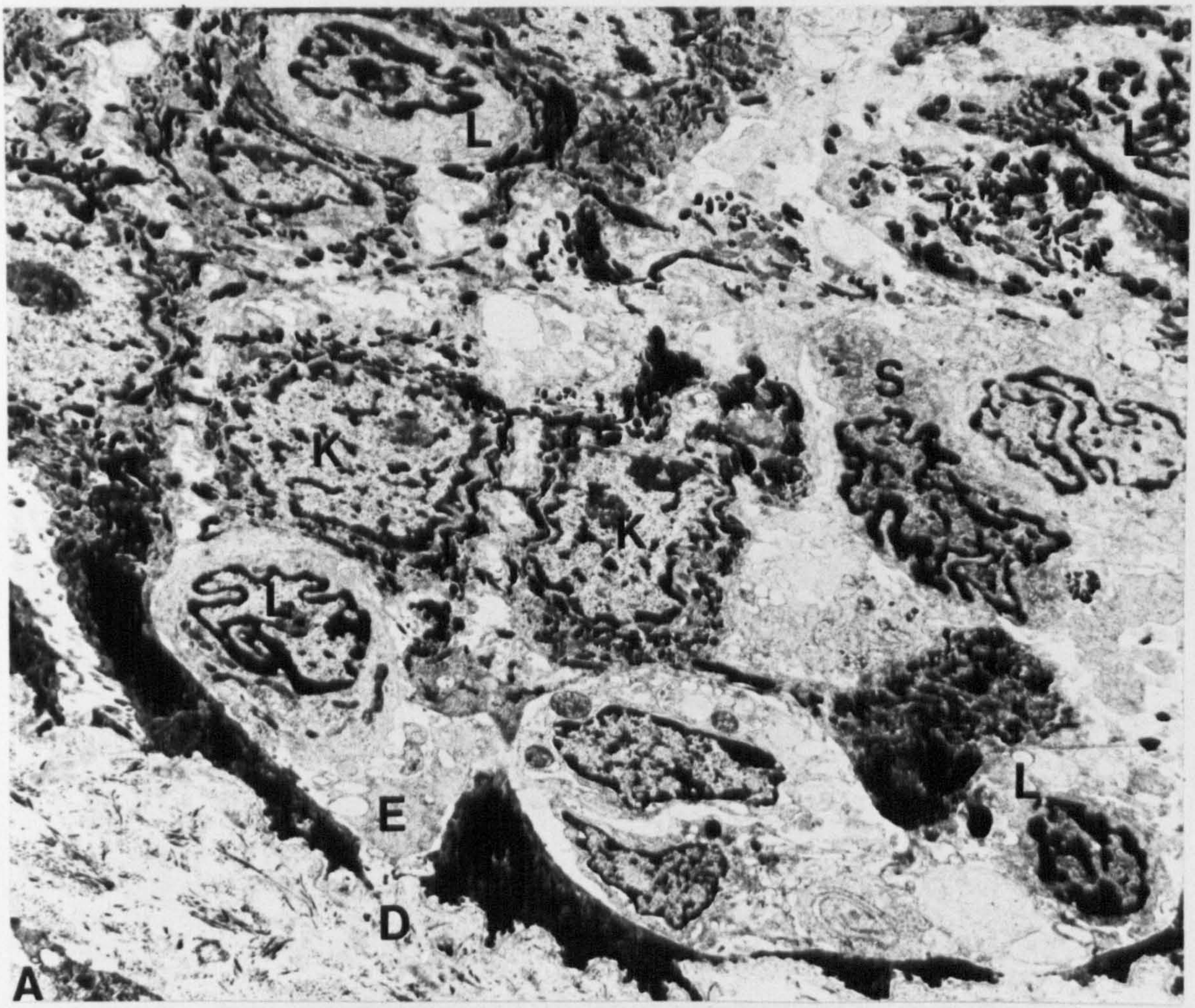


Fig **53** . A,B - Electron micrographs of an uninvolved area showing marked inflammatory changes of the epidermis, mainly lymphocytes (L) and spongiosis. S Sezary like cell, D Dermis, E Epidermis, H Histiocytes. (A, X 6250, B, X 4000)

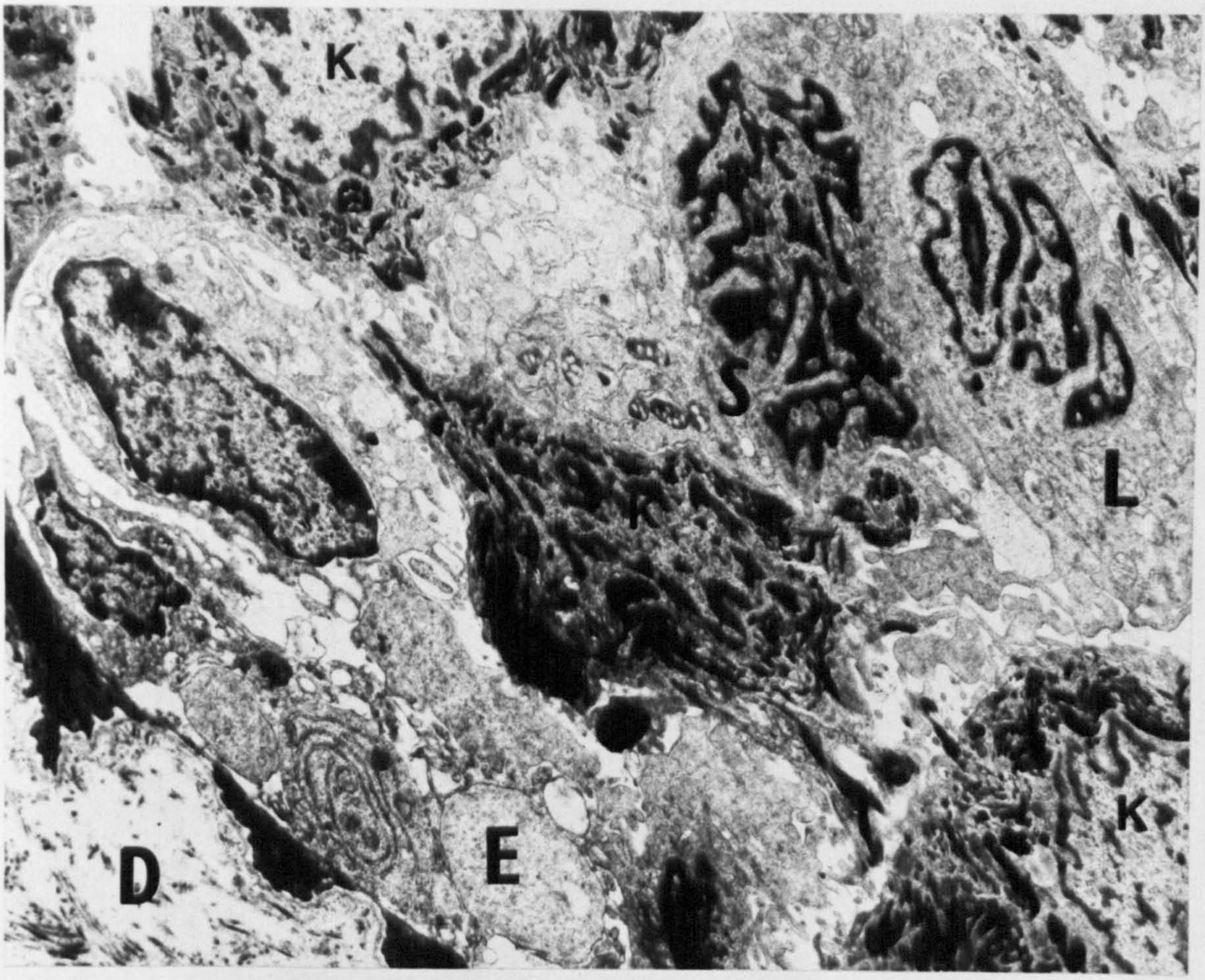


Fig 54 . Electron micrograph of an uninvolved area showing the marked inflammatory changes in the epidermis (E) like spongiosis, atypical lymphocytes (L). S Sézary like cell, D Dermis. (X 9200)

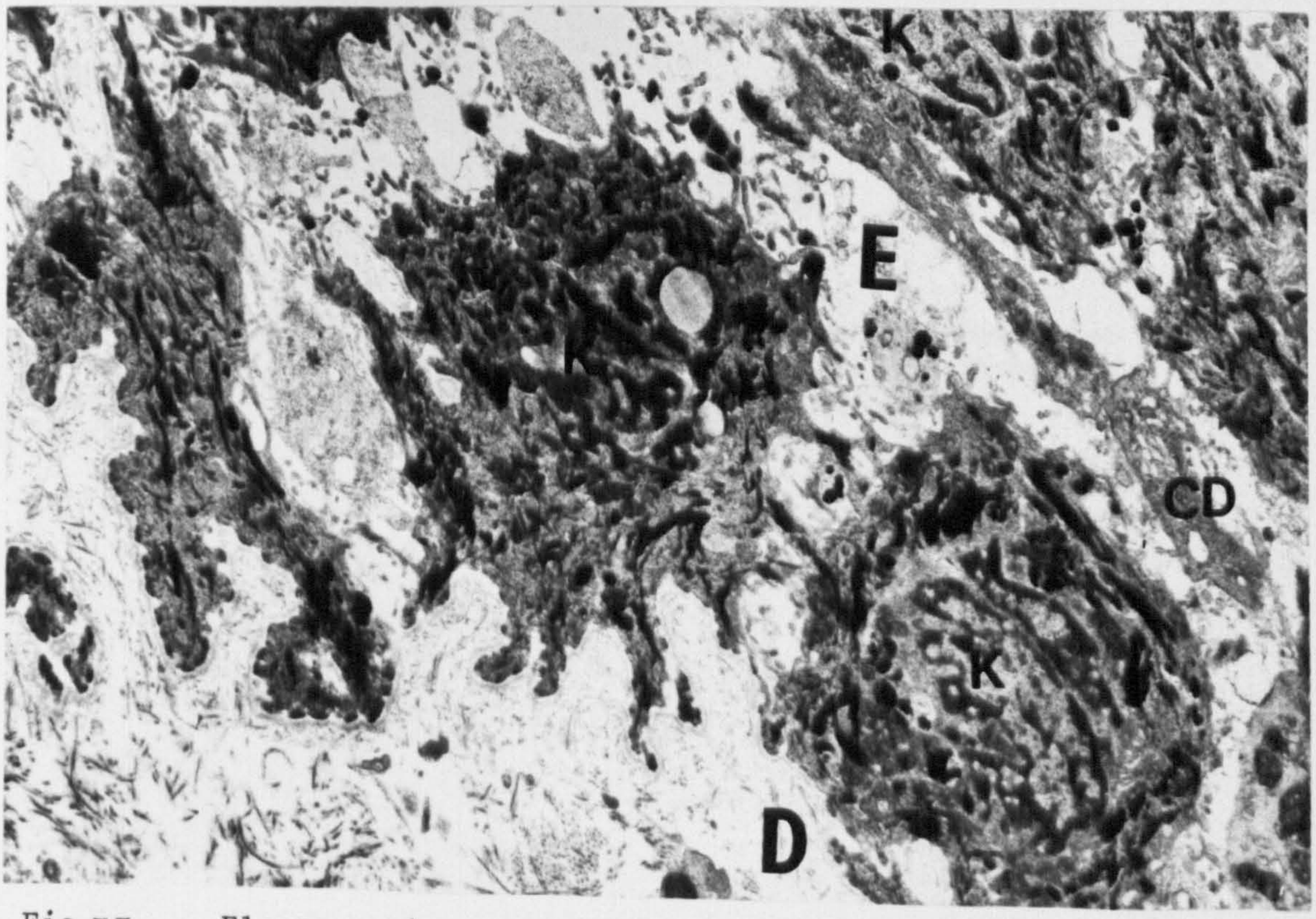


Fig 55 . Electron micrograph of a marginal area showing a marked spongiosis with cell debris (CD). D Dermis, E Epidermis. (X 9200)

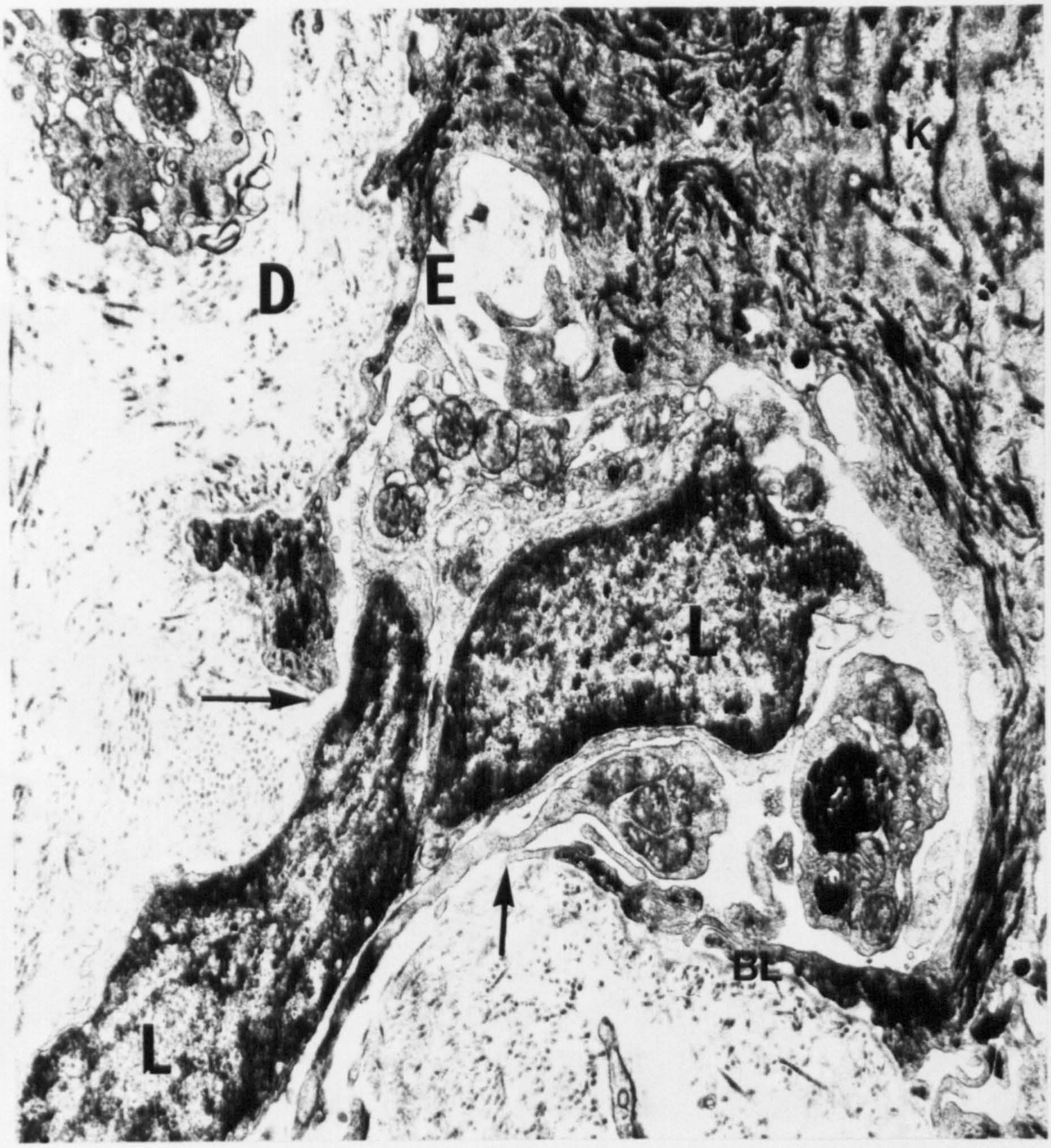


Fig 56 . Electron micrograph of a marginal area showing two lymphocytes (L) just entering into the epidermis (E) through a break (arrows) in the dermo-epidermal junction. D Dermis, BL basal lamina. (X 16434)

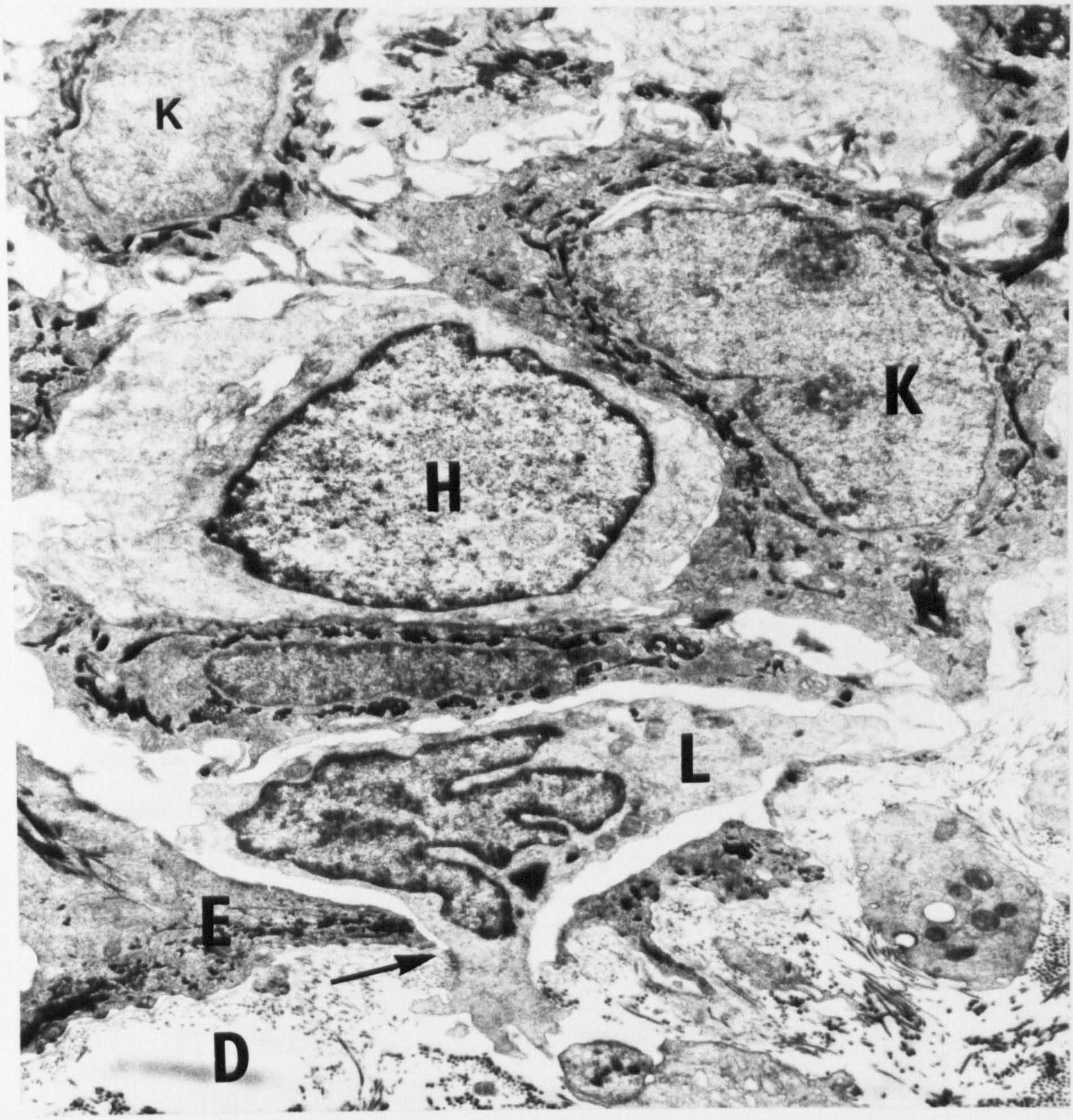


Fig 57 . Electron micrograph of a marginal area showing marked inflammatory changes, mainly spongiosis with lymphocytic cell (L) infiltrate of the epidermis (E), together with histiocyte (H). The lymphocyte seen is just passing into the epidermis through a break (arrow) in the dermo-epidermal junction. D Dermis, K Keratinocyte. (X 10000)

densed around the periphery. Some of these cells resembled those found in the Sezary cell syndrome and in mycosis fungoides. The cells identified within the epidermis were similar to those lymphocytes and histiocytes seen in the papillary dermis. In many biopsies taken from the patients with vitiligo these lymphocytes were in close contact with melanocytes (fig 38,39,40) and Langerhans cells (fig 43,44), and sometimes formed inter-digitations between them.

The population density of the epidermal mononuclear cells showed no direct relationship with the number found in the upper dermis. In some biopsy specimens there were many cells to be found within the epidermis, where as the underlying dermis contained very few lymphocytes and histiocytes.

(g) Mast Cells

Mast cells with typical cytoplasmic granules and villous processes from the cell membrane were to be found in the basal layer of the epidermis in three of the patients biopsied (fig 58). In two of the biopsies the occasional cell was found in the involved skin and in one from the marginal area. Portions of these mast cells were to be found between the keratinocytes in the basal layer. None of these cells were to be seen in the higher layer of the epidermis. In one of the specimens the mast cells

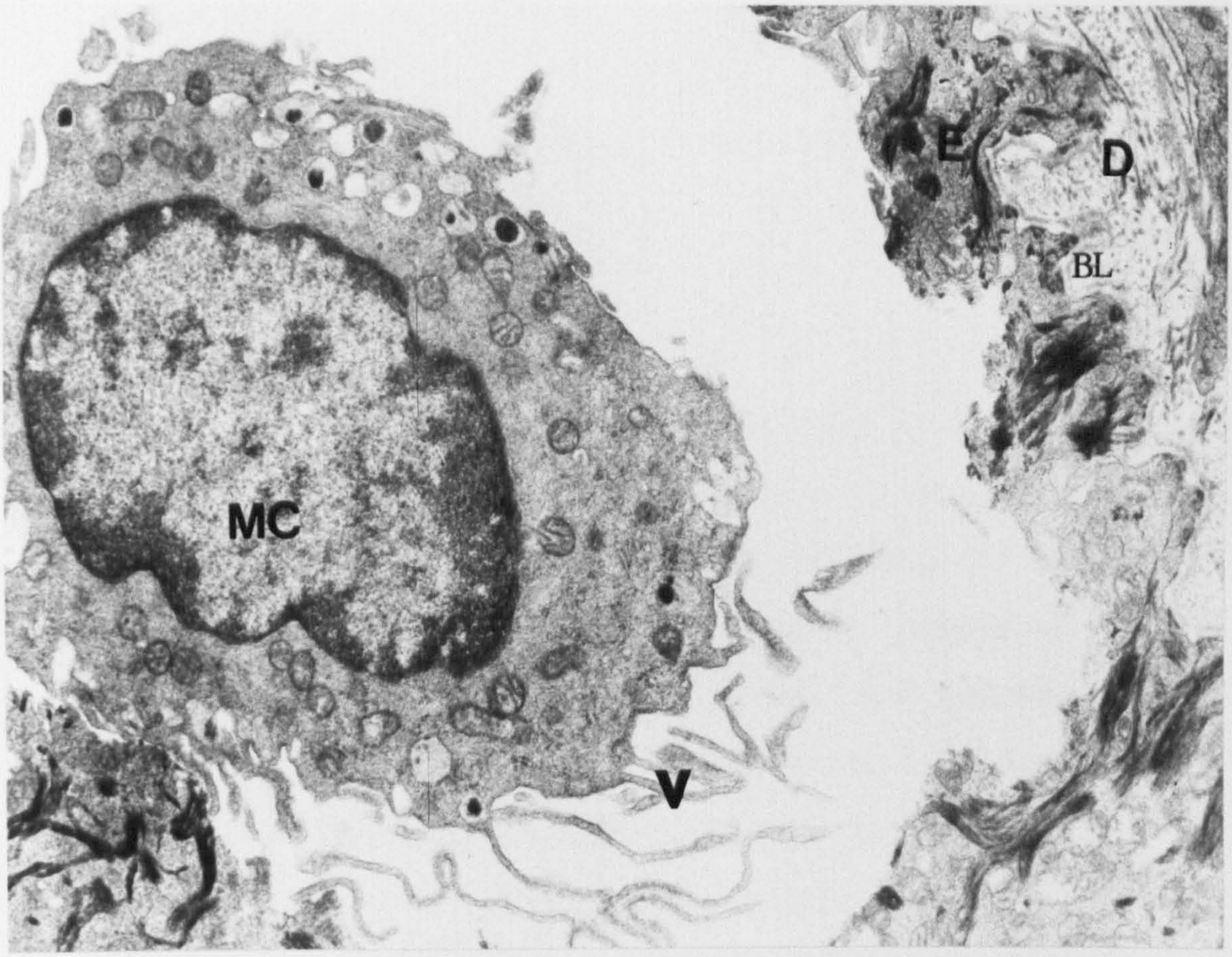


Fig 58 . A - Electron micrograph of an involved area showing intra-epidermal mast cell (MC). D Dermis, BL basal lamina, V Villous processes. (X 15120)

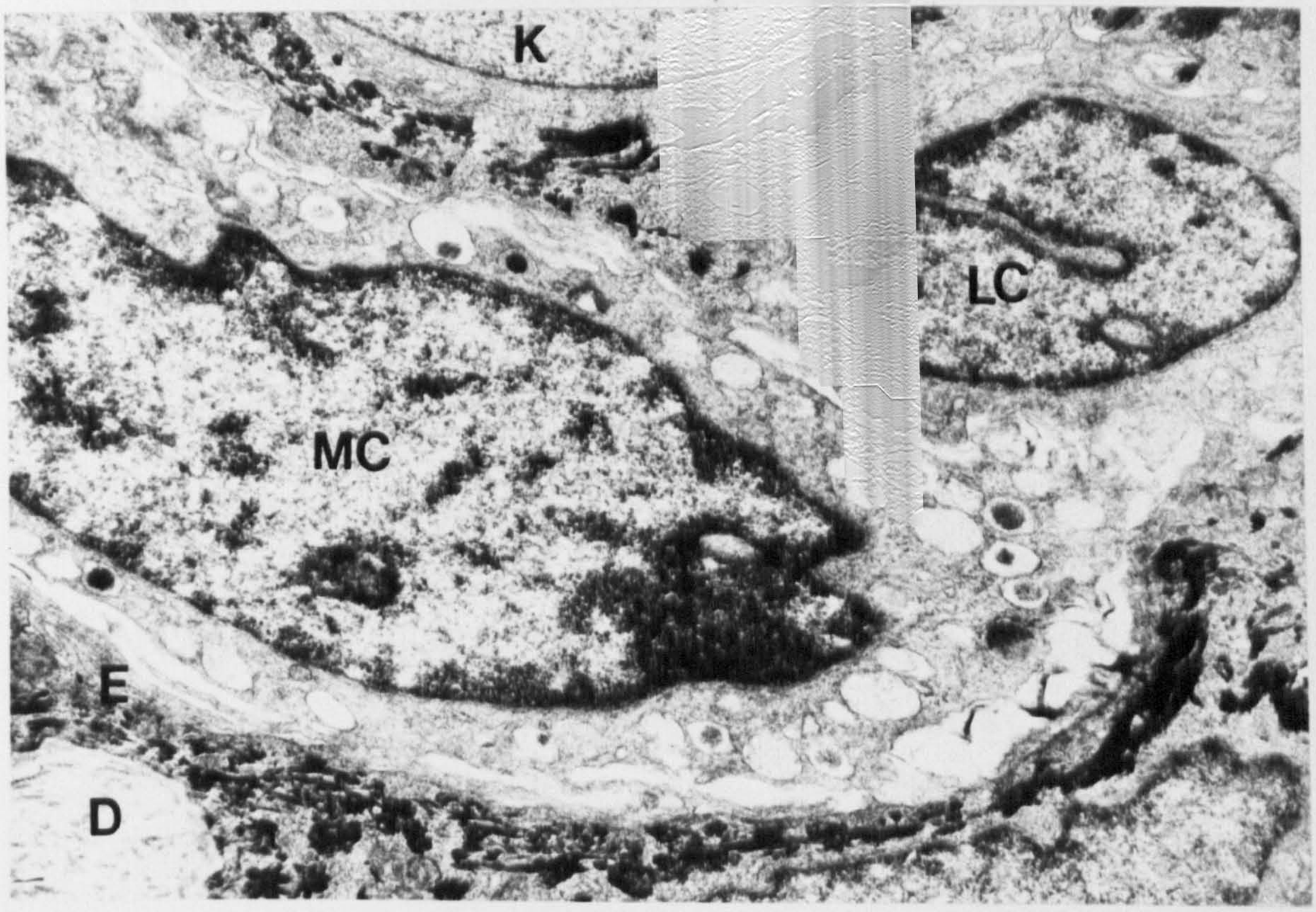


Fig 58 . B - Electron micrograph of an involved area showing intra-epidermal mast cell (MC) in contact with Langerhans cell (LC), its granules are not shown here. D Dermis, E Epidermis, K Keratinocyte. (X 16380)

were partially degranulated.

2. Dermis

(a) General Appearance

Very few alterations were noticed in the dermis and these were mainly confined to biopsies taken from the marginal areas. In a number of the patient's biopsies, there was significant oedema of the upper dermis and the collagen bundles appeared to be more widely separated. Deposition of fibrin was to be found only in the papillary dermis and mainly at the dermo-epidermal junction zone (fig 59).

The dermal cellularity consisted mainly of mononuclear cells, but also there appeared to be some increase in the number of mast cells, fibroblasts, neural elements and blood vessels. Many of the biopsies taken from the involved areas, in the blocks that were examined, very few were to be found in the dermis. In a number of the specimens there was significant replication of the basal lamina at the dermo-epidermal junction.

(b) Mononuclear Cells

Lympho-histiocytic infiltrate, particularly in the upper dermis, was noticed and appeared to be most significant in the biopsies taken from the marginal areas (fig 60). There was also increased cellularity in both the uninvolved and involved areas

(fig 61) in the biopsies taken from these patients with vitiligo. Histiocytes (melanophages) containing melanosomal complexes were seen in the marginal, uninvolved, but also occasionally in the involved areas of skin. These dermal histiocytes mostly resembled the epidermal Langerhans cells, but only one Langerhans cell was to be found in the upper dermis; this was seen in the marginal area. Lymphocytes were particularly to be found round the blood vessels in the marginal area. The cells had deeply convoluted nuclei with prominent heterochromatin pattern (fig 62). Some resembled the Sezary cell. These cells were very similar to those that were found in the epidermis.

(c) Mast Cells

Typical mast cells (fig 63) were found around the blood vessels in all the areas that were biopsied. It was difficult to quantitate these cells, but they appeared to be increased in number, particularly in the marginal and involved areas of skin in many of the patients that were biopsied. Some of the mast cell granules were to be seen lying free in the dermis and a number of the mast cells also showed early changes indicative of degranulation (fig 64). The damage to the mast cells was particularly more apparent in the marginal areas. As mentioned previously the mast cells were also to be found in a

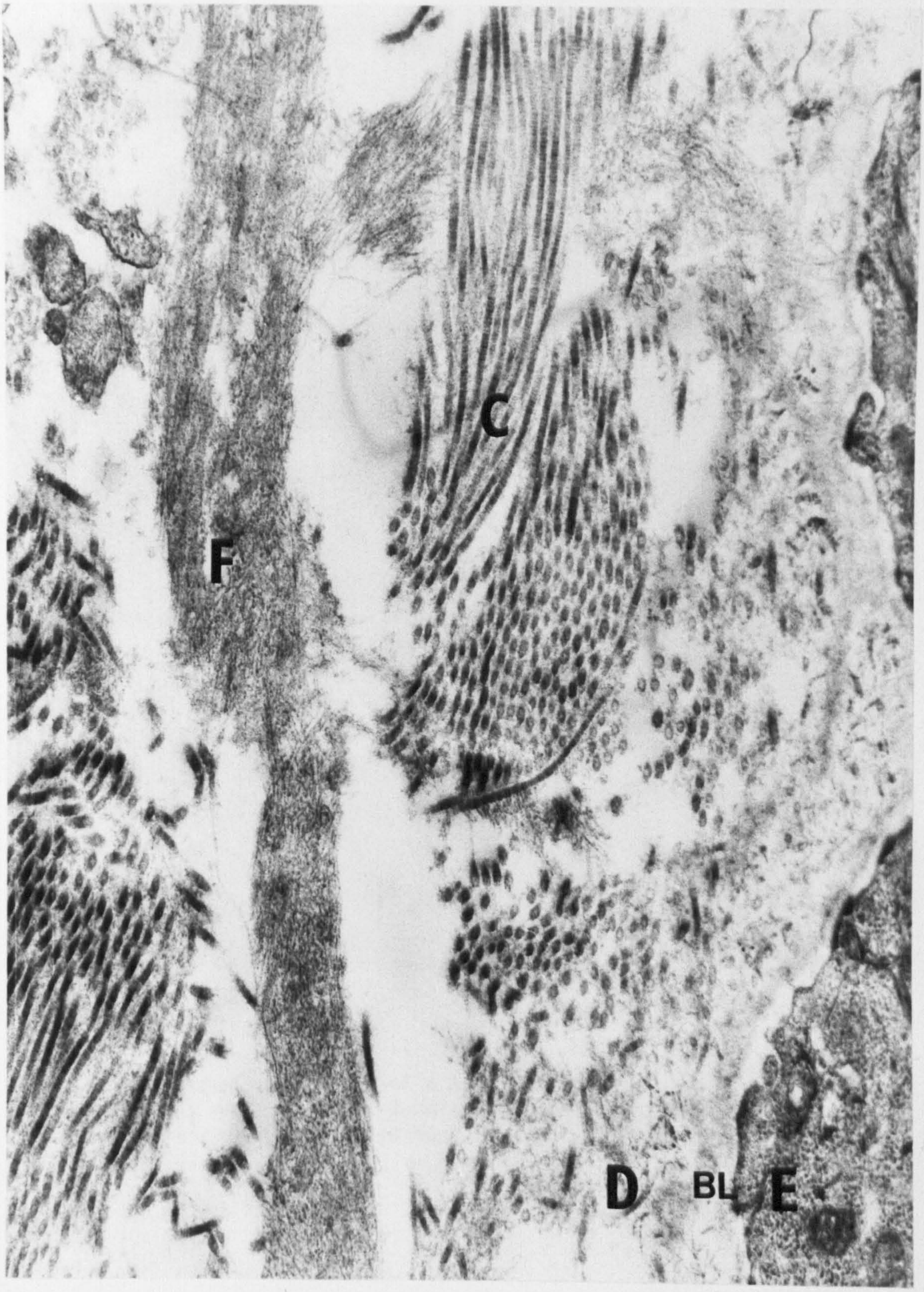


Fig **59** . Electron micrograph of a marginal area showing fibrin deposition (F) mainly in the papillary dermis. D Dermis, E Epidermis, BL Basal lamina, C Collagen fibres. (X 34687)

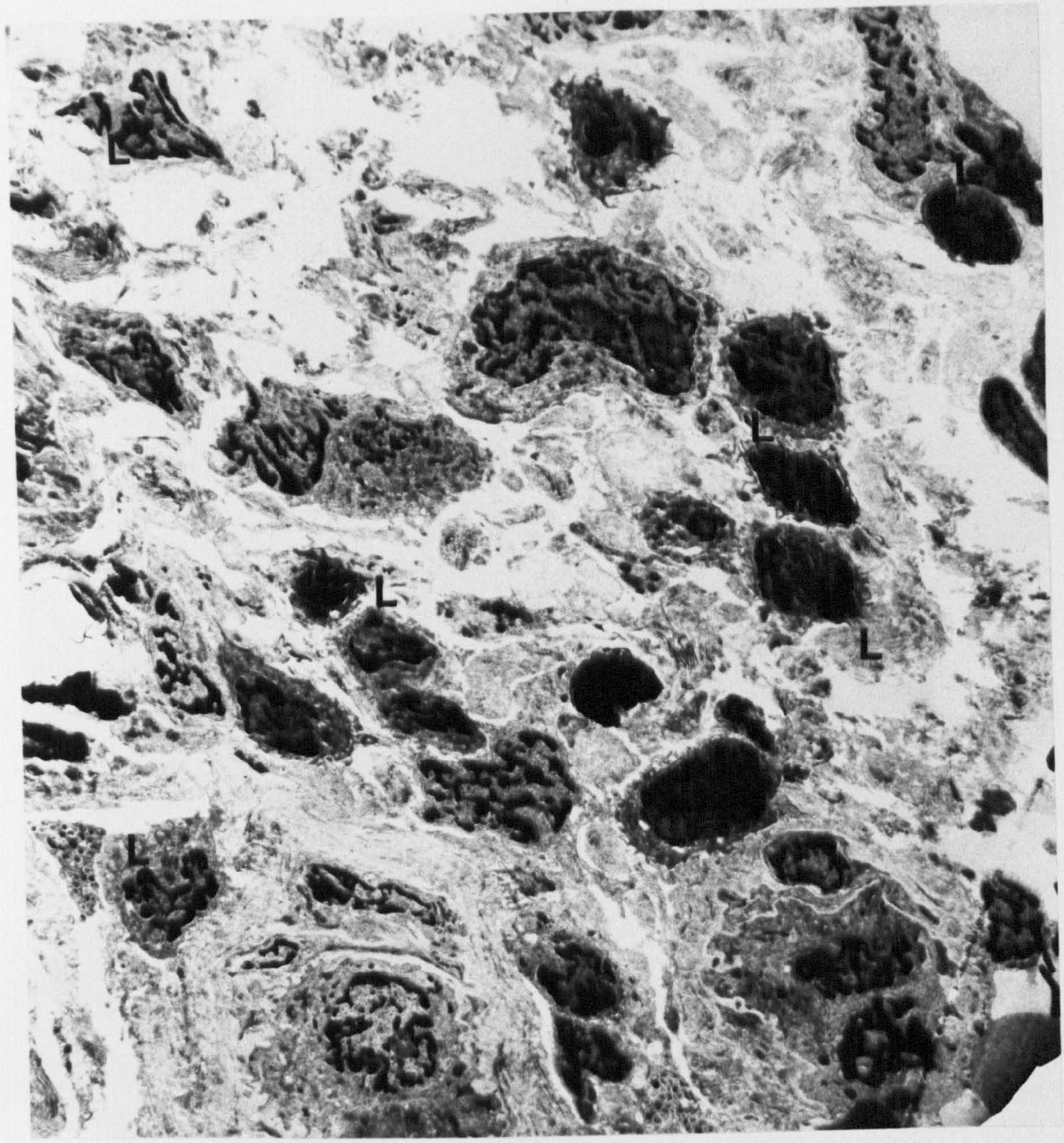


Fig 60. Electron micrograph of a marginal area showing inflammatory changes mainly oedema of the dermis with atypical lymphocytes (L). Many of these cells have convoluted nuclei and looked Sézary like cells. (X 4000)

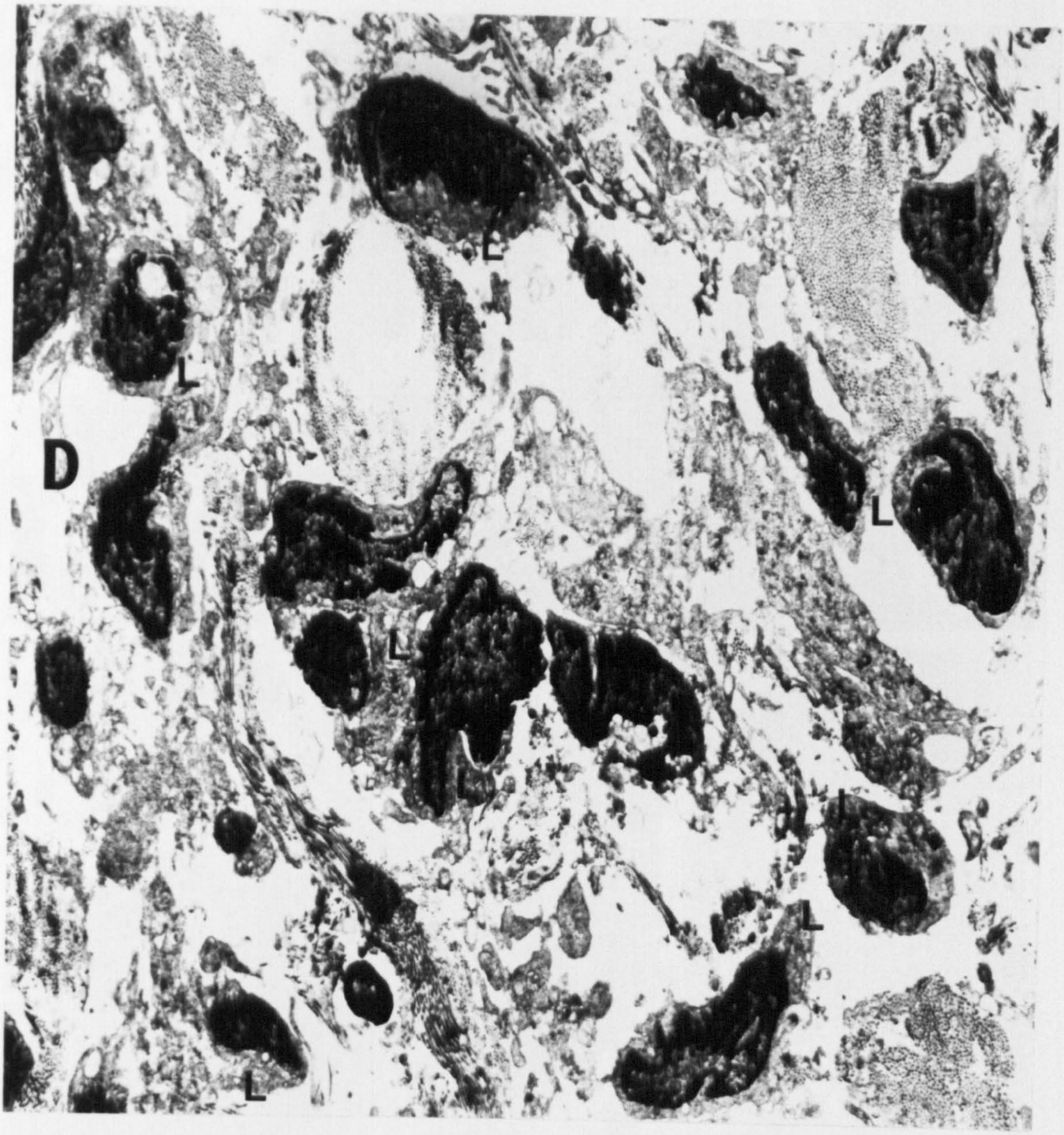


Fig **61** . Electron micrograph of an involved area showing inflammatory changes in the dermis (D), mainly oedema and atypical lymphocytes (L). (X 6000)

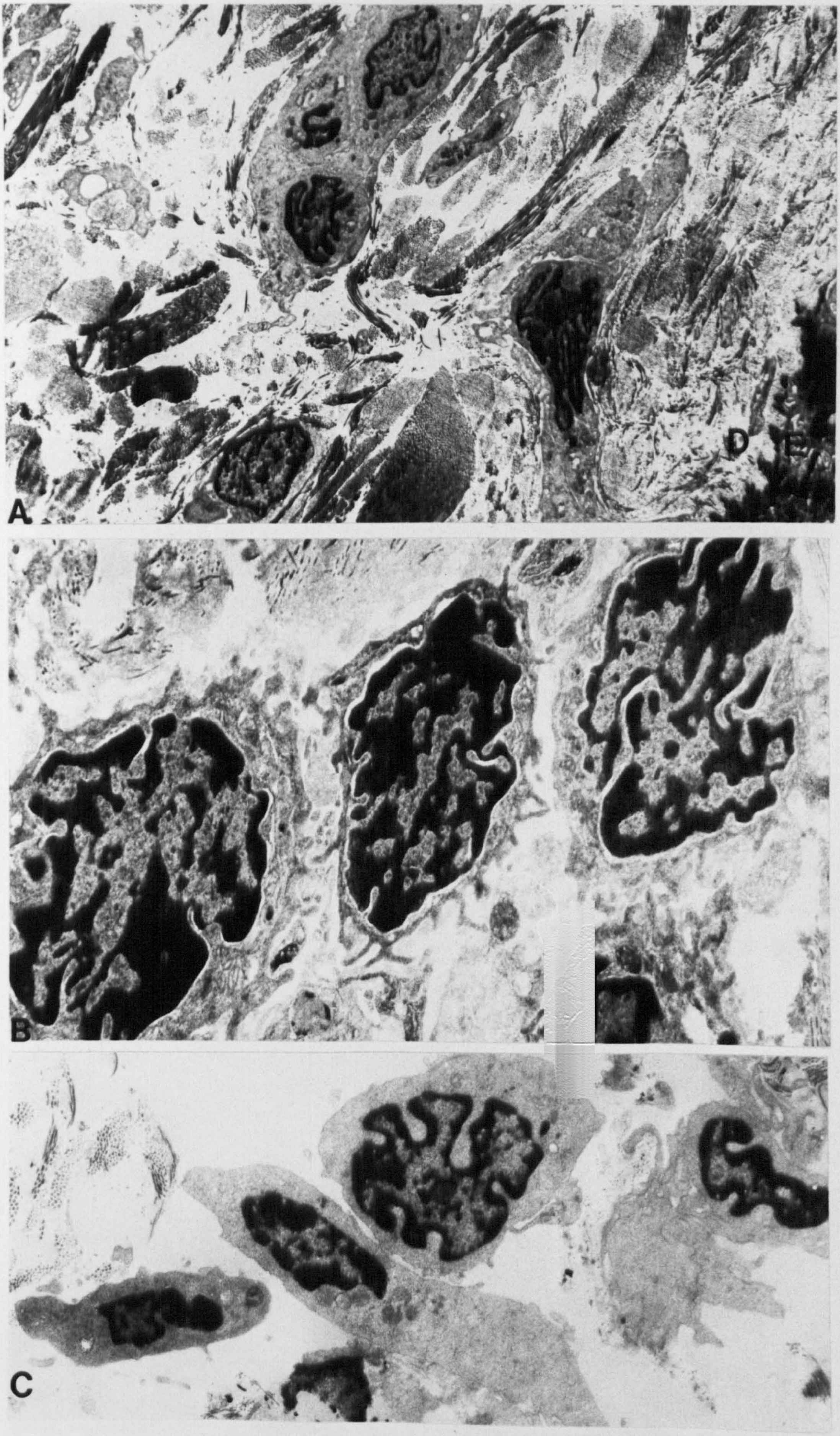


Fig62 . Electron micrograph showing the lymphocytes that can be seen in the dermis of vitiligo patients. D Dermis, E Epidermis. (A X 3840, B X 15120, C X 12000)

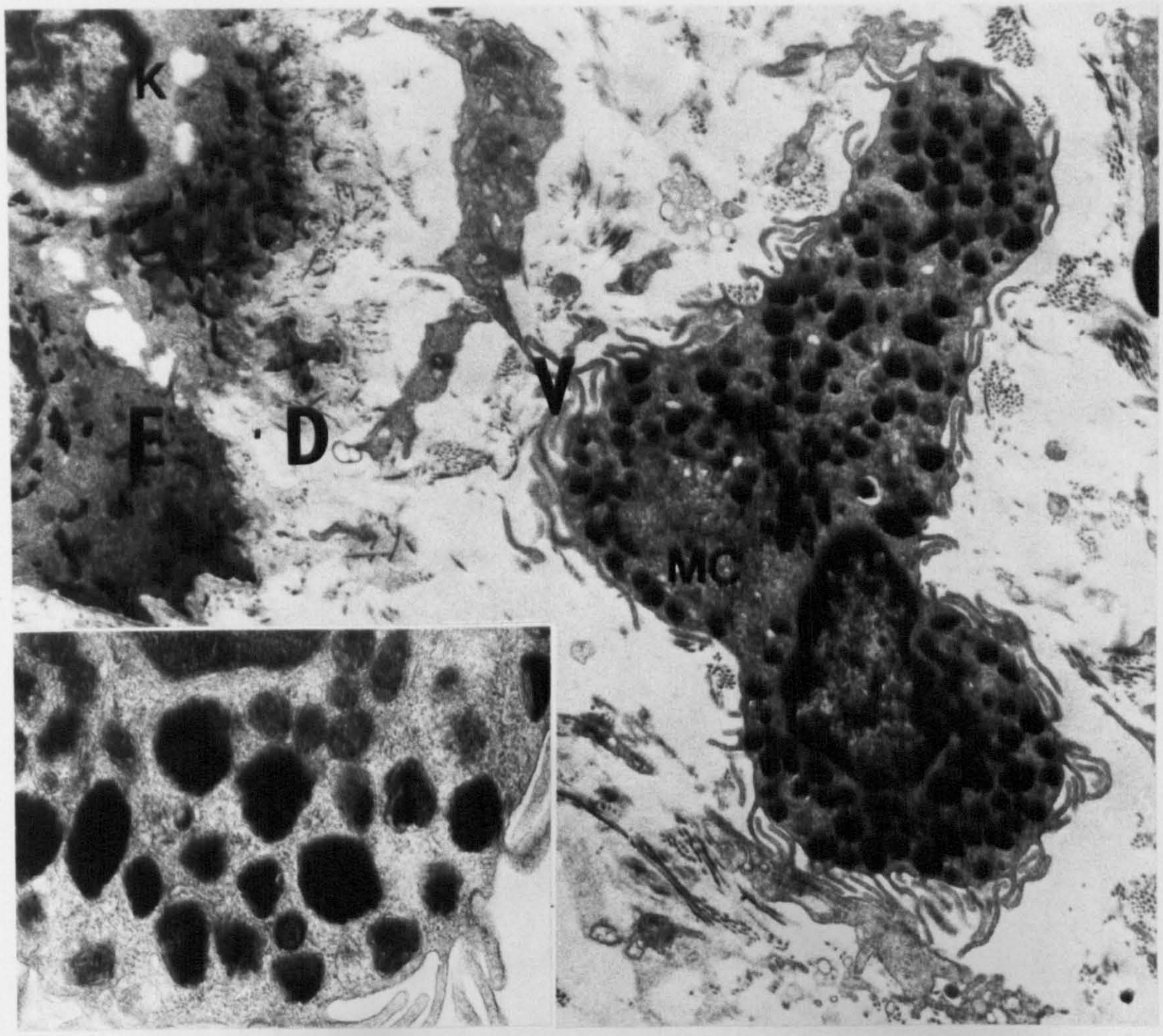


Fig 63 . Electron micrograph showing a normal mast cell (MC). Inset showing ultrastructure of its granules. V Villous processes, D Dermis, E Epidermis. (X 9600)

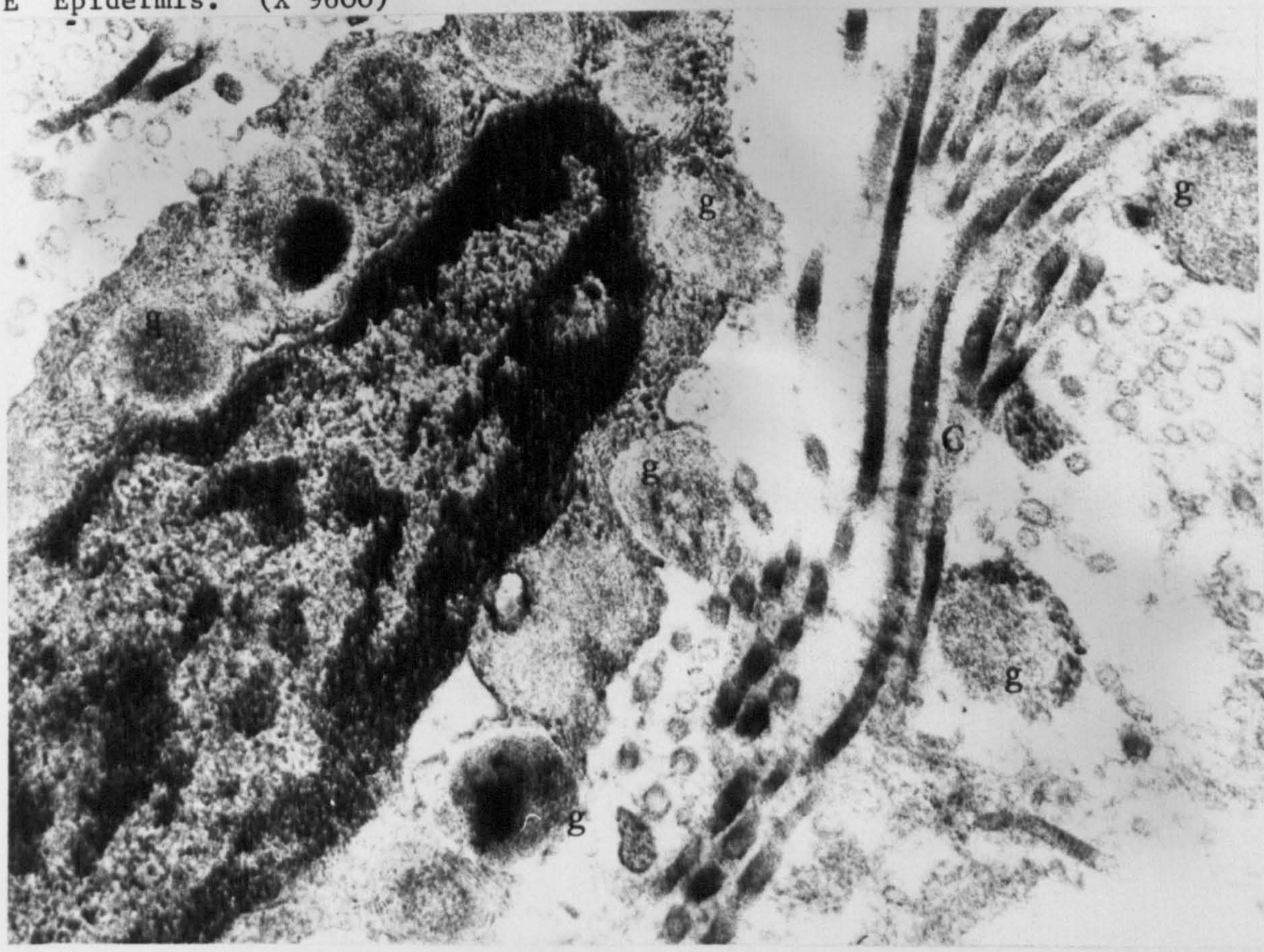


Fig 64 . Electron micrograph of a marginal area showing degranulating mast cell. g granules, C Collagen. (X 48000)

number of biopsy specimens in the most lower layer of the epidermis.

(d) Blood Vessels

Some pathological changes were noted in the blood vessels, particularly those in the papillary dermis. Capillaries and small venules showed some thickening of their wall (fig 65,66), due to replication of the basal lamina and amorphous granular material between thse lamellae. These changes were observed in all the areas biopsied, but were more noticeable in the marginal and involved areas of skin. Changes are those that are not frequently seen in the sun-exposed areas of skin.

The other changes noted were somewhat swollen endothelial cells with rather shrunken nuclei and the 'tight' endothelial junctions that are normally seen, appeared to be more widely separated than usual (fig 66,67).

(e) Cutaneous Nerves

Dermal nerves: The unmyelinated nerves were seen containing either small (60nm in diameter) and large (105 nm in diameter) dense core vesicles of adrenergic nature or small agranular vesicles of cholinergic type. Replication of basal lamina surrounding the axons and Schwann cells was seen and this occured in many of the patients biopsies and from all areas. The Schwann cells surrounding

axons in the involved areas of the skin and occasionally in other sites, not completely enveloped the axons and there appeared to be some regeneration (fig 68,69). Infrequently axons were seen that were involved in the inflammatory response around them and showed oedema (fig 70,71), but otherwise changes seen, including vacuolation of the cytoplasm of the axons, did not differ from those changes as observed in the normal controls.

Intra-epidermal nerves: In a third of the patients intra-epidermal nerves were found. They were present on five occasions in the uninvolved areas, on three occasions in the marginal skin and on four specimens from involved sites. The majority of these nerves consisted of Schwann cell processes with axons. They were either mono-axonal or poly-axonal and were never found in any other site than in the basal layer. On two occasions intra-epidermal adrenergic nerves were observed in association with a secretory melanocyte (fig 72). These nerves had typical small and large dense core vesicles. Usually there appeared to be no connection between the dermal and intra-epidermal nerves. However, in a few cases serial sections indicated that the dermal nerves crossed the dermo-epidermal junction zone and into the epidermis (fig 73). The intra-epidermal nerves found were normal and did not differ from those found

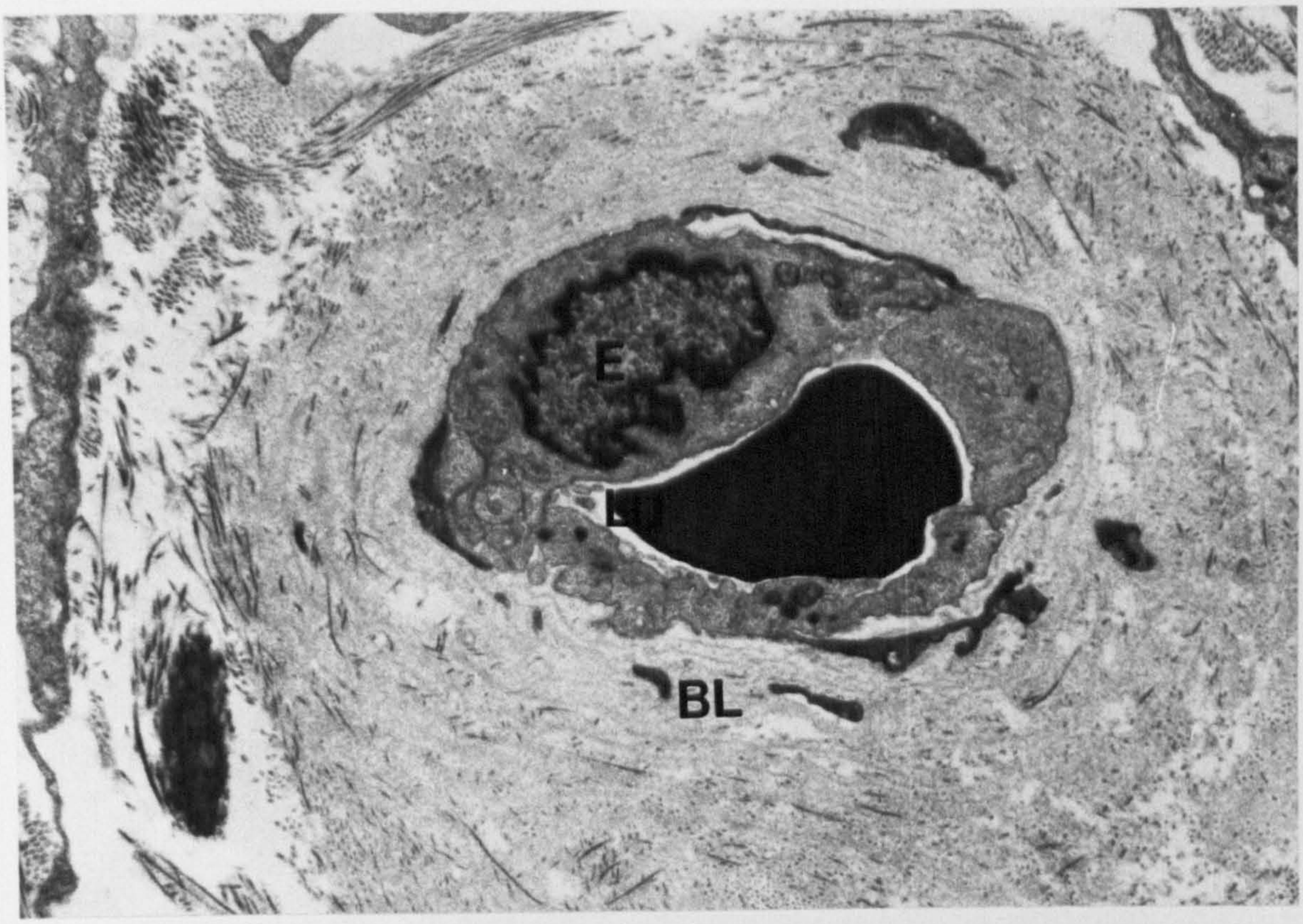


Fig 65 . Electron micrograph of a marginal area showing a blood vessel with a thickened wall, mainly due to replication of its basal lamina (BL). E Endothelial cell, LU Lumen containing red blood cell. (X 9600)

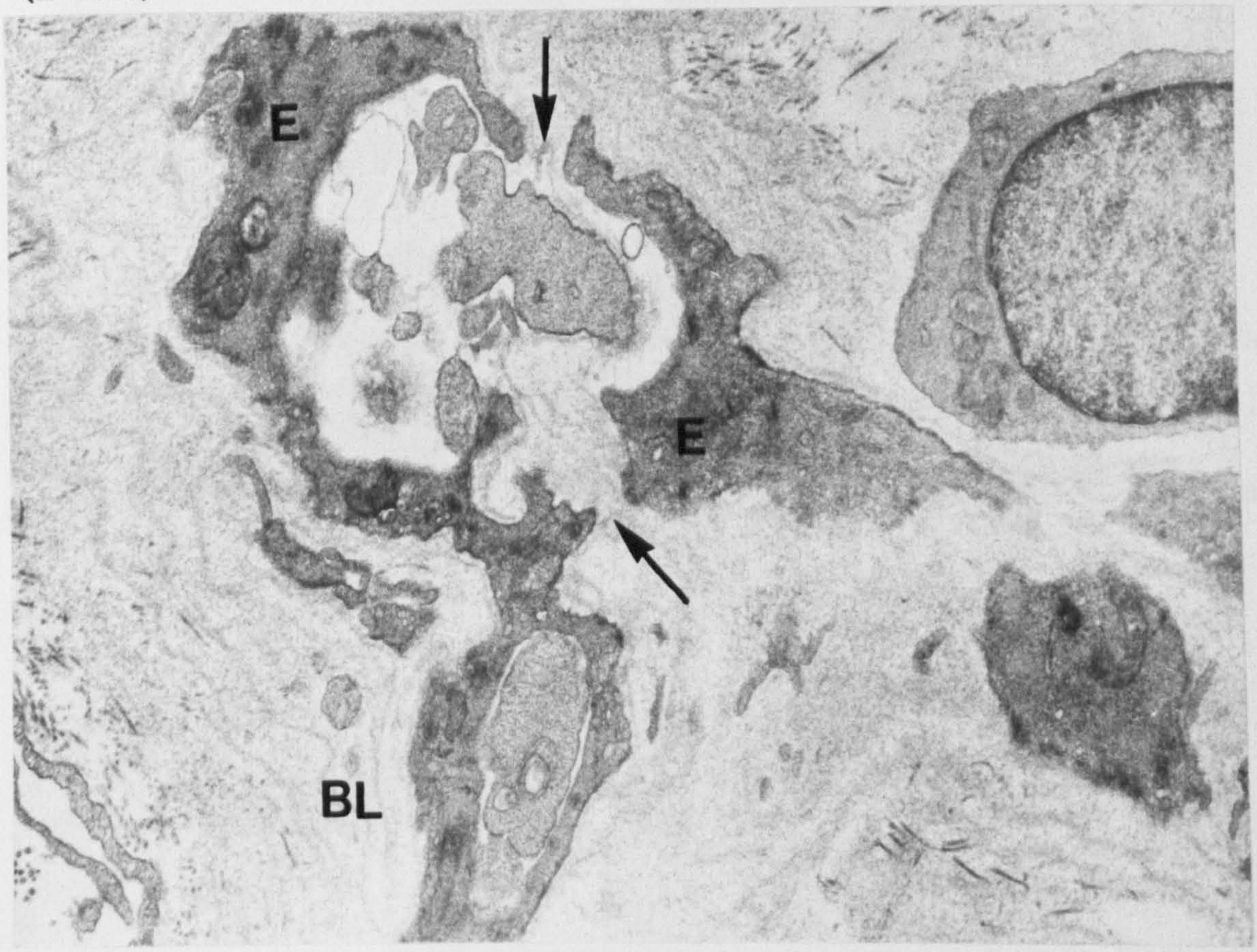


Fig 66 . Electron micrograph of a marginal area showing a blood vessel with inflammatory changes, mainly degeneration of endothelial cells (E) and interendothelial gaps (arrow). BL basal lamina. (X 15120)

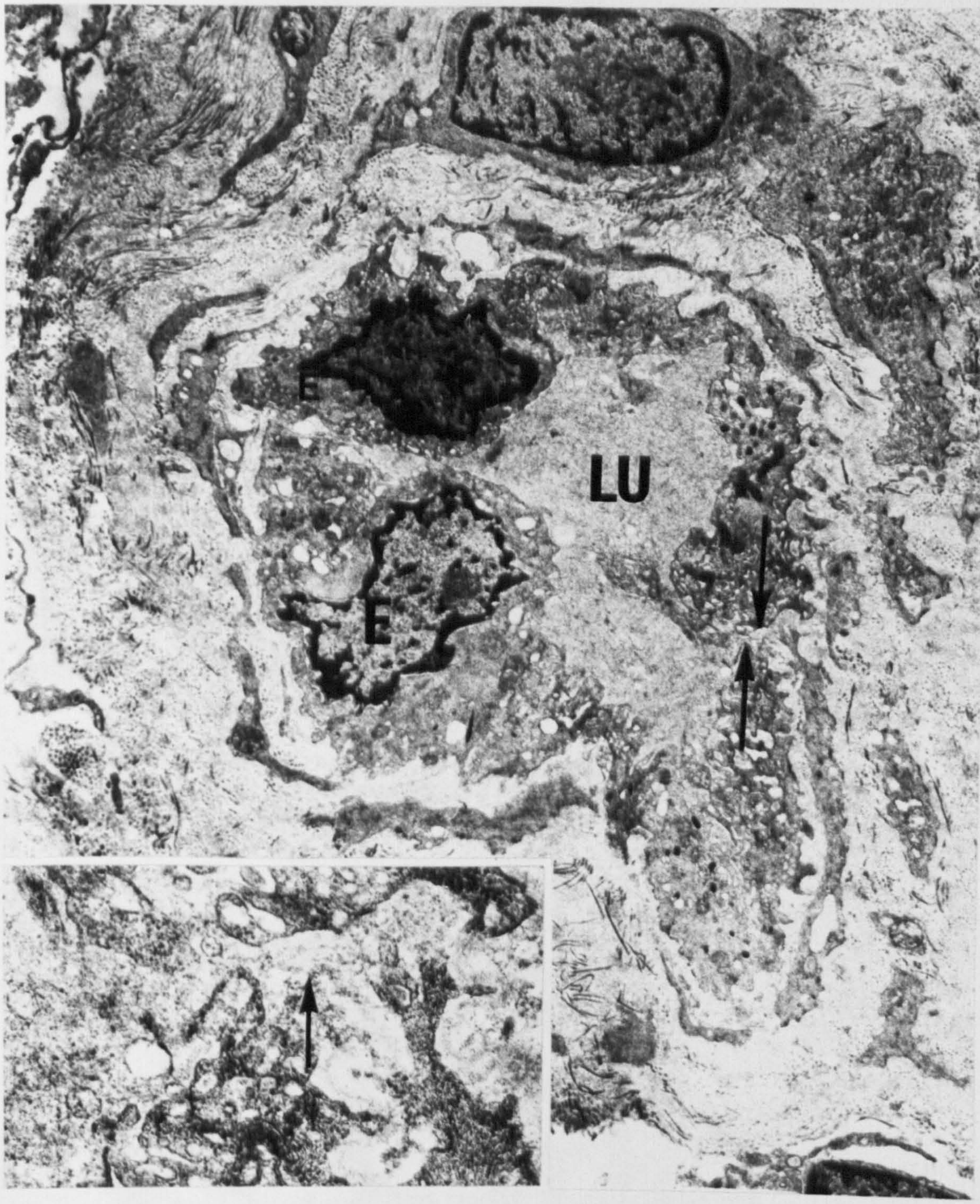


Fig67. Electron micrograph of a marginal area showing dermal blood vessel with inflammatory changes like interendothelial gap (arrows). Inset showing higher magnification of interendothelial gap.
E Endothelial cell, LU Lumen. (X 12333)

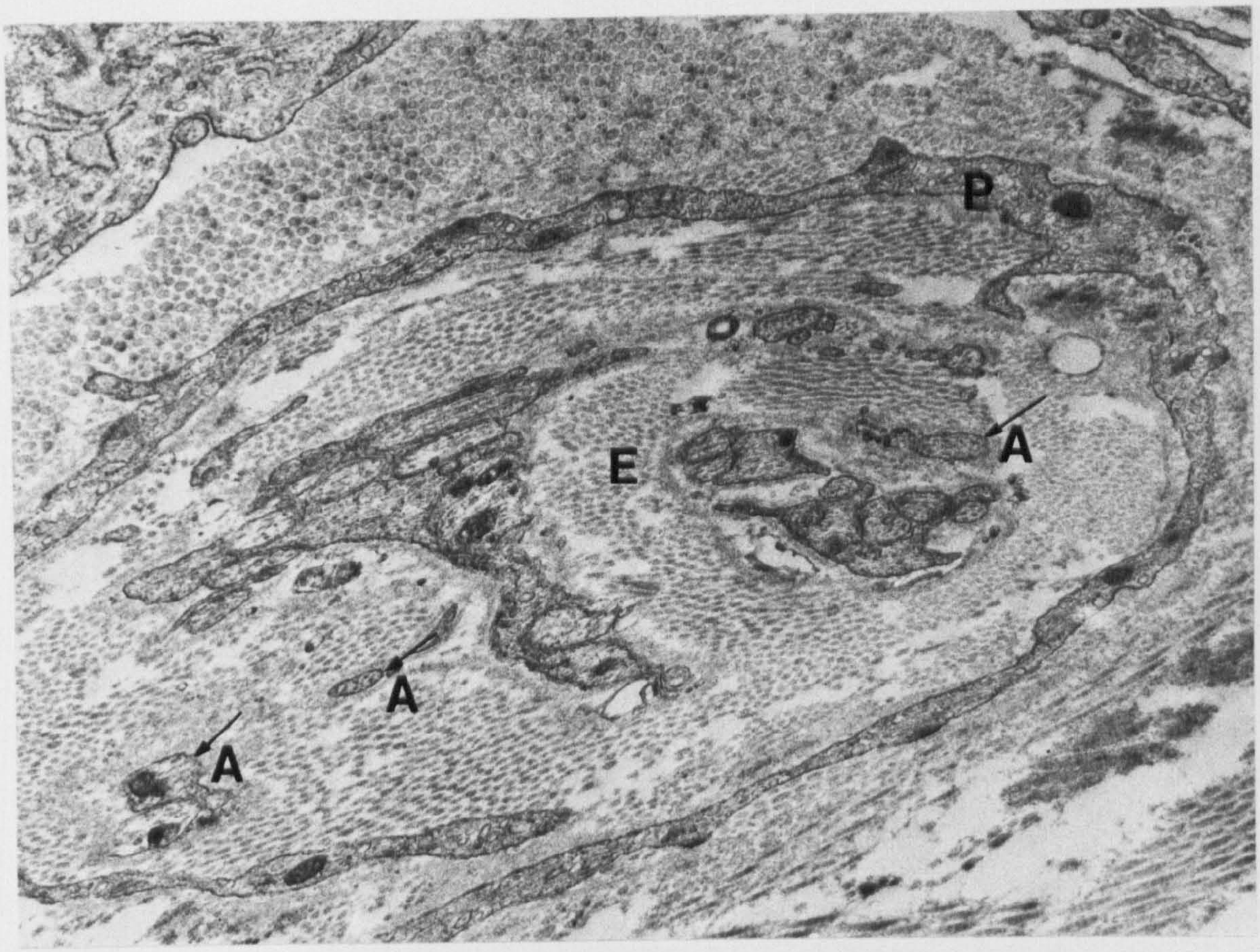


Fig 68 . Electron micrograph of an involved area showing a regenerating nerve with many of its axons (A) denuded.
E Endoneurium, P Perineural cell. (X 15400)

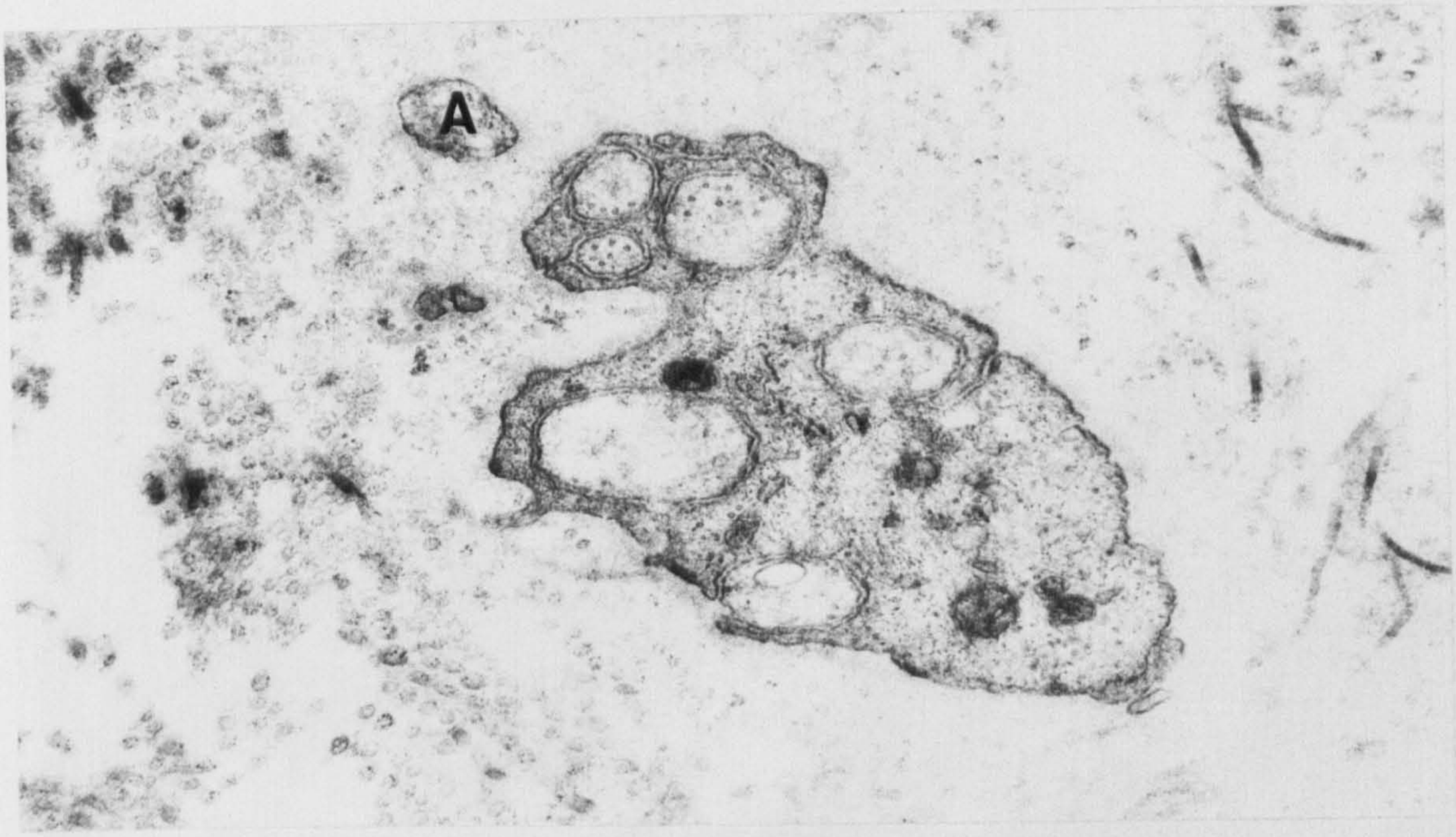


Fig 69 . Electron micrograph of an involved area showing a polyaxonal nerve with one of its axon denuded (A). (X 26000)

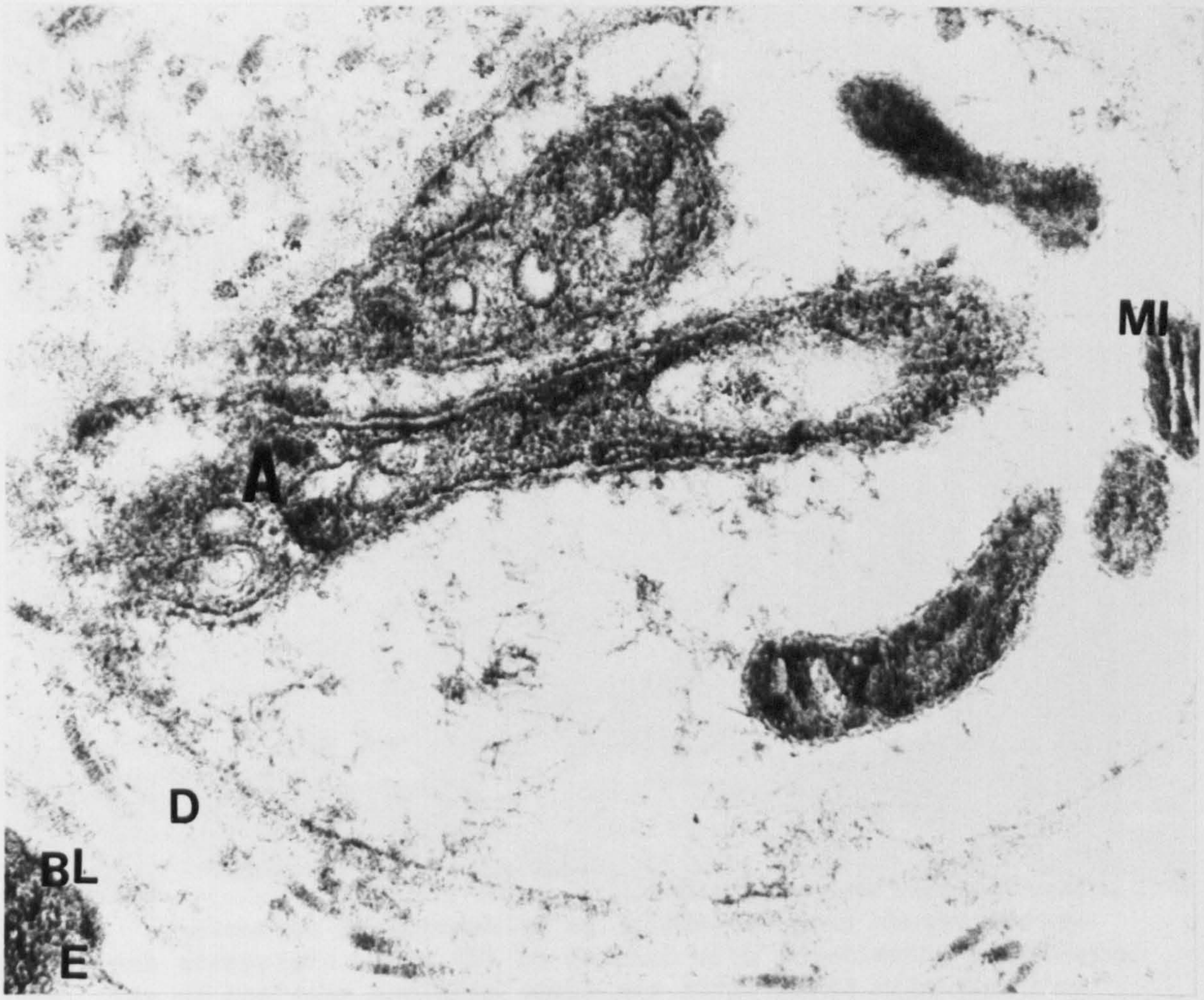


Fig **70**. Electron micrograph of an involved area showing degeneration of Schwann cell process leaving denuded axons (A) and few mitochondria (MI). BL Basal lamina, D Dermis, E Epidermis. (X 48000)

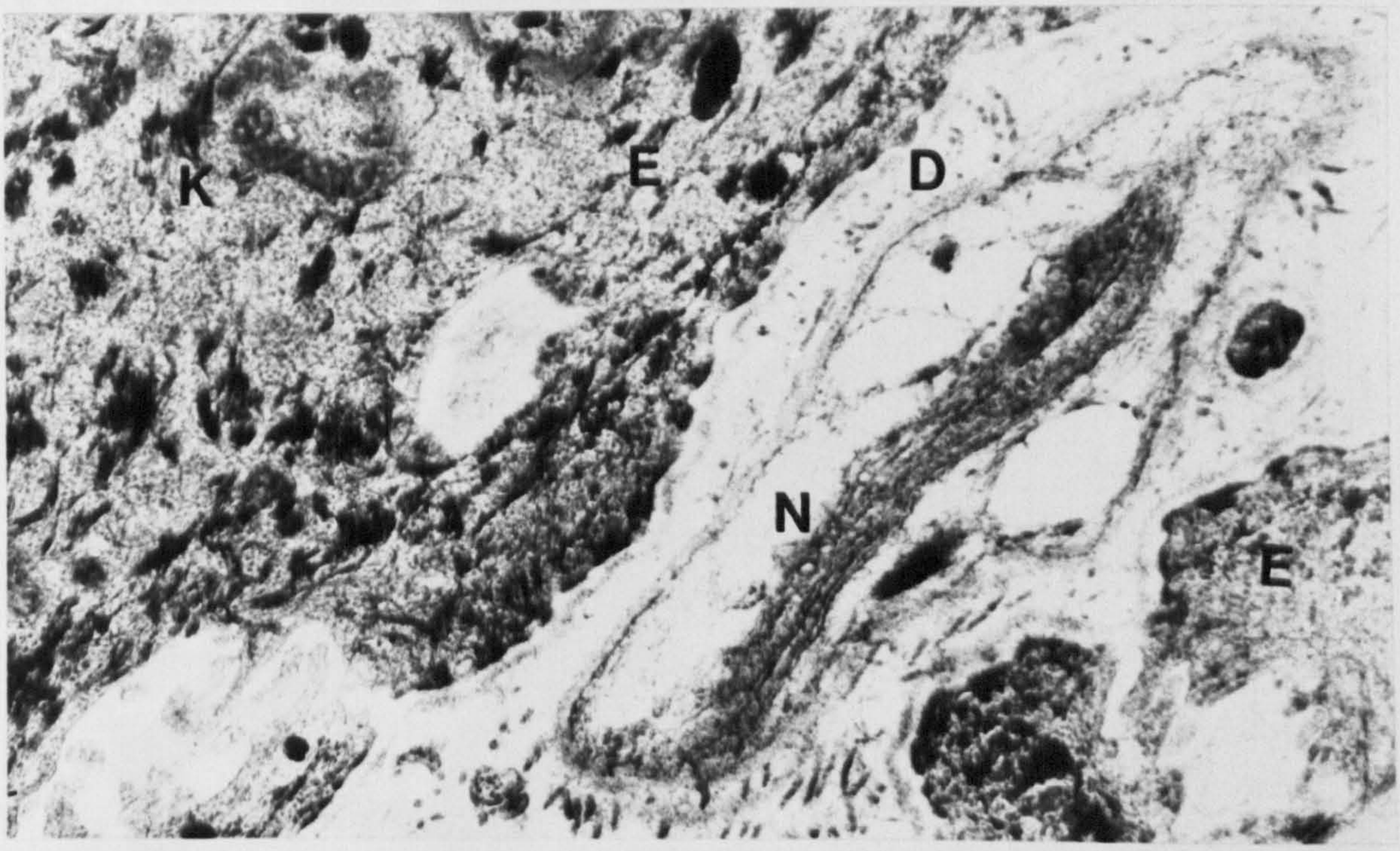


Fig **71** . Electron micrograph of a marginal area showing papillary nerve (N) sharing the inflammatory reaction in the epidermis (E) and dermis (D). (X 24000)

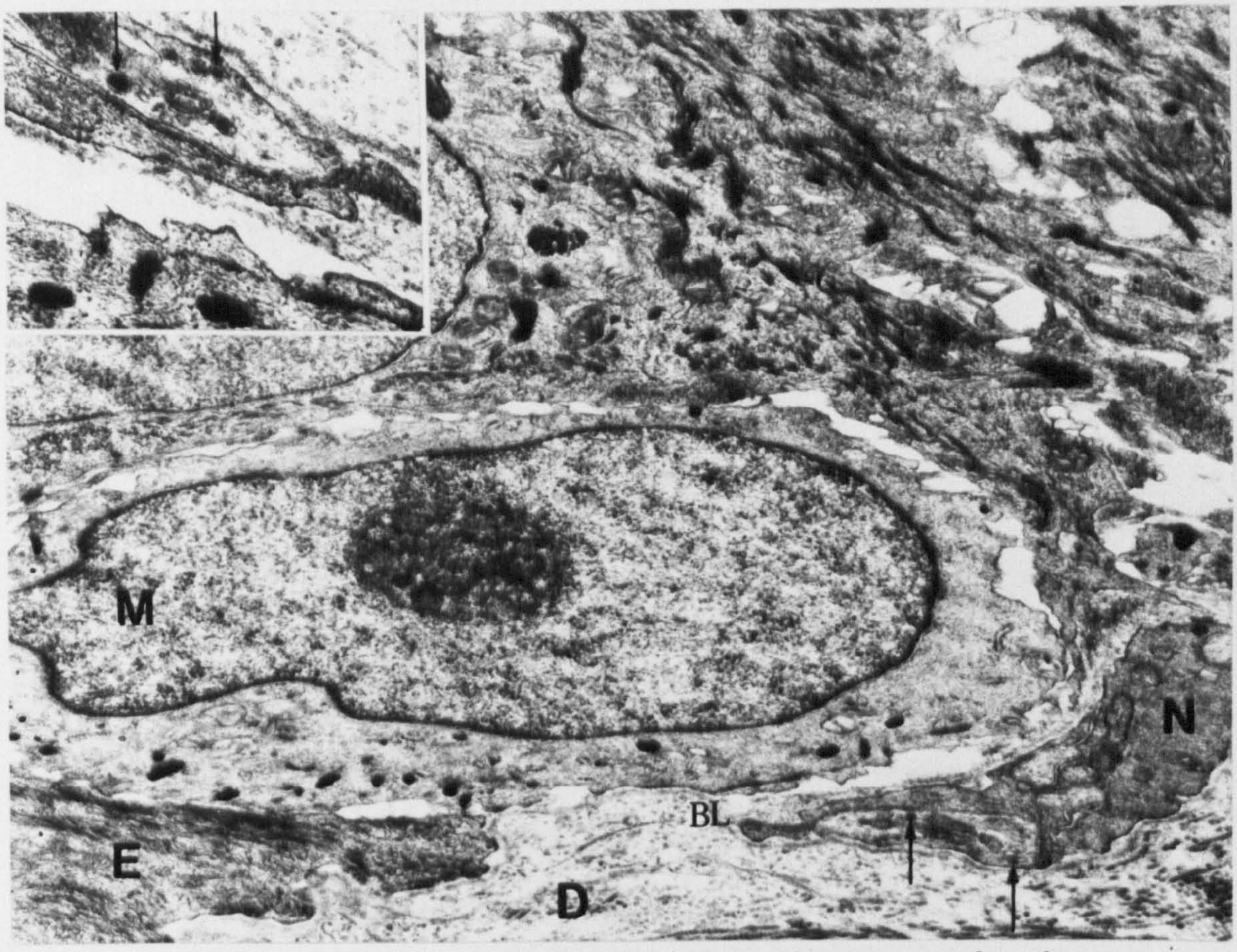


Fig 72 . Electron micrograph of an uninvolved area showing intra epidermal adrenergic nerve (N) in contact with functioning melanocyte (M). One of the axon contains small and large dense core vesicles (arrows) which are shown in the inset. D Dermis, BL Basal lamina, E Epidermis. (X 15120)

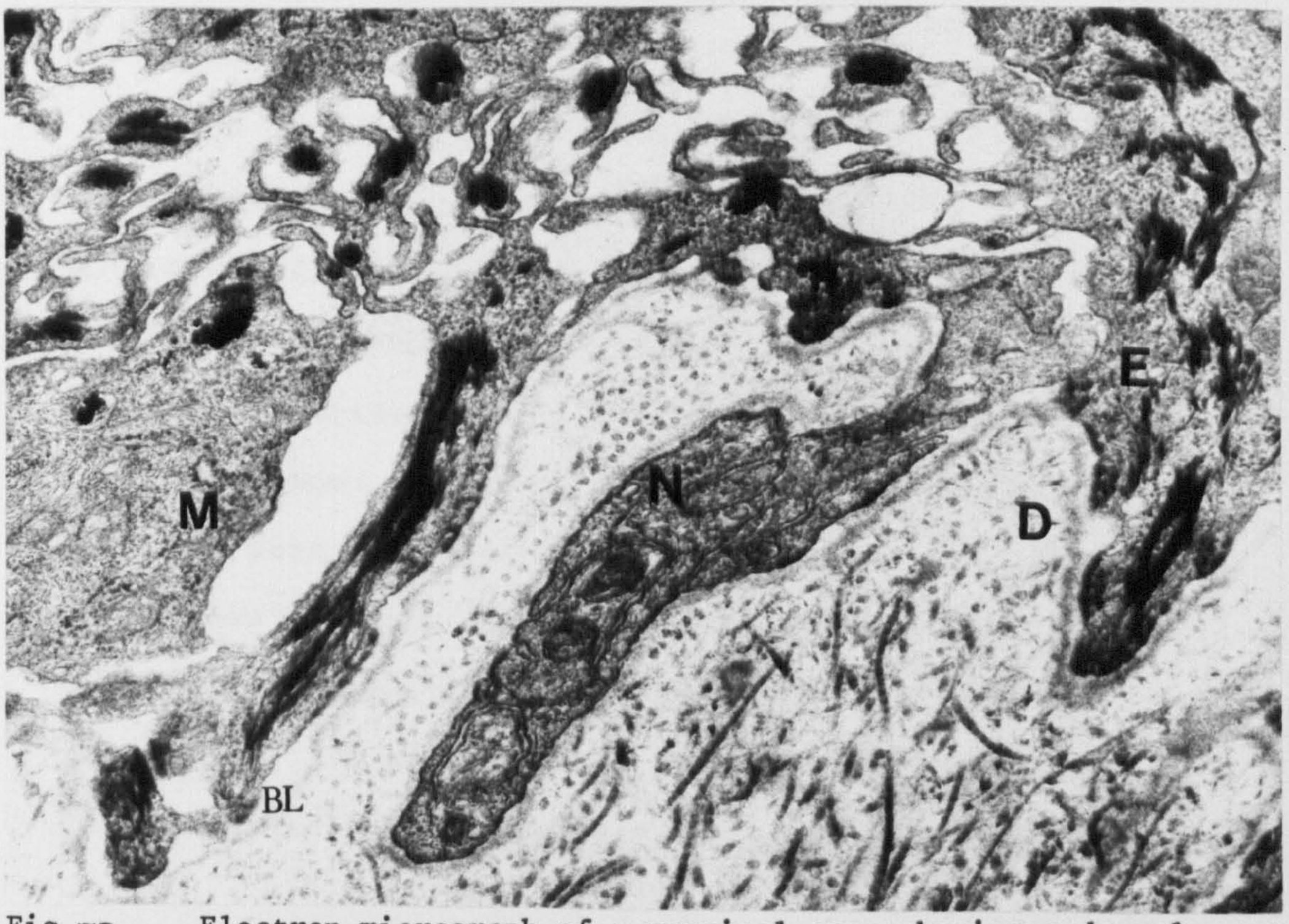


Fig 73 . Electron micrograph of a marginal area showing a dermal polyaxonal nerve (N) passing into the epidermis (E). D Dermis, BL Basal lamina, M Melanocyte dendrite. (X 24000)

in the dermis. Intra-epidermal nerves were only found in the biopsies taken from the patients with vitiligo and were not present in those biopsies taken from normal controls.

(f) Filamentous Colloid/Amyloid Bodies

These were found in nearly 50% of the patients and were present in all areas (Table XXV). They were more frequent in the marginal and uninvolved skin than in the involved vitiliginous areas. When compared with normal controls they were more frequent. Most of these bodies were rather globular in shape and varied in size from 7-20μ. They were always found in the papillary dermis close to the dermo-epidermal junction zone (fig 75). In many of the biopsy specimens they were also seen in the basal layer of the epidermis (fig 74).

The colloid/amyloid bodies consisted of filaments that formed whorls (fig 75), but were also with straight unbranched filaments as seen in amyloid (fig 76,77). However, they had not infrequently a mixed appearance of colloid and amyloid filaments (fig 74). Most of these filaments resembled more those as seen in colloid bodies and had whorled appearance. These filaments resembled those cyto-filaments seen in the melanocytes (fig 78). The diameter of these filaments ranged from 70-130Å with a mean of 100Å. Macrophages (histiocytes) were not

No.	Vitiligo			Normal Controls
	Uninvolved	Marginal	Involved	
1	--	Neg	Neg	Neg
2	-	Neg	Neg	Neg
3	-	Neg	Neg	Pos
4	Neg	Neg	Neg	Neg
5	-	Neg	Neg	Neg
6	Neg	Neg	Neg	Neg
7	Neg	Neg	Neg	Neg
8	-	Pos	Neg	Neg
9	-	Neg	Pos	Neg
10	-	Pos	Neg	-
11	Neg	Neg	Neg	-
12	Pos	Neg	Neg	-
13	Neg	Pos	Pos	-
14	Pos	Pos	Pos	-
15	Pos	Pos	Neg	-
16	Pos	Pos	Pos	-
17	Pos	Pos	Pos	-
18	Pos	Neg	Pos	-
19	-	Neg	Neg	-
20	Neg	Neg	Neg	-
21	Neg	Neg	Neg	-
22	Neg	Pos	Pos	-
23	Neg	Pos	Pos	-
24	Pos	Pos	Neg	-
25	Neg	Neg	Pos	-
26	Neg	Pos	Pos	-
27	Pos	Pos	Neg	-
28	Pos	Pos	Pos	-
29	Pos	Pos	Neg	-

Table XXV. Indicating the presence or absence of filamentous colloid/amyloid bodies on electron microscopic study in patients with vitiligo and in normal controls.

infrequently seen in relation to those colloid/amyloid bodies in the dermis.

Not infrequently those filamentous bodies contained electron dense granules that were undoubtedly melanosomes. Also remnants of other cellular organelles were to be found. A filamentous body was also seen in the papillary dermis of the involved skin and this was enveloped by a dendrite of a basal indeterminate cell that appeared to be dipping into the dermis through a break into the basal lamina (fig 79).

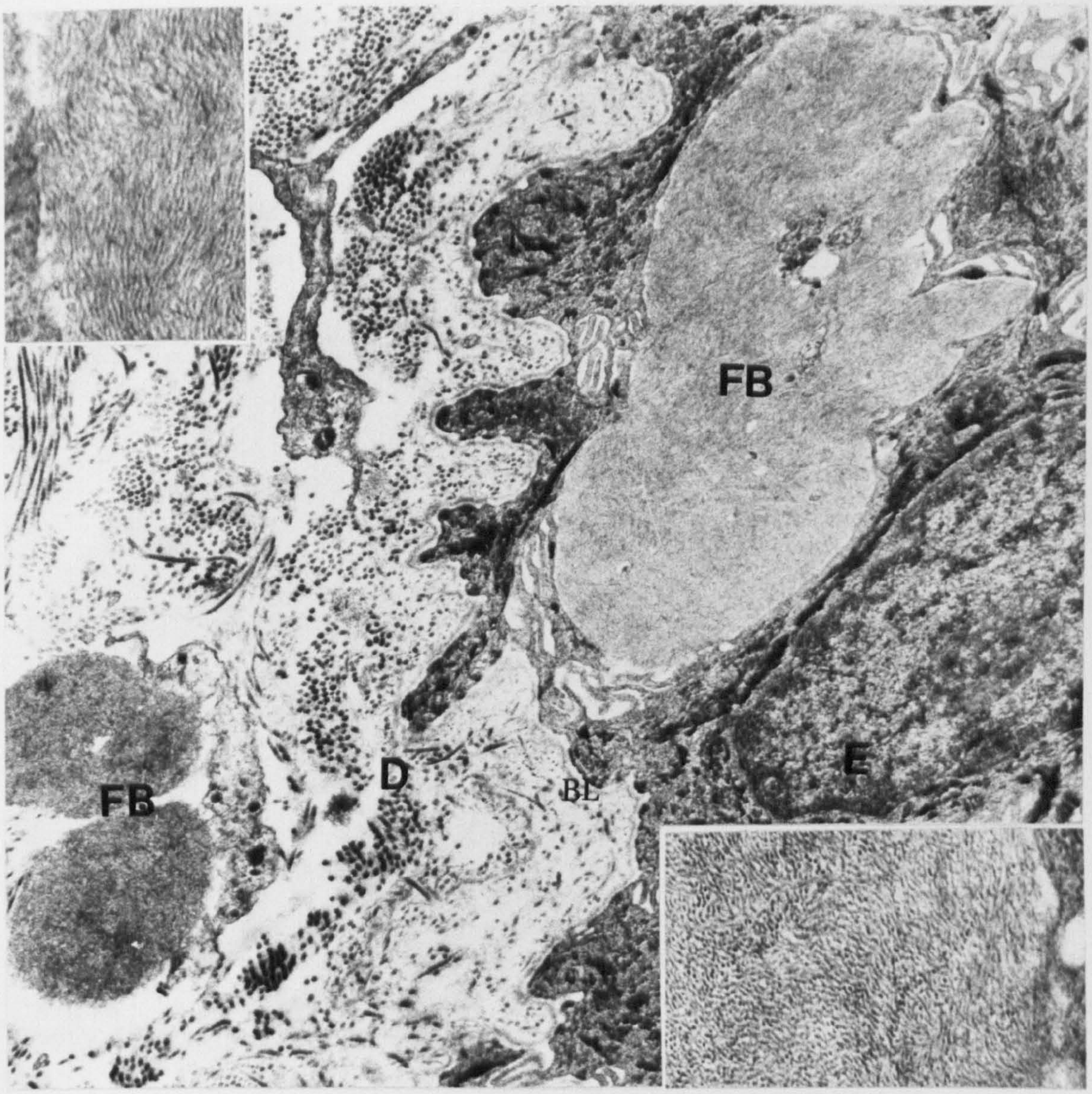


Fig 74 . Electron micrograph of a marginal area showing many filamentous bodies (FB). The intra-epidermal filamentous body is showing features of both colloid (upper inset) and amyloid (lower inset) filaments. D Dermis, E Epidermis, BL basal lamina. (X 15120)

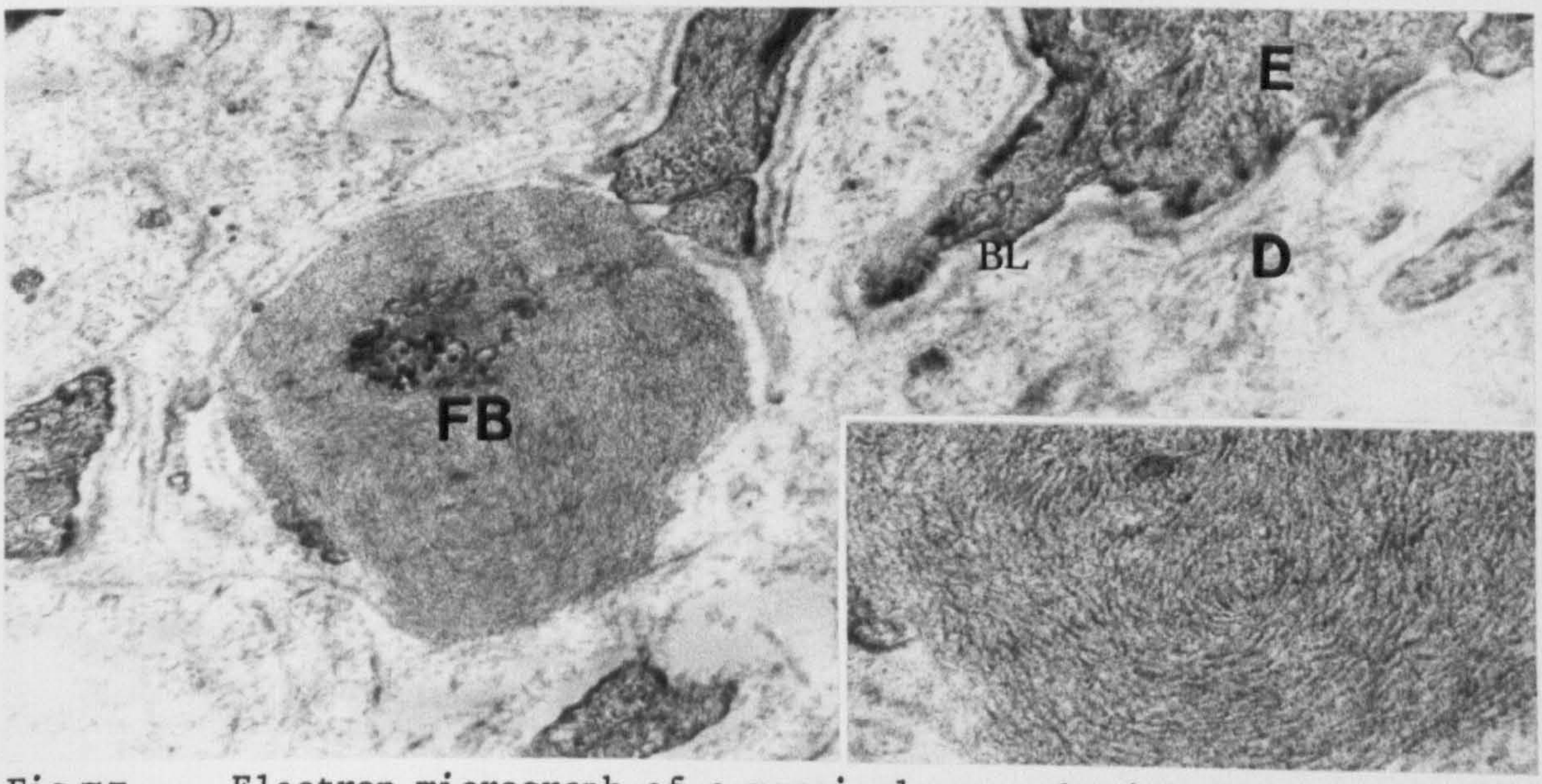


Fig 75 . Electron micrograph of a marginal area showing dermal filamentous body (FB) which is still surrounded by the basal lamina of dermo-epidermal junction. The filaments in this body mainly of colloid nature. Inset showing higher magnification of a filamentous body. D Dermis, BL basal lamina, E Epidermis. (X 24000)

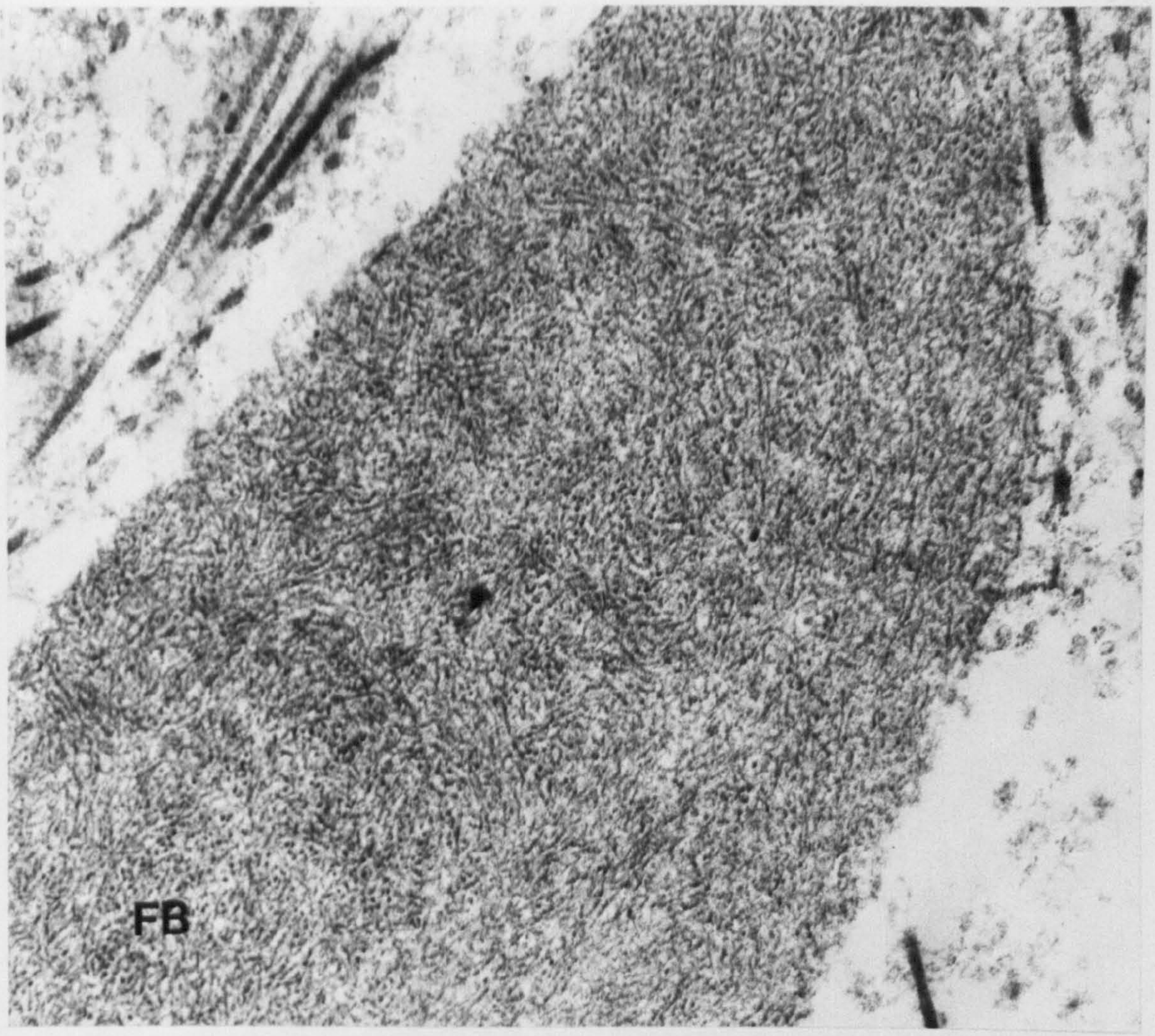


Fig 76 . Electron micrograph of an involved area showing a filamentous body (FB) of amyloid nature. (X 46000)

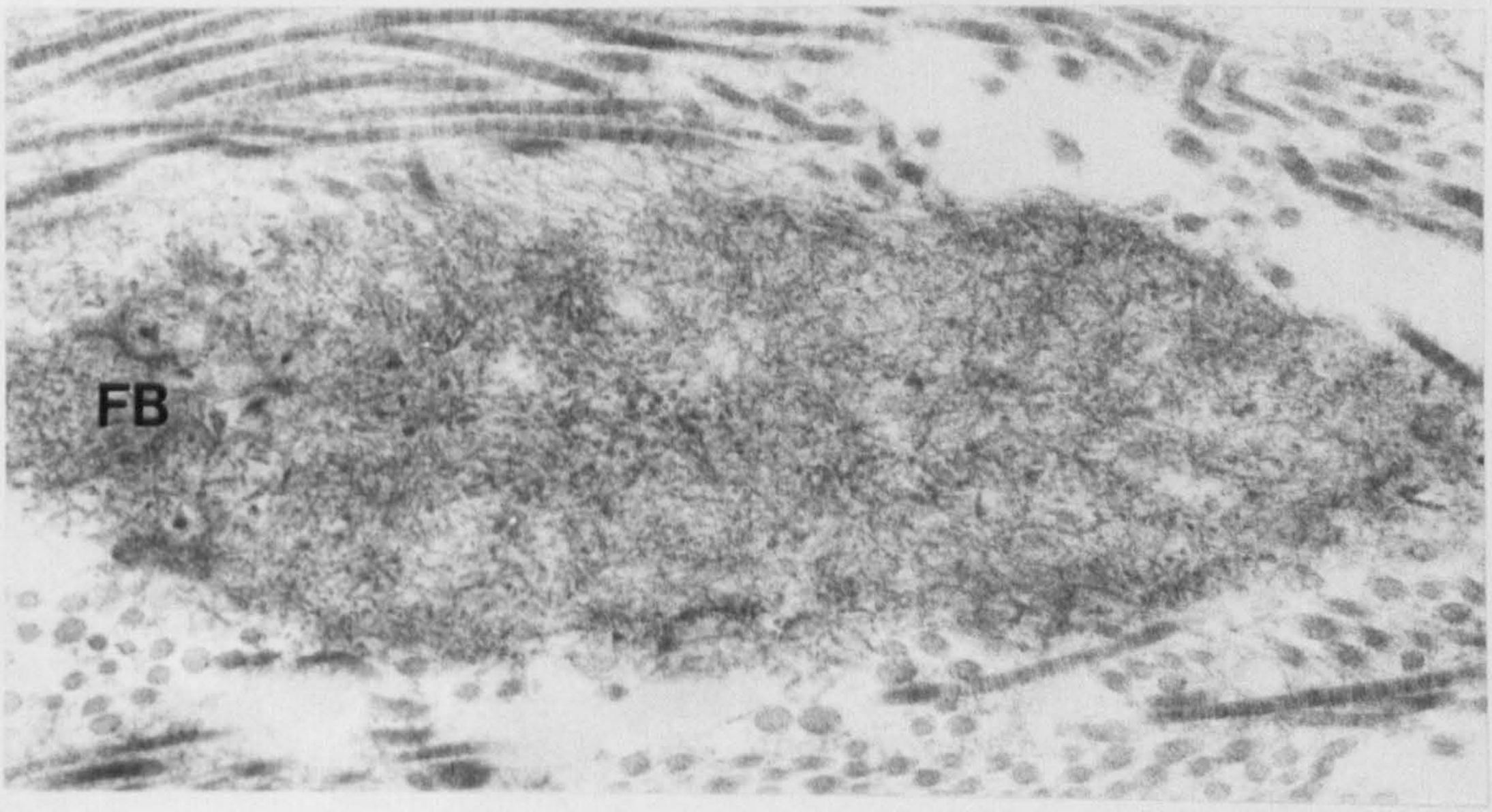


Fig 77 . Electron micrograph of an involved area showing a filamentous body (FB) of amyloid nature. (X 36800)

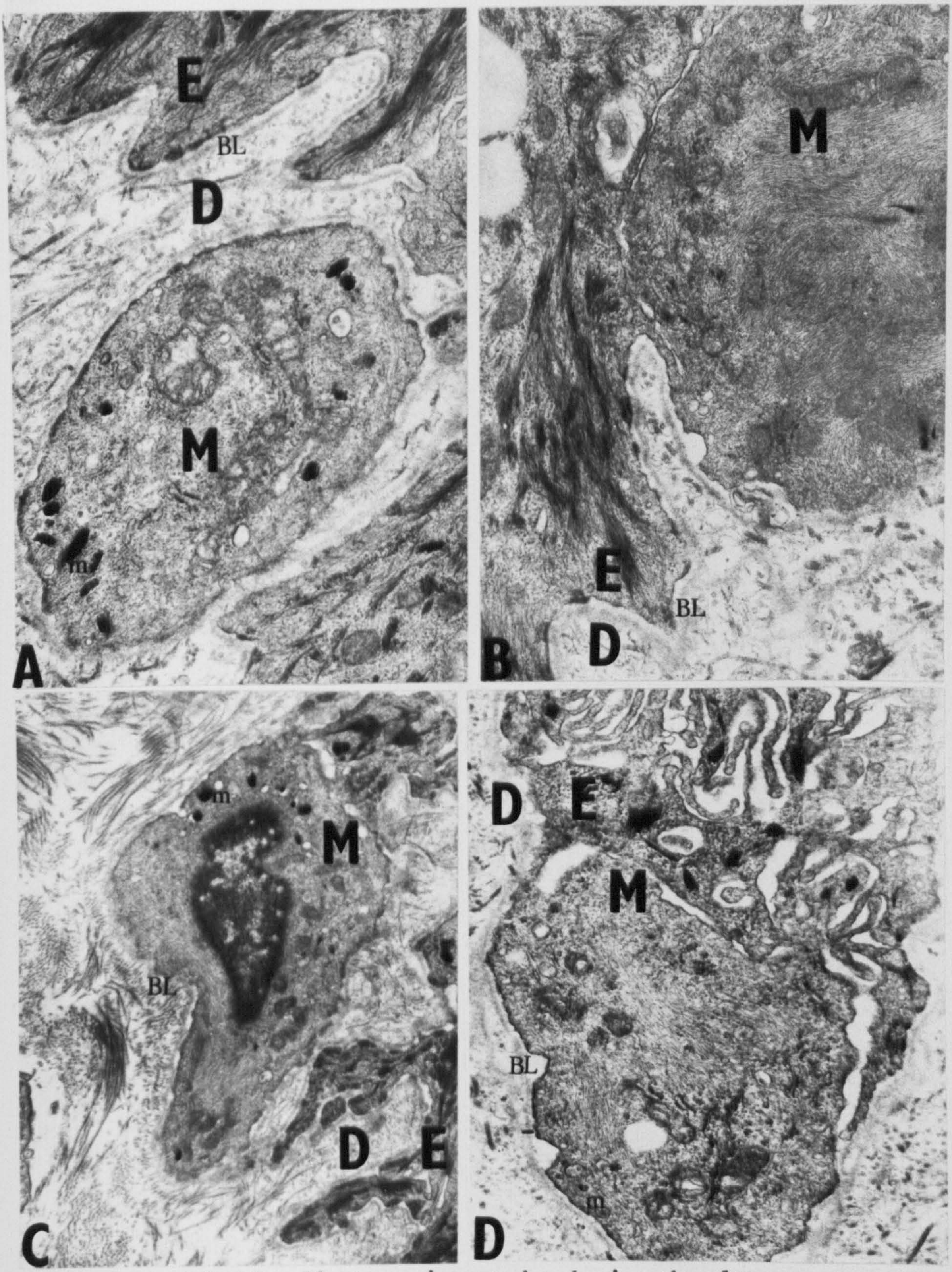


Fig 78 . A,B,C,D - Electron micrographs showing the ultrastructure of melanocytes (M) and their dendrites which are similar to that of filamentous bodies. D Dermis, BL basal lamina, E Epidermis, m melanosomes. (A, X 10588, B, X 10144, C, X 11650, D, X 3750)

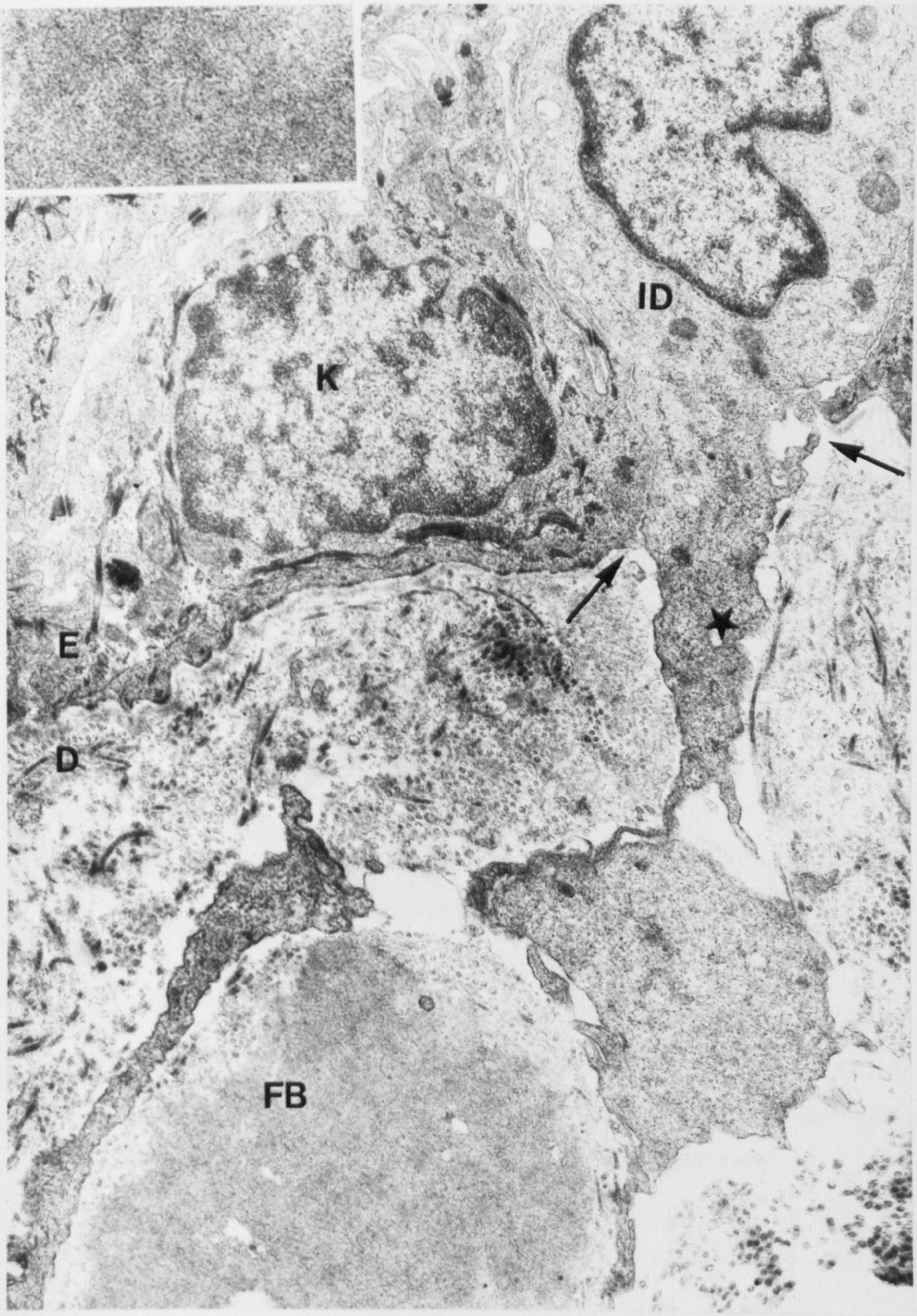


Fig 79 . Electron micrograph of an involved area showing a basal indeterminate cell (ID). Its dendrite is dropping down into the dermis (D). Filamentous body (FB) has probably originated from the dendrite (asterix) of the indeterminate cell. Inset showing higher magnification of the filamentous body. E Epidermis, arrows showing a break in the dermoepidermal junction. (X 22050)

C. OCCUPATIONAL VITILIGO (LEUCODERMA)

Biopsies were taken from two patients with occupational vitiligo who had been in contact with paratertiary butyl phenol. The results of the histological studies noted on the biopsies taken from these two patients are shown.

(a) Epidermis

Melanocytes were frequently seen in the basal layer of the epidermis of the uninvolved skin. They were markedly reduced in the marginal and completely absent in the involved areas. Melanosomes were found and tended to be singly distributed within the keratinocytes in the marginal and uninvolved areas. No melanosomes were found in the keratinocytes in the involved areas.

Langerhans cells were found mainly suprabasally, but also within the basal layer of the epidermis. They seemed to be increased in the involved areas when compared with the biopsies taken from other sites. Indeterminate cells were rarely seen.

The epidermis showed inflammatory changes (fig 80) in both patients with spongiosis, particularly in the marginal and involved areas in one and marginal in the other. There was some disruption of the epidermis and a number of degenerated keratinocytes were present. Mononuclear cell infiltrate was found within the epidermis and this occured in various foci and was

present in both patients, particularly in the marginal and involved areas. It was also seen in the uninvolved skin of one of the patients. Cells consisted of lymphocytes and histiocytes and of the same appearance as those found in the underlying dermis. Several of these dermal cells were to be seen entering the epidermis through breaks in the dermo-epidermal junction (fig 80). Many of these lymphocytes had convoluted nuclei and were to be found in association with histiocytes, melanocytes and Langerhans cells (fig 81).

(b) Dermis

Inflammatory changes were to be found in the dermis which had a marked increase in its cellularity of marginal and uninvolved areas. Cells were mainly mononuclear (fig 82,83), particularly lymphocytes that had convoluted nuclei and resembled T lymphocytes.

Blood vessels in areas that had the most marked inflammatory changes had swelling of their endothelial cells, and some damage (fig 84). Occasionally, in addition to red blood cells in the lumen, white blood cells were also found.

Filamentous bodies were also found in the biopsies taken from both patients. They resembled colloid/amyloid bodies (fig 85) seen in patients with idiopathic vitiligo.

The histological changes were identical to that found in the common (idiopathic) vitiligo. The nerves appeared to be normal although a number of regenerating axons were found. In the biopsies taken from both of these patients no intra-epidermal nerves were found.

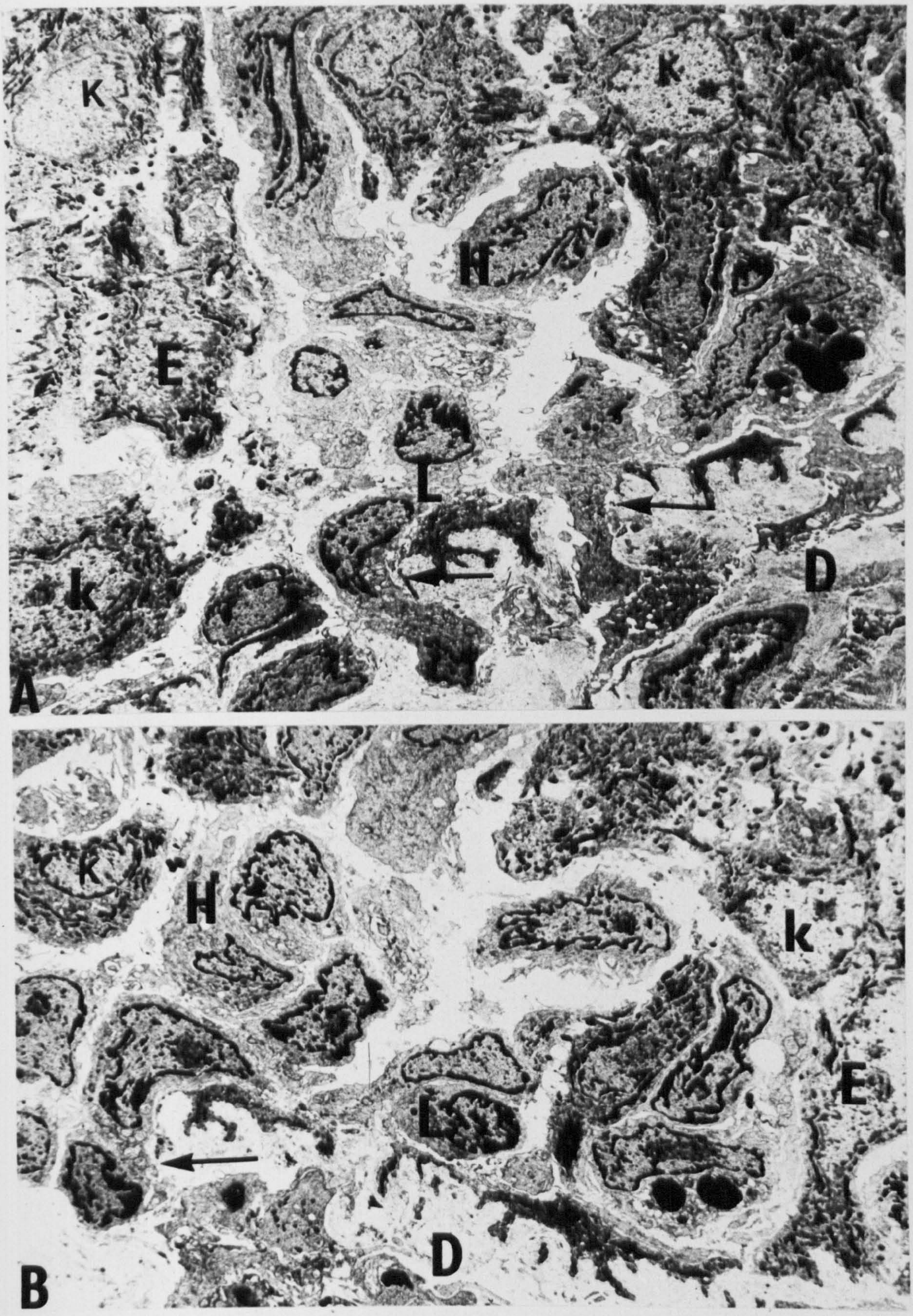


Fig 80 . A,B. Electron micrograph of a marginal area from a patient with occupational vitiligo showing lympho-histiocytic infiltrate of the epidermis. Some of these cells just going into the epidermis through a break (arrows) in the dermo epidermal junction. D Dermis, E Epidermis, H Histiocytes, L Lymphocyte, K Keratinocyte. (A, X 3754, B, X 3792)

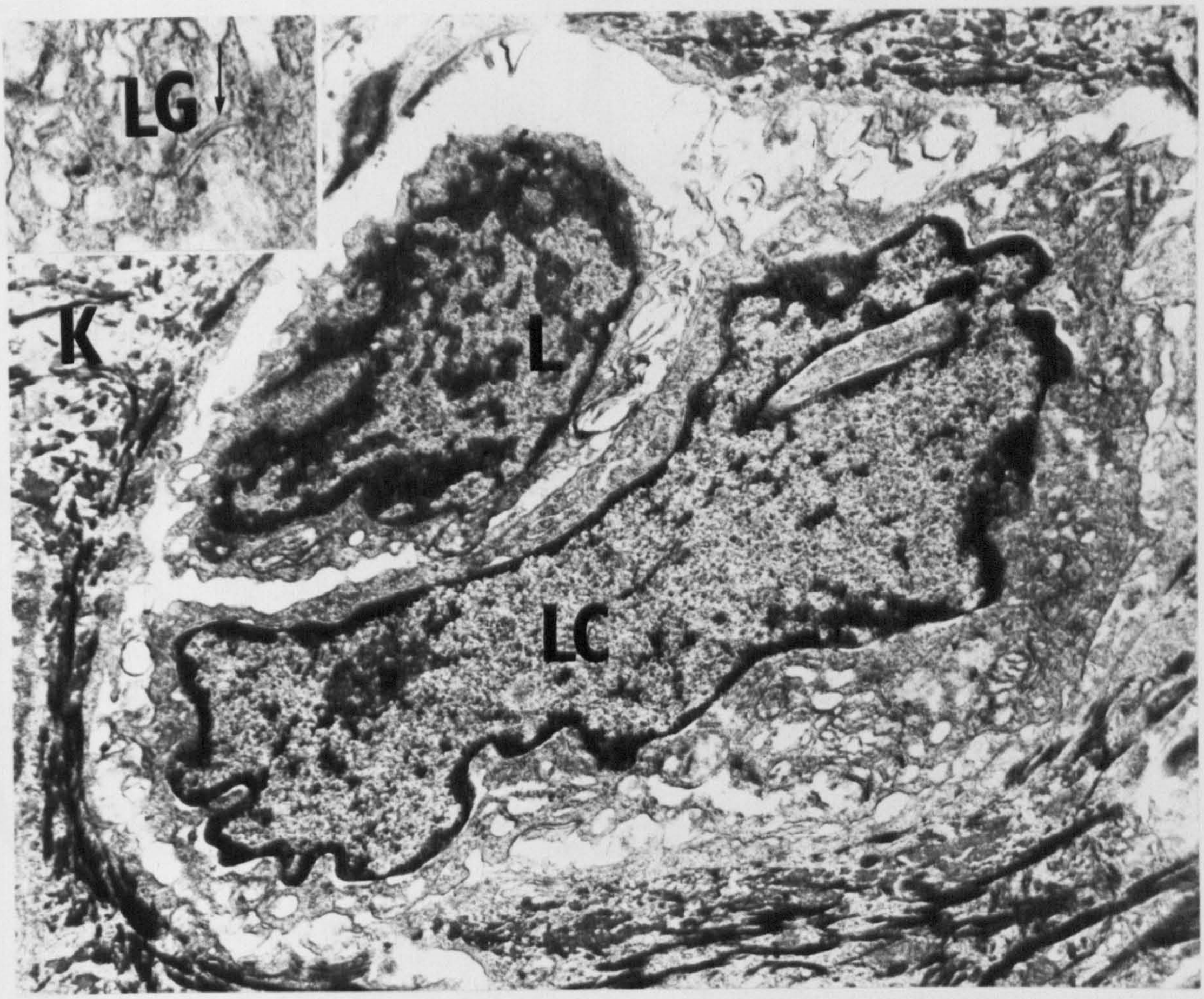


Fig **81** . Electron micrograph of a marginal area from a patient with occupational vitiligo showing Langerhans cell (LC) in contact with lymphocyte (L) in the epidermis. Inset showing a higher magnification of Langerhans granules (LG). K Keratinocyte. (X 3840)

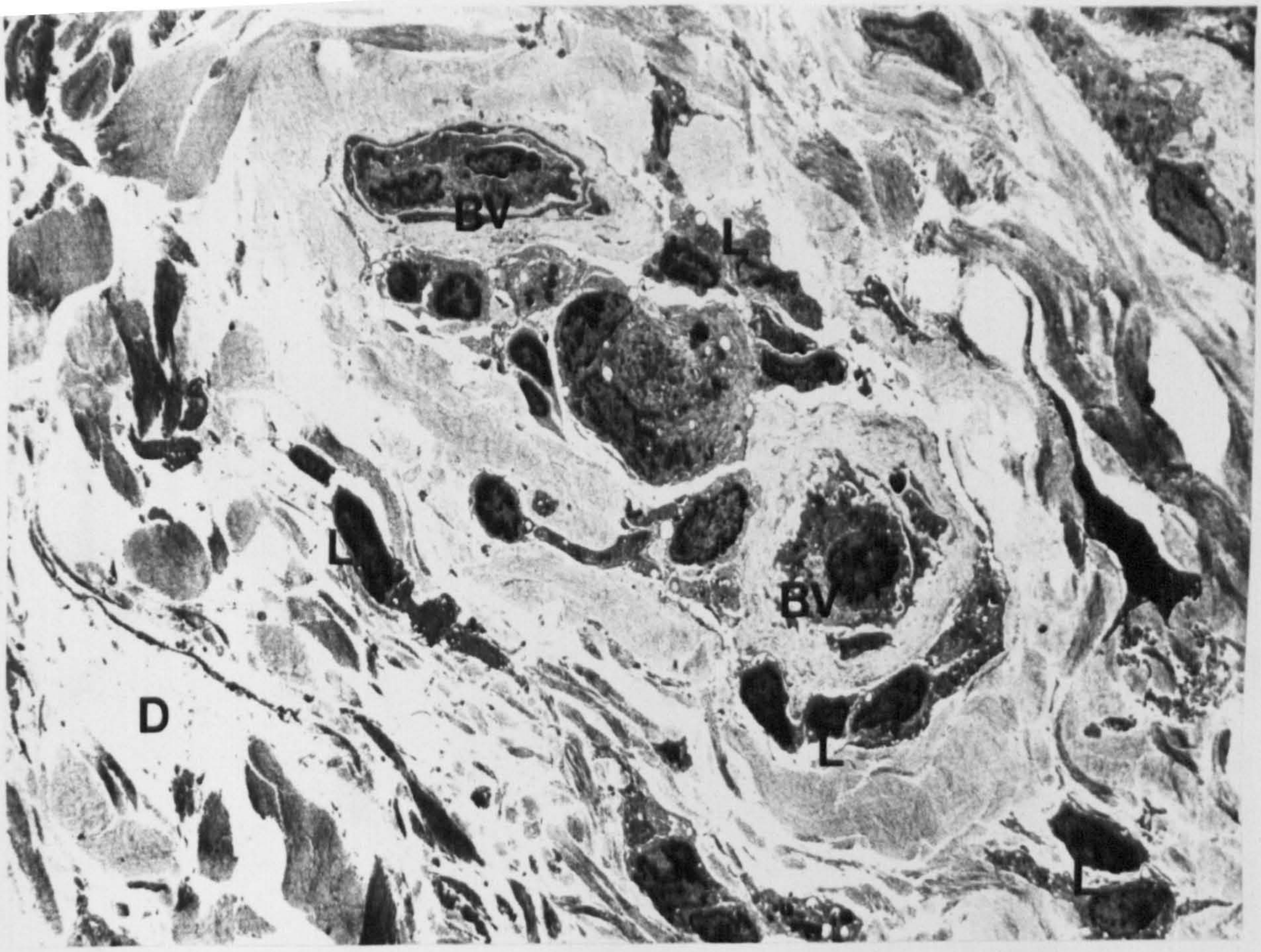


Fig 82 . Electron micrograph of a marginal area from a patient with occupational vitiligo showing blood vessels (BV) with lymphocytic cells (L) in an oedematous dermis (D). (X 2500)

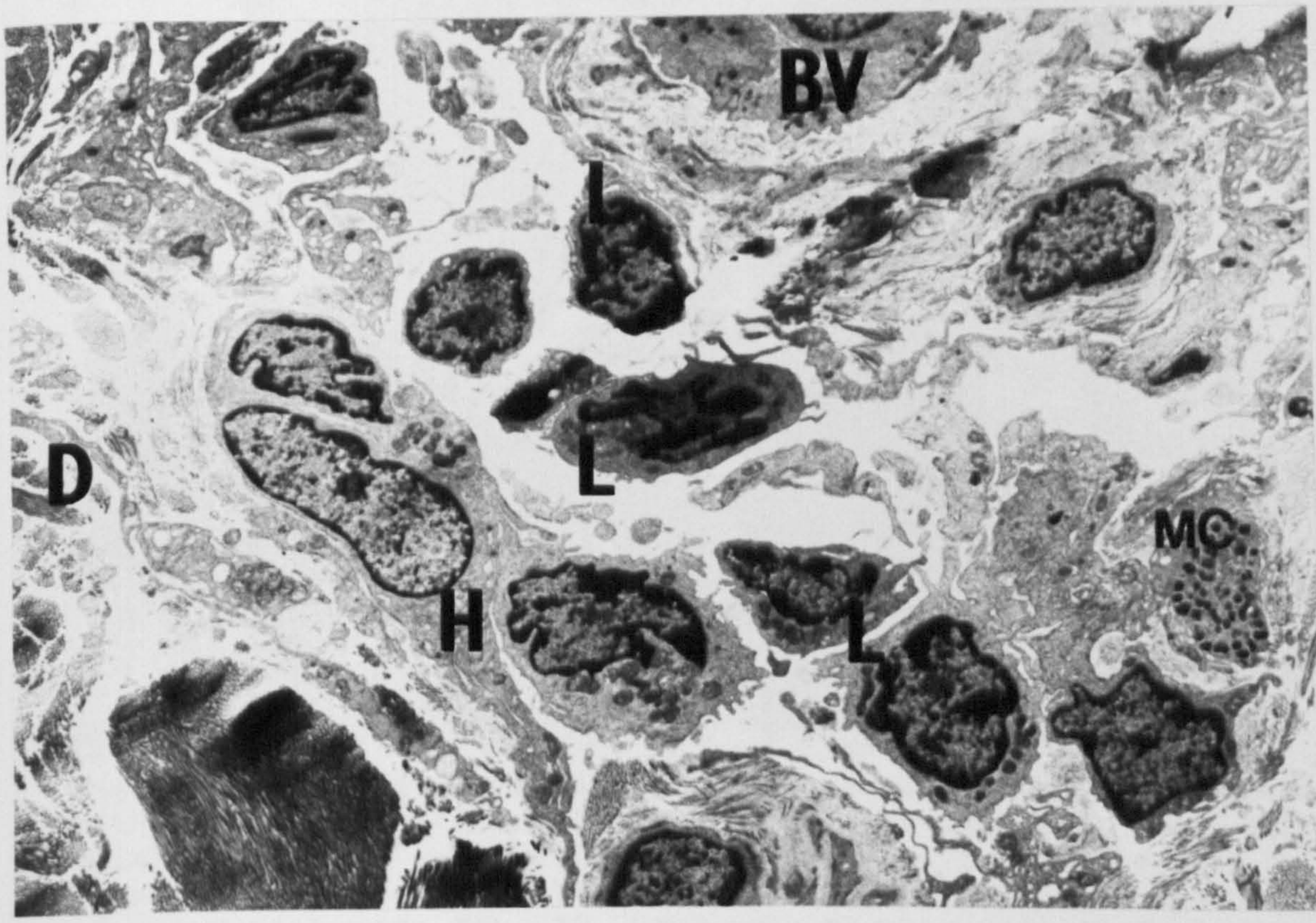


Fig 83 . Electron micrograph of an uninvolved area from a patient with occupational vitiligo showing oedematous dermis (D) with inflammatory cell infiltrate mainly lymphocytes(L). H Histiocyte, MC Mast cell, BV Blood vessel. (X 3848)

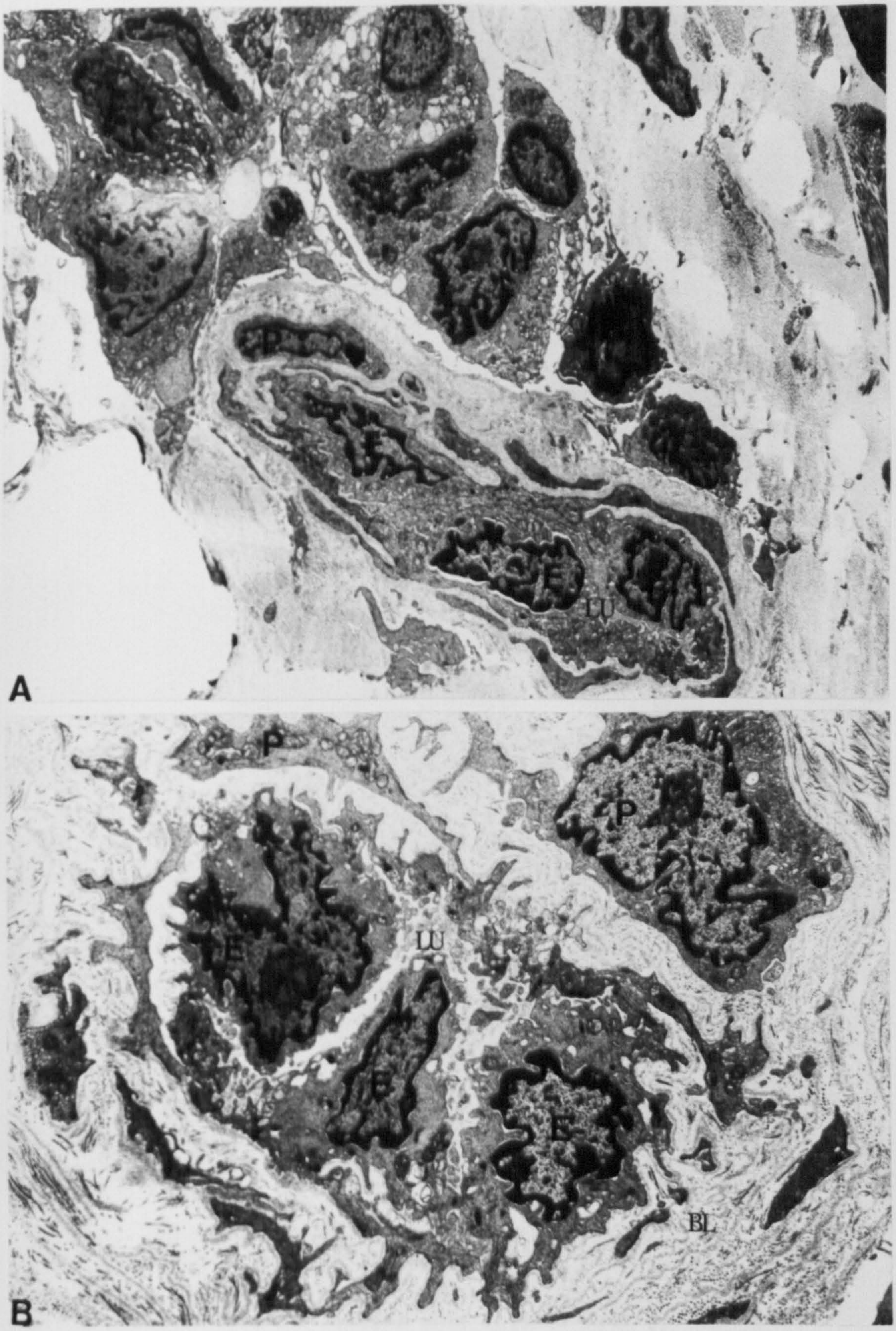
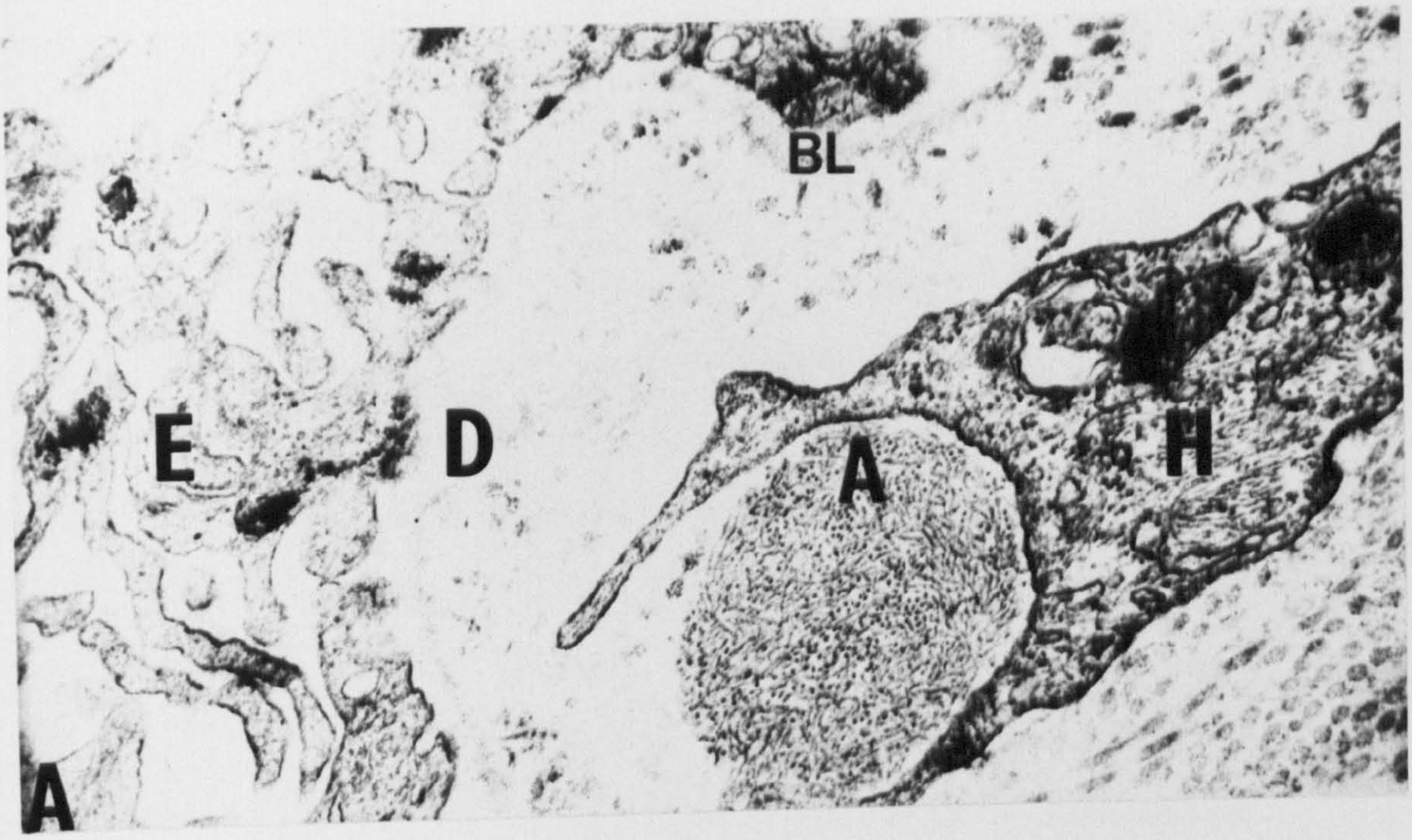


Fig 84 A,B. Electron micrograph of a marginal area from a patient with occupational vitiligo showing inflammatory changes in the dermis mainly lymphocytic cell infiltrate and damage to the blood vessel in a form of replication of the basal lamina (BL), swelling and degeneration of endothelial cells (E). LU Lumen of a blood vessel, P Pericyte. (A, X 6000, B, X 96000)



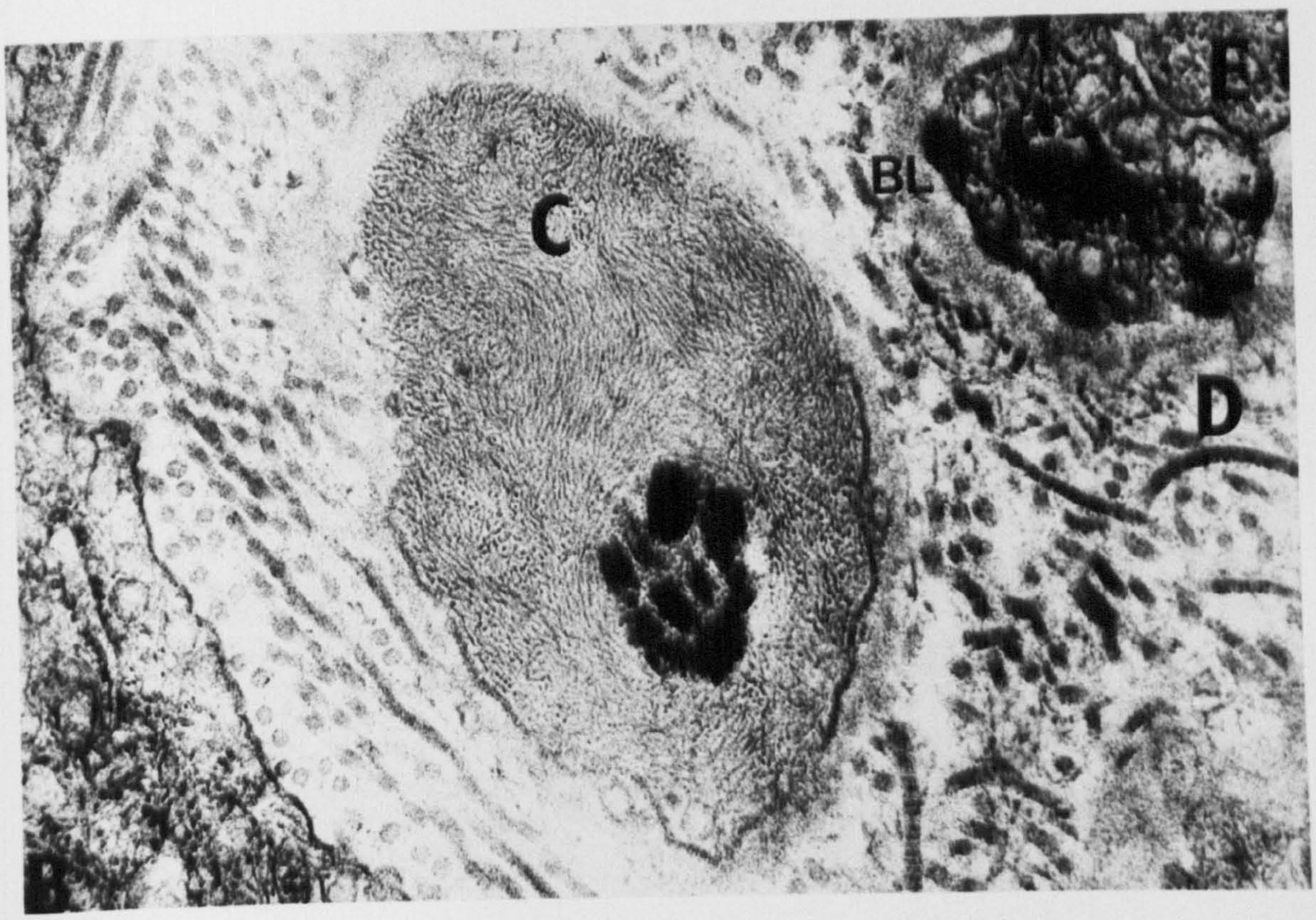


Fig 85 . Electron micrographs of filamentous bodies from patients with occupational vitiligo showing:-

A - Amyloid body (A) in the involved area (X 42142)
B - Melanocyte dendrite changed into colloid body (C) in the uninvolved area (X 49157)

D Dermis, E Epidermis, BL Basal Lamina, H Histiocyte

D. A REPIGMENTING VITILIGO

Biopsy was taken from a freshly repigmented area of vitiligo and this showed many melanocytes in the basal layer and these contained melanised melanosomes. Morphology of these melanocytes appeared to be normal and they contained melanosomes in various stages of development. No inflammatory changes were observed in this biopsy. The superficial dermis contained only a few cells in the specimens examined. Only a few filamentous bodies containing melanised melanosomes were found.

CHAPTER THREE

DISCUSSION

I. CLINICAL ASPECTS AND ASSOCIATED DISORDERS

(a) Clinical Aspects

Vitiligo is a common disease that affects about 0.38-1% (Lerner 1959; El Mofty 1968; Mehta et al 1973; Howitz et al 1977) of the population. It can start at any age and in this study, the age of onset ranged from 1-65 years with nearly half the patients developing the disease before the age of 20. This is comparable to the studies by Lerner (1959) who found that 50% developed vitiligo before the age of 20. In this study the incidence of the disease appeared to be increased in females (79.3%) and this is similar to other studies that are based on out-patient attendances rather than on studies of the general population. The incidence is probably the same in both sexes (Howitz et al 1977). A high preponderance among females is almost certainly due to the fact that these are more aware of a cosmetic disability and, therefore, seek medical treatment.

Vitiligo frequently occurs in families who are aware that it is inherited. About 30-40% of patients have a positive family history (Lerner 1959; Fitzpatrick 1964; Copeman et al 1973). This has been shown in the present study, where 24% had a positive family history. In this study the families of these patients also had an increased incidence of auto-immune disorders, e.g. pernicious anaemia,

diabetes mellitus etc. Not infrequently one of these auto-immune disorders would occur in one member of the family and another would have vitiligo.

All the patients presented with symmetrical areas of depigmentation. One only had segmental vitiligo. Many of the patients developed depigmentation at the sites of the biopsies, this being an isomorphic or Koebner phenomenon; a well known feature of vitiligo and sometimes of diagnostic value (Fitzpatrick 1964).

(b) Associated Disorders

Vitiligo is not infrequently associated with a number of disorders considered to be of auto-immune aetiology (Lerner 1959; Cunliffe et al 1968; Dawber 1968,1970; Bor et al 1969; Howitz 1971; McGregor et al 1972). In this study the incidence of auto-immune disorders seemed to be increased.

1. Pernicious Anaemia

There is a strong association between this disease and other auto-immune disorders. It is associated with increased auto-antibodies, particularly against intrinsic factor and antibodies are present in the sera of these patients to gastric parietal cells. Cell mediated immunity is also involved in the pathogenesis of pernicious anaemia (Wright 1979).

Pernicious anaemia is well recognised to occur in association with vitiligo and there are many

case reports. Francis (1931) found that achlorhydria was present in a significant number of vitiligo patients and Ishida (1954) showed that many patients with vitiligo secreted less gastric acid than normal.

Vitiligo not infrequently occur in those patients with pernicious anaemia (Allison and Curtis 1955). Cunliffe et al (1968) found that three patients among fifty-six with vitiligo (5.3%) had pernicous anaemia. This was statistically significant. Dawber (1970) found that 9% of patients with pernicious anaemia had vitiligo. In this study 3.4% of the patients had pernicious anaemia.

2. Addison's Disease

In the original description of this disorder Addison (1855) described a patient with hyperpigmentation of the skin, but also with vitiligo. This association of vitiligo in patients with Addison's disease was later confirmed (Dunlop 1963). Vitiligo is present in 15% of patients with Addison's disease (Forsham 1968). Vitiligo has also been reported in association with Addison's disease in patients with multiple glandular insufficiencies (McGregor et al 1972; Hertz et al 1977; Betterle et al 1979). In this study 6.8% of the vitiligo patients had Addison's disease.

3. Thyroid Disease

Several reports have suggested an association between vitiligo and thyrotoxicosis (Habermann 1933; Lerner 1959; Goudie, Spence and MacKie 1979). The clinical association between these two disorders has been confirmed (Cunliffe et al 1968; Ochi et al 1969). Vitiligo has been demonstrated in patients with hypothyroidism (Lerner 1955; Morgans 1964; Doniach et al 1972). Hashimoto's thyroiditis (auto-immune thyroiditis) has been shown to significantly occur in patients with vitiligo (Cunliffe et al 1968). An association between these two diseases has been reported (McGregor et al 1972). Two patients were described in this study, one with hypothyroidism and the other with thyrotoxicosis.

Although antithyroid antibodies are usually demonstrated in the sera of patients with thyroid diseases, evidence for cell mediated immune reactions to thyroid antigens have also been shown to occur in patients with Grave's disease, Hashimoto's thyroiditis and spontaneous primary hypothyroidism (Volpe et al 1974; Amino and DeGroot 1975; Calder and Irvine 1975; Tötterman et al 1977 a,b; Gordin et al 1979). The close association of vitiligo with auto-immune thyroid disease support the possibility that vitiligo is also an 'auto-immune' disease.

4. Diabetes Mellitus

Evidence that diabetes is an autoimmune disorder is accumulating and it occurs more frequently in patients with other autoimmune disorders (Cunliffe et al 1968; Bor et al 1969). Autoantibodies to gastric parietal cells, adrenal and thyroid tissue are found in patients with diabetes (Goldstein et al 1970; Irvine et al 1970). Islet cell antibodies are found in insulin dependent diabetes (Lemdrum, Walker and Gamble 1975; MacCuish et al 1974; Bottazzo et al 1974). A lymphocytic infiltrate of the islets of Langerhans in the pancreas - insulitis is an autopsy finding in insulin dependent diabetes (Gepts 1965; Warren et al 1966). Cell mediated immunity has been shown to play a role in the pathogenesis of insulin dependent diabetes (MacCuish et al 1974; Nerup et al 1971; Sensi and Pozzilli 1979; West, Boozer and Haberman 1978; Pozzilli et al 1979).

As regards the association of diabetes with vitiligo, cases have been described with both conditions (Lerner 1959; Dawber 1968; Villa Verde 1969; McGregor et al 1972; Heyman and Lustman 1971; Bleehen 1972). The association of juvenile diabetes mellitus with vitiligo was also found by Cunliffe et al (1968), where 7.1% of the vitiligo patients had diabetes. Macaron et al (1977) also reported five juvenile diabetics had vitiligo.

In this study no patients with diabetes mellitus were included. However, a family history of diabetes was not uncommon and 20.6% of the patients had close relatives with the condition. This increased incidence of diabetes in the relatives of patients with vitiligo has also been reported (Bor et al 1969; Macaron et al 1977).

5. Alopecia Areata

About 2% of all new dermatological out-patient attendances in the United Kingdom are those with alopecia areata. In 70-80% of the cases the first attack occurs before the age of 40, but it can occur at any time. Both sexes are equally affected and family history occur in 10-24% of the cases (Ebling and Rook 1979; Friedmann 1981). Alopecia areata has many similarities to vitiligo.

Light microscopy of the hair bulbs has shown a lymphocytic infiltrate (Walker and Rothman 1950; Thies 1966; Muller, Rook and Kubba 1980; Klaber and Munro 1978). Electron microscopy (Sato 1976) confirmed the presence of lymphocytes in the hair bulbs.

No antibodies to epidermal cells and to the hair follicles were found either in the patient's serum or bound 'in-vivo' in the scalp (Klaber and Munro 1978; Muller et al 1980). There are conflic-

ting observations concerning the occurrence of auto-antibodies, particularly against thyroid constituents and gastric parietal cells, in sera of patients with alopecia areata. Thus, several authors reported positive associations between alopecia areata and auto-antibodies (Kern et al 1973; du Vivier and Munro 1975; Main et al 1975; Friedmann 1981), while others reported a lack of such association (Cunliffe et al 1969; Klaber and Munro 1978; Muller et al 1980). In a recent study Messenger (unpublished data 1981) has been unable to find complement fixing antibody against hair bulb cells in sera of patients with alopecia areata using an indirect immunofluorescence complement fixation test.

The association of alopecia areata with vitiligo has been recognised. Vitiligo is associated with alopecia areata in about 4% of cases as compared with 1% control subjects (Anderson 1950; Muller and Winkelmann 1963). Cunliffe et al (1968), has shown that 16% of vitiligo patients had alopecia areata, while only 1.7% of patients with psoriasis had the condition. Bleehen (1972) also reported that 9.3% of vitiligo patients had alopecia areata. Alopecia areata and vitiligo occur together as part of multi-autoimmune disorders (Fields et al 1971; Doniach et al 1972; Tan 1974; Hertz et al 1977; Freinkel and Asrman 1977; Betterle, Peserico and Bersani

1979). Both disorders occur as part of the Vogt-Koyanagi-Harada syndrome. In this disorder a cell mediated immune reaction against melanocytes is involved, not only in the skin and hair, but also in the eye (Hammer 1974; Tagawa 1978). Alopecia areata sometimes also affects pigmented hair and not grey hair. All the hair in a regrown patch may remain white indefinitely (Ebling and Rook 1979). It is possible that melanocytes in the hair bulb are involved in the pathogenesis of alopecia areata.

In this study 6.8% of vitiligo patients had alopecia areata. The clinical association between these two diseases may have some significance as to the aetiology and favour a cell mediated immune pathogenesis.

6. Lichen Sclerosus et Atrophicus

This uncommon condition occurs mostly in females. The major histological change is a band of hyalinisation of the collagen of the papillary dermis. In addition there is lymphocytic infiltrate below this altered collagen. In this disorder there is increased autoantibodies formation to thyroid tissue and gastric parietal cells, when compared with control subjects (Goolamali et al 1974). The presence of these antibodies was not related to the severity or duration of the disease.

The association of vitiligo with lichen sclerosus is well established. Wallace (1971) found that 2.6% of patients with lichen sclerosus have vitiligo as well. This is probably significant. Goolamali et al (1979) has reported that 7.6% of patients with lichen sclerosus have vitiligo. This percentage is much higher than in a normal population. In this study this association between the two disorders has been confirmed. 6.8% of vitiligo patients had lichen sclerosus.

7. Halo Naevi

This condition is commonly associated with vitiligo and not infrequently antedates its onset. It has been reported (Lerner and Nordlund 1978) that 50% of vitiligo patients have halo naevi. Histopathological studies have shown a lymphohistiocytic infiltrate of the naevus tissue and overlying epidermis (Frank and Cohen 1964; Kopf, Morrill and Silberberg 1965; Stegmaier, Becker and Medenica 1969; Swanson, Wayte and Helwig 1968; Rowden and Lewis 1975). Ultrastructural studies of the depigmented halo have shown an absence of melanocytes in the basal layer that are replaced by Langerhans cells (Hashimoto 1974,1975: Swanson et al 1968).

Circulating autoantibodies in the sera of patients with halo naevi have been demonstrated

against the cytoplasmic antigen of melanoma cells (Lewis and Copeman 1972; Copeman et al 1973; Roenigk et al 1975; Rowden and Lewis 1975). Cell mediated immunity is involved and lymphocytes from these patients with halo naevi are cytotoxic to malignant melanoma cells (Roenigk et al 1975: Nordlund et al 1980; Mitchell, Nordlund, and Lerner 1980).

In this study 10.3% of the vitiligo patients had halo naevi.

8. Psoriasis

El Mofty (1968) reported that 0.7% of vitiligo patients have psoriasis. This incidence is even lower than psoriasis in the general population. It is likely that psoriasis is coincidental and not clinically associated. In a study of 62 patients with vitiligo 4.8% had psoriasis (Bor et al 1969). In the present study 6.8% of vitiligo patients had psoriasis. Both studies appear to confirm that there is an association between the two disorders, but it could equally be coincidental.

9. Lichen Planus

In lichen planus there is a degeneration of the epidermal cells in the basal layer and a lympho-histiocytic infiltrate in the papillary dermis. Electron microscopic studies (Ragaz and Ackerman

1980) have shown an increased number of Langerhans cells in the epidermis. These cells have been seen to be in contact with lymphocytes in the epidermis and dermis. It has been suggested by the authors that lichen planus may represent a manifestation of a delayed hypersensitivity reaction to unrecognised antigens.

The association of lichen planus with vitiligo has been recognised (Bor et al 1969; Tan 1974). In one study (Bor et al 1969) 4.8% of vitiligo patients had lichen planus. In the present study 3.4% of patients with vitiligo had lichen planus.

10. Discoid Lupus Erythematosus

There is no previous report showing the association of these two disorders. In this study 3.4% of patients have discoid lupus erythematosus.

11. Atopic Eczema

The association between atopic eczema and vitiligo has been reported in two patients where the atopic eczema spared the vitiligo lesions (MacMillan and Rook 1971). Bleehen (1972) described one case of atopic eczema among 32 vitiligo patients. In the present study 6.8% of patients have atopic eczema. This association could be coincidental as both diseases are common in general population.

II. AUTOANTIBODIES

In vitiligo there is an increased incidence of organ-specific autoantibodies such as is seen in other so called autoimmune disorders. This was confirmed in the present study. Autoantibodies to gastric parietal cells, thyroid microsomes and thyroglobulins were significantly raised in the sera of these patients as in other previous studies (Cunliffe et al 1968; Bor et al 1969; Brostoff et al 1969; Howitz and Schwartz 1971; Dobmeier and Sams 1971; Betterle et al 1976; Macaron et al 1977). Antibodies to smooth muscle were negative as found in other studies (Brostoff et al 1969; Betterle 1976; Woolfson et al 1975). Antinuclear antibodies were found in 12.5 % of the vitiligo patients in titre greater than 1/20. This finding was significant (Brostoff et al 1969). In previous studies antimitochondrial antibodies were reported to be negative (Woolfson et al 1975; Betterle et al 1976). In this study antimitochondrial antibodies were found in the serum of one patient.

III. IMMUNOFLUORESCENCE STUDIES

In 1965 Langhof et al reported the presence of precipitating antimelanin antibodies in the sera of the majority of 26 patients with vitiligo. In a subsequent study of 42 cases of vitiligo (Woolfson et al 1975) precipitating antibodies were not found. Dobmeier and Sams (1971) were unable to demonstrate antimelanocyte antibodies in ten patients using direct and indirect immunofluorescent techniques, even in vitiligo patients with other associated autoimmune disorders; e.g. pernicious anaemia etc. Similarly, in another study on 96 patients (Betterle et al 1976) these workers were unable to find anti-melanocyte antibodies to either malignant or normal melanocytes using an indirect immunofluorescent method. Bleehen (1979) studied ten vitiligo patients using direct immunofluorescent techniques on both the involved and also the uninvolved skin and found no significant deposition of immunoglobulins or complement.

Antibodies have been found in the serum of patients with halo naevi, some of them also with associated vitiligo (Copeman et al 1973). These antibodies reacted against the cytoplasm of malignant melanoma cells. However, these antibodies were not found in the sera of patients with vitiligo without resolving halo naevi (Copeman et al 1973).

Humoral antibodies were found in the patients with halo naevi (Roenigk et al 1975) using immunofluorescent techniques. These workers also demonstrated lymphocyte cytotoxicity against malignant melanoma cells and considered that cell mediated immunity may be more important than the humoral antibodies in the pathogenesis of halo naevi. Antibodies to melanin producing cells were found to be present in the sera from two patients with vitiligo who, in addition to having multiple endocrine insufficencies, also had alopecia areata and muco-cutaneous candidiasis (Hertz et al 1977). Both the direct and indirect immunofluorescent tests were negative and showed no deposition of immunoglobulins and complement. However, the in-vitro complement fixation test demonstrated a complement fixing serum factor in both patients. This factor was bound intracellularly to normal melanocytes, naevus cells and malignant melanoma cells. This serum factor was also found in one of two patients with vitiligo who also had multiple endocrine deficiencies (Betterle et al 1979). This complement serum factor was also present in the sera of patients with muco-cutaneous candidiasis without vitiligo (Nordlund et al 1981).

In the present study there was no significant deposition of immunoglobulins in the involved, marginal and uninvolved areas of skin. In many

patients there was deposition of fibrin mainly in the papillary dermis of the marginal and involved areas, and occasionally in the adjacent uninvolved skin. In a few of the patients there was a dense band of fibrin deposition of the dermo-epidermal junction in the involved areas of skin.

The absence of immunoglobulins and complement, but the deposition of fibrin in the dermis is commonly found in cell mediated immune reactions (Colvin et al 1973).

A complement fixing antibody to melanocytes was not found in the sera of all the vitiligo patients studied using an indirect immunofluorescent technique (Jordon et al 1969; Katz et al 1976; Hertz et al 1977). Even in patients with associated autoimmune disorders, such as pernicious anaemia and Addison's disease, no serum complement fixing antibody was found. However, it was not possible to study the serum of patients with multiple endocrinopathies and with several autoimmune disorders in which such a factor is sometimes present.

The difficulty in demonstrating humoral anti-bodies to melanocytes may be due to the fact that the depigmentation in vitiligo is a slow and gradual process. The marked and relatively sudden changes as seen in halo naevi, and in a few patients with malignant melanoma, are not present in the lesions

of vitiligo.

Antibodies were present in the serum of patients only when there was actively regressing halo naevi or when the patient had multiple endocrine disorders. It is likely that the anti-melanocyte antibodies are probably secondary rather than being primary and are directed against the cytoplasm rather than against the cell membrane.

In autoimmune diseases that are associated with vitiligo there is evidence that, in addition to organ specific autoantibodies, a cell mediated immune reaction is involved in these disorders (Wright 1979; Volpe et al 1974; Amino and DeGroot 1975; Calder and Irvine 1975; Gordin et al 1979; MacCuish et al 1974; Nerup et al 1971, Sensi and Pozzilli 1979; Pozzilli et al 1979; West, Boozer and Haberman 1978). A cell mediated immune reaction has been demonstrated in vitiligo patients with Vogt-Koyanagi-Harada syndrome (Tagawa 1978; Nordlund et al 1980).

The inflammatory changes, the deposition of fibrin and the presence of a lymphocytic infiltrate in the epidermis would favour that a cell mediated immune reaction directed against the melanocytes is involved rather than a humoral mechanism with a melanocytotoxic antibody. However, it is possible

that both humoral and cell mediated immune reactions are involved.

IV. INFLAMMATORY CHANGES

It is quite rare to observe inflammatory changes in lesions of vitiligo. However, there are a number of reports in the literature of lesions of vitiligo with an inflammatory, raised and palpable erythematous border (Habermann 1933; Becker and Obermayer 1937; Garb and Wise 1948; Buckely and Lobitz 1953; Shukla 1959; Pinkus 1959; Lerner 1959; Fregert et al 1959; Allende and Reed 1964; Michaelsson 1968; MacMillan and Rook 1971; Bleehen and Sharquie 1981 unpublished). Sometimes these lesions are itchy. There are several reports of histopathological changes indicative of inflammation. These were mainly observed in the inflammatory raised border, where severe spongiosis and a mononuclear cell infiltrate constituted mainly lymphocytes, were found in the epidermis (Gopinathan 1965; Michaelsson 1968; MacMillan and Rook 1971). Recently an increase in the cellularity of dermis including lymphocytes and histiocytes has been seen in vitiligo lesions (Breathnach 1975; Nordlund et al 1978; Bleehen 1979).

In this study no obvious inflammatory raised borders were seen in any of the lesions in the patients. However, the histopathological studies revealed inflammatory changes in the vitiligo skin, especially in the epidermis of marginal areas.

Spongiosis of the epidermis was commonly found in these marginal areas, together with a mononuclear cell infiltrate consisting mainly of stimulated (atypical) lymphocytes and histiocytes that were in contact with each other. Intra-epidermal lymphocytes were also seen, some of them were in direct contact with Langerhans cells and melanocytes. Lymphocytes were seen entering the epidermis through breaks in the dermo-epidermal junction. Also mild inflammatory changes were observed in the blood vessels together with deposition of fibrin in the papillary dermis. These changes resembled those seen in delayed hypersensitivity reactions (Dvorak, Mihm and Dvorak 1976; Dvorak et al 1976; Colvin et al 1973). The degree of mononuclear cell infiltration of the dermis did not parallel that seen in the epidermis; sometimes a massive cellular infiltrate in the epidermis was seen even forming Pautrier-like micro abscesses, but with only a few mononuclear cells in the dermis. This would suggest that the primary inflammatory reaction occurs in the epidermis, where an antigen is present. This primary epidermal reaction has been described in mycosis fungoides (Ryan et al 1973; Tan et al 1974), and in Behcet's syndrome (Honma, Saito and Fujioka 1981).

The skin represents a special micro environment for T lymphocytes. There seems to be proclivity for T lymphocytes to localise preferentially within the skin (Streilein 1978). This homing of cells to a special tissue is termed *ecotaxis* (De Sousa 1971; Goudie, MacFarlane and Lindsay 1974; Goos, Kaiserling and Lennert 1976), This may explain the pathogenesis and symmetry of vitiligo. In a genetically predisposed individual a clone of lymphocytes that are reactive against a melanocyte antigen may develop. The abnormal lymphocytes then home in on the skin in a focal and symmetrical fashion. They destroy the melanocytes as a result of lymphokine production. This primary immunologic damage of melanocytes will initiate an early focus of vitiligo. The melanocytes surrounding this focus will become hypertrophied and hyperactive in order to compensate for the loss of the adjacent pigment cells. The marginal melanocytes then commit suicide by "working themselves to death", probably as a result of the production of toxic melanin precursors (Lerner 1971). Thus the initiating factor is an immunological one and the progression is partly secondary.

Quantitative studies of T and B lymphocytes in patients with vitiligo did not reveal any signifi-

cant changes (Ortonne and Alario 1978). Using the microcytotoxicity tests, Tagawa (1978) and Nordlund et al (1980) reported that lymphocytes from patients with early stages of Vogt-Koyanagi-Harada syndrome, showed high cytotoxic activity against melanoma cell surface antigen. They suggested that cell mediated immunity plays a role in pathogenesis of this disease. Lymphocytes cytotoxicity has also been observed in patients with actively regressing halo naevi (Roenigk et al 1975; Nordlund et al 1980; Mitchell et al 1980). The microcytotoxicity test was negative in patients with vitiligo (Mitchell et al 1980) using allogenic melanoma cells. However, in vitiligo the process of depigmentation is very slow and this test was not very sensitive and may not detect a cell mediated immune reaction in patients with vitiligo. In addition the immune reaction may be directed against a melanocyte antigen that is not found in melanoma cells.

Psoralens and ultraviolet light have been used in treatment of vitiligo for several thousand years (El Mofty 1948, 1968). The mode of action of these compounds in promoting the repigmentation of the skin is uncertain. It is possible that these psoralens that induce photosensitivity may also promote mitosis of residual melanocytes and

induce tyrosinase activity of residual melanocytes (Bleehen 1975). Photochemotherapy using oral psoralens and exposure to long wave ultraviolet light (PUVA treatment) have been used for the treatment of many disorders including vitiligo (Bleehen 1972,1975; Ortonne et al 1979). Recent studies indicated that PUVA can alter the immune responses and depress lymphocyte function both in vitro and in vivo (Roenigk 1976; Hodge et al 1977; Bleehen, Briffa and Warin 1978; Morison, Parrish and Epstein 1979; Morhenn et al 1980; Morison et al 1981; Kraemer et al 1981). Repigmentation of vitiligo can also be induced by the application of topical corticosteroids (Kandil 1970,1974; Koopmans-Van, Dorp et al 1973; Bleehen 1976) and nitrogen mustard (Van Scott and Yu 1975). Similarly ACTH injections have been successful in promoting repigmentation in vitiligo (Gokhale and Gokhale 1976). Nitrogen mustard and corticosteroids (Webel and Ritts 1977; Wang and Zweiman 1981) are both immunosuppressive drugs and it is therefore possible these agents are effective in vitiligo by suppressing a cell mediated immune reaction.

The inflammatory changes in the epidermis in vitiligo are commonly present, but can be easily

missed. This is due to the process of depigmentation being rarely acute and usually gradual. If inflammatory changes are present, they are confined to a narrow marginal area that may not be biopsied or sectioned for histopathological examination. In order to observe the inflammatory changes, the advancing margin of lesion of vitiligo should be chosen for biopsy and histopathological studies.

V. LANGERHANS CELLS

The Langerhans cells were first described by Paul Langerhans in 1868 and since then there has been considerable speculation as to their origin and function. Paul Langerhans thought that the cells were nerve receptors. Later there were two ideas about these cells. The first, was that they had a neural function (Ferreira-Marques 1951; Niebauer 1956; Richter 1956; Wiedmann 1952). The second, that followed the concept as put forward by Masson (1951), was that the Langerhans cells were the terminal stage in the life cycle of previously active melanocytes (Billingham and Medawar 1953; Fan and Hunter 1958; Fan, Schoenfeld and Hunter 1959). Breathnach (1963) originally regarded Langerhans cells as inactive melanocytes. However, as a result of later studies both these theories were discarded, particularly as it was apparent that the Langerhans cell was of different embyronic derivation than the melanocyte, the latter being derived from neural crest (Breathnach 1968). Other workers similarly considered that the Langerhans cell and the melanocyte did have a common lineage (Hashimoto and Tarnowski 1968; Kiistala and Mustakallio 1968; Prunieras 1969).

Recently it has been shown that the Langerhans cell in humans, guinea pigs and mice have receptors

for both Fc portion of IgG and for C_3 and also express Ia immune associated antigens encoded for the major histocompatability complex (Stingl et al 1977,1978; Rowden et al 1977,1978; Klareskog et al 1977; Berman and Gigli 1979; Tamaki et al 1979; Rowden 1980). Thus Langerhans cells have the characteristic surface markers similar to cells of the monocyte-macrophage series and confirming a mesenchymal origin for these cells. Recently it has been shown that Langerhans cells are derived from and continually replenished by a mobile pool of precursors cells that originate from the bone marrow (Katz, Tamaki and Sachs 1979; Frelinger et al 1979; Tamaki, Stingl and Katz 1980).

Up to recently there has been no real idea as to the function of Langerhans cells and this has been a subject of considerable speculations and many hypotheses. There are currently two major theories. One postulates that the Langerhans cells regulate epidermal cell proliferation and differentiation (Potten and Allen 1976; Schweizer 1981). The other proposes that Langerhans cells are related to cells from macrophage-monocyte-histiocyte series and play a role in the primary immune response in the skin (Silberberg 1971,1973; Silberberg et al 1976). Langerhans cells were found in close apposition to mononuclear cells in the epidermis and dermis in

allergic contact dermatitis, but were not present in skin affected by primary irritant dermatitis (Silberberg 1971). These cells were also found in lymphatic vessels of the dermis in guinea pigs passively sensitised to dinitrochlorobenzene (DNCB). Also they were found in actively sensitised patients and guinea pigs. Some of these cells were apposed to mononuclear cells and showed some damage (Silberberg et al 1976; Silberberg, Thorbecke and Baer et al 1976). Shelley and Juhlin (1977) demonstrated the selective uptake of ten different contact allergens (Formaldehyde, Glutaraldehyde, Paraphenylenediamine, Ethylenediamine, Toluenediamine, Nickel, Cobalt, Chromium, Mercury and Gold) by the Langerhans cells and postulated that these cells form part of the reticulo-endothelial system and binding these haptens to form complete antigens and thus, they play an important part in the development of allergic contact dermatitis. Langerhans cells like macrophages, also have been shown to have the ability to present antigen to sensitised T lymphocytes and the ability to act as stimulator cells for mixed lymphocyte reactions (Stingl et al 1978,1980; Green et al 1980; Braathen and Thorsby 1980). The cells may also be a target for inflammatory cells that as a consequence lymphokines, hydrolytic enzymes and prostaglandins are released (Thorbecke et al 1980; Silberberg-Sinakin

and Thorbecke 1980).

Though there have been several studies on the population density of Langerhans cells in vitiligo, there has been a controversy as to whether they are increased in the amelanotic areas. Using an Osmium iodide technique, Mishima and Miller-Milinska (1961) observed an increased number of Langerhans cells in the involved skin in vitiligo. Though the staining of Langerhans cells is excellent with Osmium, unfortunately it also stains melanocytes and its use is limited.

Birbeck, Breathnach and Everall (1961) used a gold stain also likewise thought that there was an increase in the number of Langerhans cells in the involved skin. With the histochemical technique that showed Langerhans cells in the skin using the adenosine triphosphatase (ATPase) stain, Brown, Winkelmann and Wolff (1967) concluded that the counts of Langerhans cells were similar in both the involved and uninvolved skin.

Electron microscopy revealed that Langerhans cells replaced the basal melanocytes in the involved skin and in this quantitative study (Zelickson and Mottaz 1968) Langerhans cells were increased in the involved areas when compared with the number in the uninvolved vitiligo skin. Similarly, in another electron microscopic study (Mishima, Kawasaki and

Pinkus 1972) the Langerhans cells were to be increased in the basal layer, but their total number remained constant in the involved skin. Langerhans cells were also reported to be encountered with much greater frequency in the involved skin than normal epidermis in patients with vitiligo (Breathnach 1975). Recently Bleehen (1979) studied vitiligo by electron microscopy and has found that melanocytes appeared to be replaced by Langerhans cells, but he did not comment whether these cells were increased in number.

It is difficult to make any definite conclusion from these somewhat conflicting studies. The small number of skin biopsied, the variability of the techniques used and especially the capricious staining methods employed, make one most uncertain about the validity of the findings. Most of these studies did not comment on the alterations in the fine structure of the Langerhans cells and on their function.

In this study the Langerhans cells were counted in 1μ Epon-embedded vertical sections of skin. All the suprabasal clear cells in the uninvolved skin and all the clear cells in the involved areas were considered to be Langerhans cells. This was largely confirmed by subsequent electron microscopy. The number of these cells in the uninvolved skin of

vitiligo was compared with that found in the normal control skin and was significantly increased. Also when the number of Langerhans in involved skin was compared with that of the uninvolved areas, it was found to be significantly increased. However, in a few patients the Langerhans cells were either not seen at all or were found in only few of the sections in the involved areas.

On electron microscopy the Langerhans cells were seen mainly suprabasal in the uninvolved areas in most of the patients. In some of the uninvolved areas the Langerhans cells were only found in the basal layer. In the marginal areas the Langerhans cells were mainly basal in location with a few being suprabasal. In many patients the cells were few or absent, in particular when there was a mononuclear cell infiltrate in the epidermis. In the involved areas, Langerhans cells appeared to replace melanocytes and it seemed that they were increased in number compared to that found in the uninvolved areas. Their position was mainly basal, but in some patients they were only suprabasal. In a number of patients the Langerhans cells were not seen at all.

The increased number of Langerhans cells in the uninvolved areas and the absence of these cells from the involved skin as seen in few patients will account for the varying results as seen by different

workers.

Very little attention has been paid to the structural and functional changes in the Langerhans cells in patients with vitiligo. In this study the Langerhans cells showed a variety of ultrastructural changes suggesting activity and cytotoxic damage. These included the development of bizarre nuclear shapes. In many of the patients these cells had very convoluted nuclei with prominent Golgi system and lysosomes. Some of the Langerhans cells had vacuolated cytoplasm with a shrunken nucleus. Cells in direct contact with lymphocytes in the marginal areas also were seen, particularly in the basal layer of the epidermis. These changes were similar to those immunological reactions where delayed hypersensitivity are involved, such as allergic contact dermatitis (Silberberg 1973; Silberberg et al 1974, 1976). The apposition of mononuclear cells with Langerhans cells was also reported in mycosis fungoides (Rowden and Lewis 1976) and in lichen planus (Ragaz and Ackerman 1980). The apposition of lymphocytes with Langerhans is not seen in primary irritant dermatitis (Silberberg 1971). The presence of this phenomenon in vitiligo indicates that an inflammatory reaction is a primary immunological event rather than a secondary one. All these findings do suggest that vitiligo is a manifestation of a delayed hyper-

sensitivity reaction to melanocyte antigens.

VI. LANGERHANS CELL GRANULES AND DESMOSOMES

Langerhans cells contain granules within their cytoplasm that are considered specific for these cells. Recently it has been shown that these cells are part of macrophage-monocyte system and they are part of mobile pool of monocytes that originate from the bone marrow (Katz, Tamaki and Sachs 1979; Frelinger et al 1979; Tamaki et al 1980). It is possible that Langerhans cells get their specific granules only after these have gained access to the epidermis. In active or recent onset lesions of vitiligo intra-epidermal histiocytes can be found exactly similar to Langerhans cells except the former lack of Birbeck granules. If these granules are endocytotic (Breathnach 1964; Zelickson 1965) or secretory (Birbeck; Breathnach and Everall 1961; Breathnach 1964; Wolff 1967; Zelickson 1965,1966) in origin, they should be present all the time and not only when these cells are in the epidermis.

Langerhans cells are usually confined to epithelia that are composed of stratified squamous cells (Wolff 1972). It has even been suggested (Wolff and Winkelmann 1967) that these epithelial cells, together with the Langerhans cell form an Epidermal - Langerhans Cell Unit.

Langerhans cell granules are similar in structure to desmosomes apart from these having an

attachment plaque. Desmosomes could also have a vesicular portion as in the Langerhans cell granules due to an enfolding of the plasma membrane. It is not infrequent to see a collection of desmosomes, particularly near Langerhans cells and often these cells appear to be partly phagocytosing these desmosomes. On one occasion an intra-Langerhans cell desmosome was seen changing into Langerhans cell-like granule. In addition on many occasions desmosomes were found to be changing into Langerhans cell-like granules in the intercellular areas. These findings would suggest that Langerhans cells can phagocytose desmosomes and alter them into Langerhans cell granules, possibly dissolving the attachment plaque as a result of their lysosomal activities. These changes may occur within the cytoplasm or within lysosomes or even outside the cell membrane.

It is perhaps speculative that the Langerhans cell granules are derived from desmosomes.

VII. MAST CELLS

Recent studies (Eady et al 1979; Cowen, Trigg and Eady 1979) have shown that mast cell population density varies considerably in the dermis, even in different sections from the same biopsy sample. The mast cells are mainly found in the superficial dermis particularly around blood vessels. In this study mast cell counts were performed on the upper (papillary) dermis. The counts on normal controls were variable, but comparable to other studies (Eady et al 1979; Cowen et al 1979). The figures in this present study were lower than in these other studies.

Mast cells have been identified in the lesion of vitiligo for a long time (Marc 1894). Sometimes the lesions of vitiligo may be associated with itching (Asboe-Hansen 1954). Marc (1894) found that in the corium of involved areas mast cell count was increased. Gentele (1949) described a case of drug induced urticaria occuring only in the depigmented areas of patients with vitiligo and has found a high histamine content in these areas when compared with the pigmented skin. Similarly, it was reported (Quiroga et al 1949) that there was an increase in the histamine content of the involved areas of skin in vitiligo. Breathnach (1975) noted that mast cells were prominent in the dermis of the depigmented areas. More recently (Nordlund et al 1978) has reported that mast cells

are increased in number in the dermis of vitiligo patients and in one study (Bleehen 1979) the mast cells were significantly increased in the marginal skin. Mast cells were also increased in the dermis of depigmented area of patients with halo naevi (Hashimoto 1974). The problem with these studies was that the observations were of a limited nature and rarely quantitative.

In this study, a quantitative method was used on the biopsies to assess the number of mast cells and in many biopsies samples. In many of the patients there was an increase in the mast cell counts, as found both in the marginal and involved areas of skin. This number was greater than that of uninvolved areas of patients with vitiligo and normal controls. However, the total mast cell counts of all the patients compared to each other and with controls did not attain statistical significance. This can be attributed to; mast cell number being variable, even in the same site; pathological changes have been observed in the uninvolved areas; the number of mast cells decrease in number and may temporarily disappear during acute inflammatory conditions (Michels 1938; Riley 1959); the mast cell number may vary with the duration of the lesion.

Intra-epidermal mast cells have been reported in vitiligo skin (Breathnach 1966; Bleehen 1979).

These cells were observed in the basal layer. In this study basal mast cells were seen in a few patients and some of them showed degranulation within the spongiotic epidermis.

Mast cells have been shown to play a role in cell mediated immune reaction in addition to immediate humoral type (Dvorak et al 1976; Dvorak et al 1976; Dvorak et al 1974). In this study degranulating mast cells were seen in many patients as a part of the general inflammatory reaction.

VIII. MELANOCYTES

Previous histochemical studies, (Hu et al 1959; Bleehen 1979) have shown that in the involved areas of vitiligo there was almost a complete absence of dopa-positive melanocytes. Electron microscopic studies (Birbeck, Breathnach and Everall 1961; Zelickson and Mottaz 1968; Breathnach, Bor and Wyllie 1966; Breathnach 1975; Nordlund et al 1978; Bleehen 1979) have confirmed that in the vitiliginous areas of skin there was a complete lack of secretory melanocytes that appeared to be replaced by Langerhans cells. Jarret and Szabo (1956) found in their histochemical studies of biopsies taken from involved areas of vitiligo, that there were a number of residual dopa-positive melanocytes and described these cases as 'relative' vitiligo.

Some of the residual melanocytes were large as well as being dopa-positive. Clinically, some of these areas of 'relative' vitiligo were pigmented and appeared somewhat brown in colour rather than the ivory-white appearance of the amelanotic areas of vitiligo.

The involved areas in all the patients in this study were ivory-white in colour when examined with a Wood's lamp. All the biopsies were taken from these ivory-white areas. However, residual dopa-posivite melanocytes were found in most of the

biopsy specimens from these areas. The residual cells were usually small and weakly dopa-positive. The presence of these residual melanocytes was confirmed on microscopy of the Huber-stained, plastic embedded 1μ thick sections and also on electron microscopy of the ultrathin stained sections. Not only were melanocytes found, but also melanosomes were not infrequently seen in the keratinocytes of the epidermis in the involved areas of skin. The number of melanosomes were few when compared with that in the adjacent uninvolved skin.

Thus, the main histological findings in the epidermis, as a result of the histochemical, light and electron microscopic studies, were an almost complete lack of melanocytes in the involved areas of skin and an almost complete absence of melanosomes in the epidermal cells. These findings and the marked reduction in number of melanocytes in the marginal areas, confirmed those findings in previous studies (Jarret and Szabo 1956; Birbeck, Breathnach and Everall 1961; Bleehen 1979).

Histochemical studies of dopa-incubated epidermal sheets showed that in the marginal areas, there was frequently a sudden drop in the population

density of melanocytes at the margin of the areas of vitiligo.

The melanocytes in this marginal region showed a variable morphology. The first type probably represented degenerating melanocytes and were weakly dopa-positive small and rounded cells. These cells had a few dendrites. The second type, the melanocytes, were large and strongly dopa-positive, but they too had lost some of their dendrites. The third type of residual melanocyte in this marginal area was large and poly-dendritic, but was weakly dopa-positive. Electron microscopy indicated that the melanocytes in the marginal areas were few in number and those that were found were large and some showed degenerative changes.

The uninvolved areas in these patients with vitiligo have always been considered to be normal. However, in this study although all the biopsies were taken from apparently normal skin, it was observed that pathological changes occured. Examination of dopa-incubated epidermal sheets showed the presence of microfoci of vitiligo. This finding was confirmed by electron microscopy of the uninvolved skin.

Many theories have been proposed to explain the pathogenesis of vitiligo and the lack of melanocytes and melanosomes in the affected areas of skin. The findings in this study would support that a cell mediated immune mechanism is involved in the destruction of the melanocytes. Intra-epidermal lymphocytes were seen, many of them in contact with melanocytes and Langerhans. Melanocytes are damaged by lymphokines and lysosomes from the lymphocytes and Langerhans cells and this was suggested as a mechanism in the pathogenesis of vitiligo (Nordlund et al 1978). The lymphocytes may only require temporary contact with the melanocytes (Don et al 1977) and only a few of them may be involved in producing the damage. The damaged melanocytes may drop down into the dermis and become mammified and form colloid/amyloid bodies. These colloid/amyloid bodies may be later removed by macrophages. This dropping of melanocytes into the dermis may be the normal fate of effete cells. The degeneration of melanocytes that occur normally may be exaggerated in vitiligo as a result of an immunological mechanism.

The progression of the areas of vitiligo could be explained on the basis of an autocytotoxic theory. Marginal melanocytes become more active and hypertrophic and as a consequence of an accumulation of toxic melanin precursors result in the death of

these cells (Lerner 1971). It is also likely that in the patients with vitiligo they lack a protective mechanism that results in this accumulation of melanocytotoxic precursors.

IX. MELANOSOMES

The difference in colouration of the skin in various races is believed to be due to variations in the number, size, degree of melanization and also the distribution of melanosomes in the keratinocytes of the skin. Melanosomes in Caucasoids, Mongoloids and American Indians are small and aggregated in groups of two or more as membrane-bound packages within the epidermal keratinocytes (Szabo et al 1969). In Negroes (Szabo et al 1969) and in Australian Aborigines (Mitchell 1968) and to a lesser extent in racially pigmented individuals with less heavily pigmented skin (Oslon, Gaylor and Everett 1973; Toda et al 1973) the melanosomes are larger in size and remain unaggregated. This distribution pattern seems to be a sized dependent phenomenon. Larger melanosomes tend to be disposed individually and are not aggregated, whereas small ones occur in complexes (Toda et al 1972; Konrad and Wolff 1973; Wolff 1973; Zaynoun et al 1977).

Melanosomal size is under genetic control, but can be affected by external factors (Quevedo 1973). It has been shown (Toda et al 1972) that exposure of Caucasoid skin to longwave ultraviolet (UVA) radiation following application of psoralen caused an increase in the number and size of melanosomes and a switch from the aggregated to the non-

aggregated distribution pattern. After application of topical nitrogen mustard (Flaxman, Sosis and Van Scott 1973) to the skin and oral therapy with bleomycin (Perrot and Ortonne 1978) the pattern change of melanosomes from complexes to single dispersion was noted. These changes were not necessarily related to the size of melanosomes.

In this study, although all the patients were Caucasoids, most of their melanosomes were singly dispersed within the keratinocytes. In a few patients there was a mixed pattern. The change in the melanosomal arrangement may be due to the reduced number of melanocytes and as a consequence of the sparse number of melanosomes, particularly in the marginal area. A sort of compensatory mechanism is operating. However, this pattern was also seen in the uninvolved areas where if there was a reduction in the number of melanocytes, it was not very marked. Melanosome complexes were also seen in the involved area where there was an almost complete absence of melanocytes and melanosomes.

X. INDETERMINATE CELLS

Attention was first drawn to the presence of these indeterminate cells in the epidermis by Breathnach et al (1963). Since then the nature of these cells has been debated. They are dendritic non-keratinocyte cells that lack desmosomes and tonofilaments. They have cytoplasmic features that are similar to those of Langerhans cells and melanocytes, but they lack Langerhans cell granules and do not contain melanosomes.

Over the years the origin of these indeterminate cells has been a matter of speculation. It has been considered that these cells are premelanocytes. Also that they could be precursors of the Langerhans cells. It has been also suggested that they are 'effete' melanocytes or they are completely unrelated to the melanocytes and Langerhans cells (Breathnach 1964; Snell 1965; Brown, Winkelmann and Wolff 1967; Tsuji, Sugai and Saito 1969).

Using an electron microscopic quantitative method (Zelickson and Mottaz 1970), it was reported that there was an increase in melanocytes and a decrease in the number of indeterminate cells following ultraviolet irradiation to the skin. They suggested that some of the indeterminate cells are dormant melanocytes. In another study (Miyazaki

et al 1974) it was found that following application of Dinitrochlorobenzene (DNCB) that there was marked increase in the number of Langerhans cells and a decrease in the number of indeterminate cells. It could be that the indeterminate cells following the application of DNCB develop granules and become mature Langerhans cells. Indeterminate cells are also found in the skin experimentally deprived of neural crest (Breathnach et al 1968).

Very recently, it has been shown (Rowden, Philips and Lewis 1979; Rowden 1980) that a high proportion of indeterminate cells in the epidermis express Ia antigen. This is similar to that of the Langerhans cells. Therefore, it seems unlikely that these indeterminate cells are related to melanocytes which are from the neural crest. It is more likely that they are related to the Langerhans cells.

In this study, indeterminate cells were only occasionally seen, particularly in the uninvolved areas of patients with vitiligo. Their morphology was more related to that of Langerhans cells and histiocytes. There were a few that could have been melanocytes, but without recognisable melanosomes. The number of these cells was not increased in the recently depigmented marginal areas. However, other workers (Mishima et al 1972) found an increase in the indeterminate cells in the newly depigmented

areas in vitiligo. Also in the older lesion of vitiligo the number of indeterminate cells dropped to zero and in these areas there was a complete absence of melanocytes. This increase of indeterminate cells in the recently depigmented areas may be explained by the presence of mononuclear cell infiltrate in the epidermis. Some of these cells are histiocytes that do not possess the distinctive Langerhans cell granules as has been shown in the present study.

XI. CUTANEOUS NERVES

There is evidence that a neurogenic factor may play an important role in the pigmentation of some animals. In fish and many other cold-blooded vertebrates, it has been demonstrated conclusively that skin pigment cells are under direct control of the nervous system (Pouchet 1876; Frisch 1911; Gray 1956). Unmyelinated nerve fibres were found in direct apposition to pigment cells in Fundulus (Bikle, Tilney and Porter 1966). Also catecholamine containing fibers found in anatomic proximity to conjuctival and dermal melanocytes of a Teleost fish. In the frog, Rana pipiens, the pigment cells are not innervated directly (Snell and Kulovich 1967). Yet these cells, like those in the lizard (Goldman and Hadley 1969; Hadley and Goldman 1969) respond to pharmacologically active adrenergic and cholinergic chemicals as well as to their blocking agents (Lerner 1971). Also it has been shown clearly that the melanocytes of fish, frogs, toads and lizards have alpha and/or beta adrenergic receptors (Goldman and Hadley 1969; Hadley and Goldman 1969; Scott 1965,1967; Scott and Wong 1966; Gupta and Bhide 1967; McGuire 1970).

It is apparent that human melanocytes are predominantly under endocrine control (Lerner et al 1966). To what extent those cells are also under

neurochemical regulation is as yet unknown. The only evidence in mammals that catecholamines affect melanocytes is that injection of adrenaline into rat skin produced grey hair (Munan 1953; Shelley and Öhman 1969).

In vitiligo a number of clinical observations suggest a relationship between the activity of human epidermal melanocytes and the nervous system. A patient having transverse myelitis with paralysis from waist down developed vitiligo on the face and upper part of the body, but no depigmentation occured below the level of the cord damage (Lerner et al 1966). Repigmentation in one patient with extensive vitiligo and diabetes occured concomitantly with development of severe diabetic neuropathy (Lerner et al 1966). In two human subjects with cervical sympathectomies, the development of grey hair with age was retarded on the denervated sides (Lerner et al 1966). In vitiligo hypopigmented areas frequently are confined to skin regions supplied by a nerve segment in a dermatomal distribution (Lerner 1959; Koga 1977).

Morphological changes of the nerves in vitiligo skin have been looked for in order to explain the pathogenesis of this disorder and to implicate a neurogenic factor. Breathnach et al (1966) investigated five vitiligo patients by electron microscopy

and found a minor degenerative change in the few nerve terminals, mainly of the marginal area. However, those changes are not convincing, especially when compared with the changes found in this study in normal controls. Bleehen (1979) has also found minor degenerative changes in the nerves of the upper dermis, but did not comment on the nature of these degenerative changes.

In a number of conditions in which cutaneous depigmentation occurs, compensatory proliferation of nerves may be present rather than degenerative changes. These nerves may enter into the epidermis. Although intra-epidermal nerves in a normal skin are seldom observed (Breathnach et al 1966; Breathnach 1971; Orfanos and Mahrle 1973), increased dermal nerves with intra-epidermal axons have been observed in halo naevus (Hashimoto 1974), Vogt-Koyanagi syndrome, vitiligo and incontinentia pigmenti achromians (Morohashi et al 1977). Ultraviolet light with or without psoralen can induce dermal and intra-epidermal nerve proliferation (Kumakiri and Hashimoto 1978; Kumakiri, Hashimoto and Willis 1978). Many of these dermal nerves were partly denuded and not completely enveloped by the Schwann cell process.

The association of melanocytes with nerves seem to be very rare in the normal skin. However, such an association has been reported. However,

the close proximity of nerves with melanocytes in vitiligo skin and incontinentia pigmenti achromians has been noted (Morohashi 1977). Also direct contact of nerve with melanocyte in the basal layer of the epidermis in a patient with Vogt-Koyanagi-Harada syndrome (Morohashi 1977). Similarly, this association and direct contact between melanocytes and axons has been reported in PUVA-treated skin (Kumakiri et al 1978; Kumakiri and Hashimoto 1978).

The following obervations were noted in this study with regards to the nerves in the skin. There were a number of partially denuded and regenerating axons, particularly in the dermis of the involved areas. There were an increased number of intra-epidermal nerves, particularly in the adjacent pigmented skin with a close association of dermal nerves with the basal melanocytes in a few of the patients. There was a direct contact between several of these intra-epidermal adrenergic nerves (Grillo 1966; Orfanos and Mahrle 1973) and functioning melanocytes in two of the patients. All these findings are similar to what have been previously observed in PUVA-treated skin. Minor pathological changes that were observed in two of the nerves were probably part of the general inflammatory reaction

in the skin which is similar to what has been seen in atopic dermatitis (Mihm et al 1976).

The so-called "minor degenerative" changes previously observed by other workers can be attributed to the inflammatory changes as seen in vitiligo skin.

A neurogenic theory has been suggested (Lerner 1959; Chanco-Turner and Lerner 1965; Lerner 1971) to explain the pathogenesis of vitiligo. They postulated that there was an elevated level of norepinephrine or related catecholamines at the peripheral nerve terminal endings. According to this theory, the increased nerve activity will enhance the cutaneous depigmentation. Surprisingly, there is an increased number of dermal and intra-epidermal nerves seen in PUVA irradiated skin (Kumakiri et al 1978; Kumakiri and Hashimoto 1978) and this associated with enhanced pigmentation. However, it has been noted in long term PUVA-treatment areas of hyperpigmentation and hypopigmentation of the skin develop (Gschnait et al 1980; Kanerva, Niemi and Lassus 1981). It seems unlikely this neurogenic hypothesis of the pathogenesis of vitiligo can be correct.

XII. COLLOID/AMYLOID BODIES

A variety of names have been used for these bodies in the past, e.g. hyaline bodies, Civatte bodies, Sabouraud cells or filamentous bodies (Ebner and Gebhart 1977). These filamentous bodies, usually globular or ovoid in shape, about 7-20μ in size are to be found in the upper papillary dermis, especially in relation to the dermo-epidermal junction. They can also be found within the basal layer of the epidermis. In haemotoxylin and eosin stained sections they appear eosinophilic and stain well with PAS and basic fuchsin. Direct immuno-fluorescence is usually positive especially with anti-IgA, anti-IgM, anti-IgG and less frequently with anti-C_3, and antifibrin antibodies. The immunoglobulin could be 'washed out' overnight in O.1M glycine at pH3 without changing the positive histochemical reactions of amyloid. Therefore, it was felt that the immunoglobulins were nonspecifically adsorbed by the amyloid (MacDonald, Fergin and Black 1980).

Electron microscopy reveals the filamentous nature of these bodies, which are composed of mainly of a dense network of 80-100Å filaments. Most of these bodies are composed of wavy filaments such as is seen in colloid bodies, but also in some of these, a network of straight filaments 70-100Å that are

characteristic of amyloid (Ebner and Gebhart 1975, 1977). In the present study the diameter of these filaments in the colloid/amyloid bodies ranged from 70-130Å with a mean of 100Å. This is similar to the cytofilaments of the melanocytes in patients with vitiligo. Not infrequently there is admixture of both types of filaments. Hence the use of the description colloid/amyloid bodies, though the majority of these cytoid bodies were more that of the colloid bodies in these patients with vitiligo.

It is likely that both colloid and amyloid bodies in the skin have the same origin and a sequential change of epidermal keratinocytes to amyloid via filamentous degeneration has been observed in primary localised cutaneous amyloidosis (Kumakiri and Hashimoto 1979) and also in PUVA-treated human psoriatic patients skin (Hashimoto and Kumakiri 1979). Similarly other workers have made this observation (Black and Wilson Jones 1971; Ebner and Gebhart 1975; MacDonald, Black and Ramnarain 1977).

Degenerating epidermal cells contain bundles of filaments and have a fibrillar ultrastructure such as is seen in that colloid/amyloid bodies. Electron microscopic and histochemical studies (Anton-Lamprecht and Teligen 1972,1978; Ebner and Gebhart 1972,a,b) and more recently histochemical

staining for sulphhydryl (-SH) groups and disulphide (S-S) bonds (Danno and Horio 1981) support the view that amyloid is derived at least partially (a weak S-S fluorescence but not -SH was detected in dermal amyloid) from epidermal cells. While the colloid bodies are completely derived from keratinocytes (positive staining for both -SH groups and S-S bonds). From these studies it seems likely that there is little difference between colloid and amyloid bodies and that both can coexist in the same location. It is also possible that melanocytes form these cytoid bodies as a result of degenerating. Fine cyto-filaments 100Å in diameter are to be found within the melanocytes and these often have a wavy pattern and are arranged in bundles similar to those of colloid/amyloid bodies.

Colloid/amyloid bodies are most frequently to be found in PUVA treated skin in both psoriasis (Hashimoto and Kumakiri 1979) and in mycosis fungoides (Bleehen, Vella Briffa and Warin 1978). These bodies are also found in normal skin. The number of these colloid/amyloid bodies as detected by direct immunofluorescence and electron microscopy seemed to be increased in vitiligo when compared with that found in normal controls. This was a surprising finding in the present study.

Colloid bodies have been described in a variety

of other pathological conditions. In lichen planus (Weedon 1974; Hashimoto 1976) in basal cell epithelioma (Kerr and Searle 1972) and in graft vs host reaction (De Dobbeleer, Ledoux-Corbusier and Achten 1975). Also these bodies have been seen in regressing plane warts (Weedon and Roberston 1978). Immunofluorescence studies have demonstrated cytoid bodies in discoid lupus erythematous, fixed drug eruption, lichen planus and mycosis fungoides (Ueki 1969; Gogate et al 1980).

Amyloid bodies have been observed in primary cutaneous amyloidosis (Hashimoto and Yoong Onn 1971; Black and Heather 1972; Black and Wilson Jones 1971; Black 1971). Secondary localised cutaneous amyloidosis has been described with basal cell epitheliomas (Sumegi, Goreczky and Roth 1954; Malak and Smith 1962; Brownstein and Helwig 1970; Hashimoto and Brownstein 1973; Weedon and Shand 1979), Cylindroma (Freudenthal 1930; Heyl 1966), seborrhoeic keratosis (Malak and Smith 1962), actinic keratosis (Hashimoto and King 1973), Bowen's disease (Brownstein and Helwig 1970), disseminated superficial actinic porokeratosis (Piamphongsant and Sittapairoachana 1974), PUVA treated skin (Hashimoto and Kumakiri 1979) and in melanocytic naevi (MacDonald and Black 1980).

As a regard the origin of amyloid deposits in the skin (Hashimoto, Gross and Lever 1965) suggested

that the amyloid fibrils in lichen amyloidosis were secreted by fibroblasts and then deposited in the papillary dermis. Since then several other investigators (Ebner 1968; Rodermund and Klingmüller 1970; Pierard and Kint 1971; Hashimoto and Yoong Onn 1971) have stressed the role of dermal fibroblast in the pathogenesis of lichen amyloidosis. In a light microscopic study of macular amyloidosis (Black and Wilson Jones 1971), it was suggested that damage to the lower epidermis might be the primary pathology and that degenerating epidermal cells could be converted into the deposits of amyloid. In a further enzyme histochemical study of lichen amyloidosis (Black 1971) it was considered that the epidermis was most likely to be involved in the pathogenesis of lichen amyloidosis.

Both amyloid and colloid bodies have been observed in conditions where there is hyperpigmentation of the skin and an associated degeneration of the basal layer. In addition pigmentary incontinence is present in the upper dermis, such as in lichen planus and discoid lupus erythematosus (Ueki 1969), macular amyloidosis (Black and Wilson Jones 1971; Black and Heather 1972; Eng 1977), Riehl's melanosis (Nagaos and Iijima 1974), PUVA-treated skin (Hashimoto and Kumakiri 1979), also in fixed drug eruption. Black and Wilson Jones (1971) emphasized the morphological

similarities between colloid and amyloid bodies and in both conditions a reduction in the number of basal melanocytes was noted. The number of DOPA positive melanocytes have been shown to be markedly reduced in the epidermis overlying the amyloid deposits in lichen amyloidosis (Black 1971),(even may be associated with leukoderma, Sharquie unpublished 1978) and that overly the colloid bodies in lichen planus (Black and Wilson Jones 1972).

Melanocytes are probably also involved and destroyed by the disease process. Amyloid deposition has been identified within the tumour mass of melanocytic naevi and it was thought that amyloid is likely to be derived from degenerating naevus cells (MacDonald and Black 1980). This is in agreement with this present study where the presence of these filamentous bodies was more related to the presence or absence of melanocytes. In addition in melanocytes 100Å filaments are well developed, particularly in the inactive cell and these cytofilaments resemble these seen in the colloid/amyloid bodies.

Not infrequently there is an admixture of wavy and straight filaments in the same filamentous body - those being colloid/amyloid bodies. Portions of dendrites of the melanocytes were seen in the papillary dermis and appeared to be very similar

to the adjacent colloid bodies. The absence of 'effete' melanocytes high up in the epidermis points to the fact that the fate of these cells is that they drop off into the dermis and become apoptotic cells. These will be later changed into filamentous bodies. However, the change of these degenerating melanocytes into colloid/amyloid bodies occured inside the epidermis in the basal layer. This natural process of apoptosis is exaggerated in pathological conditions, especially when there is hyperpigmentation. Thus, in macular and lichen amyloidosis, PUVA-treated skin and also in vitiligo these colloid/amyloid bodies are more apparent than normal skin.

The primary pathological process causing this secondary phenomenon of colloid/amyloid deposits differs according to the individual disease process. In a group of diseases a lymphocytic cell infiltrate is present as a form of immunological reaction causing damage to the basal layer of epidermis. This is seen in lichen planus, discoid lupus erythematosus graft versus host reaction (De Dobbeleer et al 1975) and regressing plane warts (Weedon and Roberston 1978), causing deposition of colloid bodies. The colloid bodies were also observed in an in vitro cell mediated immune reaction (Don et al 1977). This immunological reaction might also explain the colloid/amyloid formation in vitiligo, in particular, the presence of a lymphocytic cell infiltrate in

the epidermis.

XIII. OCCUPATIONAL VITILIGO

A vitiligo-like leukoderma that is often progressive is not infrequently seen in patients who have been in contact with a number of substituted phenols that are known to have a melanocytotoxic effect (Bleehen 1981). These compounds have a selective lethal effect on functional melanocytes, both in vivo (Bleehen et al 1968; Riley 1969) and in vitro (Riley 1970; Bleehen 1976; Mansur et al 1978). The mode of action in having a selective lethal effect on melanocytes is most probably that they are oxidised by the tyrosinase of the cells to give rise to free radical derivitives that are highly toxic.

Outbreaks of occupational vitiligo have occured due to contact with the monobenzyl ether of hydroquinone (Oliver et al 1940), P-tertiary butyl phenol (Kahn 1970; Malten et al 1971; Chumakov, Babanov and Smirnov 1962; James, Mayes and Stevenson 1977; Calnan and Cooke 1974), P-tertiary amyl phenol (Kahn 1970) and 4-tertiary butyl catechol (Gellin, Possick and Perone 1970). Often in these cases the depigmented areas fail to repigment and frequently metastatic areas of leukoderma develop a long time after the exposure to these phenolic compounds has ceased. Similarly, patients with various hypermelanotic conditions treated with

these compounds can develop confetti-like areas of depigmentation and often a progressive vitiligo-like leukoderma that may be more cosmetically disabling than original pigmentory disorder (Bleehen 1980). Patients with occupational vitiligo do not usually have an increased incidence of auto-immune disorders and autoantibodies (James et al 1977). However, several cases have reported (Gold-man and Thiess 1976) in which organ specific auto-antibodies are present.

Histopathological studies on biopsies from patients with occupational vitiligo have shown a picture similar to true (idiopathic) vitiligo (Kahn 1970; Malten et al 1971; Bleehen 1981). The finding in this present study confirms those previous ones. In addition, there were marked inflammatory changes in both the dermis and epidermis, with mainly atypical lymphocytes and histiocytes infiltrating the epidermis both in the uninvolved and marginal areas. Langerhans cells seemed to be increased in number and some of these cells were seen in contact with lymphocytes. Filamentous bodies of colloid and amyloid nature were also seen in the papillary dermis of these patients.

Immunofluorescence studies were also negative on the biopsies from these patients. All these findings do also suggest that a cell mediated reaction is involved in some of the cases of occupational

vitiligo.

Histopathological studies on biopsies from experimentally (Bleehen et al 1968) and therapeutically (Bleehen 1976) induced depigmentation did not show any inflammatory changes. The inflammatory changes in this present study suggest that a primary immune reaction is involved rather than being secondary to melanocyte destruction. However, it could be argued that the inflammatory response was secondary though this does seem unlikely.

It is also quite likely that the patients who readily develop occupational vitiligo are indeed 'latent' cases of idiopathic vitiligo which is unmasked by the exposure to these chemicals (Richter 1980; Bleehen 1981)

XIV. REPIGMENTATION IN VITILIGO

Repigmentation of the skin in vitiligo can occur spontaneously or may be the result of therapy. It is most frequently perifollicular and also the pigmentation can occur around the margin of the lesions. The precise mechanism in which repopulation of the skin takes place is uncertain, but several possibilities have been suggested.

It has been suggested that there is reactivation of weakly dopa-positive residual melanocytes in the vitiliginous area of skin (Jarret and Szabo 1956). Similarly inactive melanocytes in the areas of vitiligo become functional and produce pigment (El Mofty 1968). A number of studies suggest that the melanocytes involved are responsible for the repigmentation originate from the hair follicles, from melanocytes that may remain in the normal pigmented hair in their external hair root sheath (El Mofty 1968; Parrish et al 1976; Bleehen 1976; Ortonne, Sannwald and Thivolet 1978). This repigmentation around hair follicles occurs either spontaneously or as a result of treatment. Areas devoid of hair follicles such as the mucous membrane, palm and soles, if affected with vitiligo rarely repigment. Similarly, involvement of the hair in the affected areas 'leukotrichia' is a bad prognostic sign (Dutta and Mandall 1969) and rarely repigment.

In a study of hair follicles in PUVA-induced repigmentation of vitiligo (Ortonne et al 1979; Ortonne, Schmitt and Thivolet 1980) these workers found on split dopa preparations and on electron and scanning microscopy hypertrophic melanocytes in the hair follicles of the pigmented area. They suggested that melanocytes migrate from the external root sheath to the basal layer of the interfollicular epidermis to recolonise the areas of vitiligo.

In the present study residual melanocytes were seen in the interfollicular areas of the epidermis in most of the biopsies from the involved areas of skin. Thus it seems likely that the interfollicular, as well as the follicular residual melanocytes, are involved when vitiligo repigments. No melanocytes were seen to be in mitosis in any of the biopsies that were examined in this study. However, the melanocytes that repopulate the areas of vitiligo when they repigment, almost certainly undergo mitosis and proliferate.

APPENDICES

1. DIRECT IMMUNOFLUORESCENCE TECHNIQUE

The biopsies are frozen as soon as possible following their excision. Each block is sectioned as soon as possible allowing one hour after mounting for temperature equilibrium in the cryostat. The total time being approximately 1½ hours. Maximum number of specimens stained at one time is 4 (12 slides).

1. Cut cryostat sections 6-8μ thickness, 2/slide, labelled. Storage slides are placed back to back and wrapped in foil (a layer of foil between each pair of slides) numbered and placed in the deep freeze.
2. Label three slides in sequence with a glass marker as follows; leave the slides out to dry at room temperature for fifteen minutes.
3. Remove diluted conjugates *** from the deep freeze to thaw out.
4. Place the slides in a staining rack and immerse in a bath of 1 part veronal buffer*, 1 part distilled H_2O. Agitate slowly (at 2-3 using large magnet) on magnetic stirrer for fifteen minutes.
5. Remove slide racks, drain slides and wipe around the marked sections so as to prevent excess dilution of the conjugates, but do not allow the sections to dry out. (put the lid

on the buffer bath). Using plastic pipettes put on sufficient diluted antiserum*** to cover the section (usually 1 drop) and leave in a moist box for thirty minutes, i.e. wipe the slides, place in the box which has been rinsed with water and drained. Use a separate pipette for each conjugate. Place lid on the moist box and leave undisturbed. Replace the conjugates in the deep freeze. Stain in sequence as in 2 above.

6. Put the buffer on to stir vigorously a few minutes before putting the slides in. Drain the slides sideways so that the drop of conjugate drains into the moist box, then put slides into the slide rack. Place the rack into the buffer with the magnetic stirrer at 2. Leave for fifteen minutes.

7. Remove the slide rack, drain the slides and carefully dry around the sections. Place 1 drop of sodium barbitone/glycerol buffer** onto each section and place a clean coverslip on top, ensuring no air bubbles are present by lowering the coverslip from the side. The stained slides are placed in the cardboard holders (in order as in 2 above), a label written out containing the number, name of patient and disease attached and kept in the deep freeze.

Veronal Buffer*

20.6g	sodium bicarbonate
85g	NaCl
5L.	H_2O

The powders are dissolved separately in measured amounts of distilled water, e.g. 500ml. each. Then added to the container and made up to 5 litres. The pH is adjusted if necessary to 7.2 using concentrated HCl. The above solution is diluted 1:1 with distilled water for us.

Mounting Medium**

Sodium barbitone	0.1030g
NaCl	0.85g
H_2O	10ml.
Glycerol	90ml.

The powders are dissolved separately in measured amounts of distilled water, then made up to 10ml. when put together. The glycerol is added, shaken and allowed to settle.

Conjugates (antiserum)***

These were obtained as powder from the manufacturer (Behringwerke). 1ml. of distilled water is added to the ampule and elute 0.1m. of this solution into each of 10 bijou bottles, place these into the freezer. When needing to stain, remove one of the 0.1ml. bottles from the freezer and add 1.4ml. of normal saline. Mix well and use for the method***, One set of all the conjugates should be kept in

the above form***, replenishing when necessary from the O.1ml. eluents. If received as a liquid (as in C_4) elute O.1ml. into the 10 bijou bottles and continue as before.

Freezing Skin Biopsies for Direct Immunofluorescence Technique

1. Biopsies are wrapped in gauze and immersed in normal saline for transport to the laboratory. The biopsy must be frozen immediately. The tissue is trimmed, taking care not to damage it.
2. The tissue is frozen using Bright cryospray. A blob of OCT compound is placed on the frozen chuck and the frozen tissue is correctly aligned and embedded on the freezing medium using the tweezers and the cryospray. More medium is added if necessary so as to just cover the tissue. Water is used to seal the chuck to the block and the whole is wrapped in foil, numbered and stored in the cryostat until it is sectioned. After sectioning the block is removed and kept in numbered foil, either within the cryostat or in the deep freeze.

II. INDIRECT IMMUNOFLUORESCENCE COMPLEMENT FIXATION TEST

Procedures:

1. Cut frozen human skin 6μ thick.

2. Dry at room temperature for ten minutes.

3. Wash in phosphate buffered saline (pH 7.2) for fifteen minutes. Phosphate buffered saline (PBS).

PBS	One litre
NaCl	6.798 gm.
Na2HPO4 (anhydrous)	1.478 gm.
KH2PO4	0.43 gm.

Add salts to portion of distilled water and mix on magnetic stirrer until dissolved, then make volume with distilled water.

4. Cover the sections with patients and control sera (in different dilutions) in moist box at room temperature for 45 minutes.
5. Wash sections with PBS for fifteen minutes and fan dry.
6. Cover the sections with human complement (fresh human sera as source of complement) for 45 minutes at $37^{o}C$ temperature in moist box.
7. Change PBS, wash for fifteen minutes and fan dry.
8. Incubate the sections with fluorescin conjugated antihuman complement (C_3) for 30 minutes in moist box at room temperature.

9. Wash for 30 minutes in PBS. Wipe around the sections.
10. Mount the sections in sodium barbitone/glycerol buffer on ordinary slides.
11. Examine with fluorescent microscope as early as possible otherwise the fluorescence fades.

The antigens as human skin were taken from normal skin, involved vitiligo skin, pigmented naevus and blue naevus.

For each vitiligo patient's serum that was studied, positive pemphigoid serum and that from a normal person were used as controls. All patients and control sera were diluted with PBS in dilutions of 1:1, 1:2, 1:4 and 1:8. The heating of sera of patients and controls at $56^{o}C$ for 30 minutes made no difference.

The human serum as source of complement was diluted with either complement diluent or PBS in 1:5.

III. HISTOCHEMISTRY STUDY (DOPA METHOD)

L-Dopa - 1/1000 L-Dopa (0.25g. in 250 mls. water). This is made fresh each time and not stored for longer than one week in the refrigerator. The tissue is incubated in 7 ml. of 1/1000 L-Dopa to which is added 3 ml. of phosphate buffer. The pH of the solution being 7.2. The phosphate buffer is made up of 8 ml. 0.1M Na2HPO4 and 2 ml. 0.1M NaH2PO4.

Procedure

1. Incubate the shave biopsy in 2N NaBr at 37°C for one hour.
2. Separate dermis from epidermis under the dissecting microscope using tweezers and a long needle.
3. Partially fix in 5% cold formaldehyde saline for 5-10 minutes and then rinse thoroughly several times with distilled water.
4. Incubate tissue in L-Dopa solution at 37°C for 4-5 hours. If there is insufficent time that day, keep it in a refrigerator at 4°C until next morning and then incubate.
5. Fix tissue in 10% formaldehyde saline at 37°C overnight.
6. Dehydrate the tissues in 70%, 80% and 90% ethanol for one hour in each and then in absolute alcohol for two hours.

7. Place in cedar wood oil to clear for at least 24 hours.
8. Put in xylene for two hours.
9. Mount in depex on an ordinary slide, putting the dermal surface of the epidermis (curled side) up and cover with a glass coverslip without getting too many bubbles of air. For 24 hours a small weight is applied on top of the coverslip until the mounting medium has set. The slide is then ready for examination.

IV. EPON EMBEDDED TISSUE FOR LIGHT MICROSCOPY

(a) Processing of Tissue

The tissue is kept in the glutaraldehyde for 24 hours at $4^{o}C$. It is then stored in phosphate buffer until processed.

1. Wash tissues in distilled water. Empty the buffer and wash with distilled water twice. Drain.
2. Osmium tetroxide fixation (in a fume cupboard). Place 1 ml. of 2% OsO_4 into each pot (make sure all the tissues are in the liquid). Screw tops on and place in the fridge for 1½-2 hours.
3. Drain OsO_4 and wash with distilled water (as in No. 1).
4. Drain water. Fill the pots with 70% alcohol. Drain. Add more 70% alcohol and leave for ten minutes.
5. Drain 70% and add 90% alcohol for ten minutes.
6. Drain 90% and add absolute alcohol for ten minutes.
7. Drain absolute and add more absolute alchol for ten minutes.
8. Drain absolute and add absolute alcohol dried over Calcium Sulphate for ten minutes.
9. Drain Calcium Sulphate alcohol. In a fume cupboard immerse tissues in epoxy propane (propylene oxide) for fifteen minutes with tops

on. Make up Epon*

10. Drain epoxy propane and replace with fresh epoxy propane for a further fifteen minutes.

11. Add epoxy propane to Epon* solution and mix well giving a 1:1 solution. Epon**

12. Drain epoxy propane from the tissues and add Epon**. Make sure the tissues are in the Epon. Leave for one hour with the tops off.

13. Mix Epon*** forty minutes later.

14. At the end of the one hour add epoxy propane to give 1:3 mixture. Epon****.

15. Drain the 1:1 mixture well and add the 1:3 Epon. Leave overnight.

16. Make up the day's Epon, i.e. four pots of epon minus DMP30. Keep covered with a petri dish.

17. Drain 1:3 Epon and immerse tissues in full strength Epon*****. Place the tissues in a fresh change of Epon***** at 10 a.m., 12 noon and 2.00 p.m.

18. Embed tissues in Epon******. Place numbered labels in the flat embedder. Put the Epon on to mix. Remove the tissues from the epon and allow the excess Epon to drain from the tissues. After the Epon has mixed for 10-15 minutes place one drop of the prepared Epon into each mould at the tissue end using a 2 ml. syringe. Place the drained tissue into the mould and line

up as follows.

Tissue [redacted]113/81

19. After 20 minutes of mixing the Epon, syringe it into the moulds to that it adequately covers the tissue and does not protrude over the sides of the mould. Place mould in 60°C oven overnight.

20. Remove tissues at 9.00 a.m. the next day.

(b) Epon Preparation

Epon 812	ml.	90	18	9	4.5
DDSA	ml.	70	14	7	3.5
MNA	ml.	50	10	5	2.5
DMP30	ml.	4.2	0.84	0.42	0.21

Above are quantities for full strength Epon, depending on how many specimens are being processed.

Example for 6 specimen pots

1:1 Epon

Epon 812	13.5 ml.	)	)
DDSA	10.5 ml.	)	)
MNA	7.5 ml.	) *	) **
DMP30	0.63 ml.	)	)
Epoxy propane (E.P.)	32 ml.		)

Use glass measuring cylinder for E.P. and syringes for resins, green needles for DMP30. Prepare Epon. Mix (folding action) with glass rod. Place magnet

in, cover with petri dish lid, spin at 4-6 with no heat for twenty minutes.

1:3 Epon

Epon 812	18 ml.		
DDSA	14 ml.		
MNA	10 ml.	***	****
DMP30	0.84 ml.		
E.P.	14 ml.		

Prepare as above

Epon for 3 full strength changes

Epon 812	22.5 ml.	
DDSA	17.5 ml.	*****
MNA	12.5 ml.	
DMP30	1.05 ml.	

Prepare as above.

Epon 812	4.5 ml.	
DDSA	3.5 ml.	******
MNA	2.5 ml.	
DMP30	0.21 ml.	

Prepare as above.

(c) Staining Technique for Plastic Embedded Tissue

'Huber' - Staining Method

1. Cut 1μ Epon sections.
2. Place a small drop of H_2O on a clean glass and with a wire loop, place 4-5 sections on the surface of the water. Usually two slides are prepared.
3. Dry the sections on a hot place at 80°C for five minutes.

4. Ring the sections on the reverse side of the slide with a felt pen.

5. Prepare an aqueous solution of 3% basic fuchsin and allow to mix well on a hot plate at 80°C for approximately thirty minutes before use. Filter the stain through a millipore filter into a bijou bottle and leave on the hot plate for as long as it is in use. This may be retained overnight in a 37°C incubator and used the following morning. However, the stain must be discarded after 24 hours and a fresh batch prepared.

6. Stain sections for 0.5-1 minutes with basic fuchsin - cover the stain with a bottle top to prevent evaporation and precipitation.

7. Destain the section for approximately 30 seconds with distilled water at 80°C.

8. Air dry until cool.

9. Stain with 2% alkaline methylene blue at pH 12.0.

<u>Alkaline Methylene Blue</u>

(a) Prepare a 0.1N NaOH solution, i.e 1gNaOH: 250 ml. distilled H_2O.

(b) Prepare a 2% aqueous solution of methylene blue.

(c) Just prior to use, mix 5 units 0.1N NaOH + 1 unit of methylene blue.

10. Stain progressively at 15-30 second intervals until the desired effect is obtained.

11. Dry at room temperature and mount in Lenzol. Seal the edges of the coverslip with glyceal. Preparation of glyceal = 50 ml. glyceal/50 ml. methanol.

* slides may be stored in a dry, light tight box for approximately one year after which time the sections will start to lose their true colour.

V. ELECTRON MICROSCOPIC STUDY

(a) Processing Tissues for E.M.

Processing can be carried out on either a Monday or a Wednesday. Pre-fix tissues in glutaraldehyde 24 hours in fridge, then stored in PO4 buffer until processed.

1. Wash tissues with distilled water. Empty buffer out and fill the bottle with distilled water twice. Drain.
2. Osmium tetroxide fixation (in fume cupboard). Place 1 ml. of 2% OsO4 into each pot (making sure all tissues are in the liquid). Screw tops on, place in the fridge for 1½-2 hours.
3. Drain OsO4. Wash with water as in 1.
4. Block staining with Uranyl Acetate. (0.33g/11ml. of 50% C2H5OH dissolved using magnetic stirrer; covered with parafilm. Centrifuge. Keep at room temperature away from light). Place more than 5 ml. into each pot. Screw tops on and keep dark at room temperature for two hours.
5. Drain Uranyl Acetate. Fill the pots with 70% alcohol, drain, add more 70% for 10 minutes.
6. Drain 70%, then 95% for 10 minutes.
7. Drain 95%, then absolute for 10 minutes.
8. Drain absolute, then absolute for 10 minutes.
9. Drain absolute, then absolute dried over Calcium Sulphate for 10 minutes.

10. Drain. CaSO4/alcohol. In fume cupboard immerse tissues in epoxy propane (propylene oxide) for 15 minutes with tops on. Make up the araldite *.

11. Drain epoxy propane. Immerse in more epoxy propane for 15 minutes.

12. Add epoxy propane to araldite mixture, then drain epoxy propane and add ** araldite. Make sure all tissues are immersed. Tops off 24 hours.

13. Drain araldite. Immerse in *** ½ accelerator araldite, tops on overnight.

14. Make up the days araldite minus the DMP30 (keep covered with petri dishes).

15. Make araldite and immerse tissues in full strength **** araldite (three changes).

16. Embed tissues in ***** araldite. Place numbered labels in the flat embedder. Put araldite on to mix. Extract the tissues from the araldite so that they stick to side of the bottle and any araldite on them drains away. After 10-15 minutes of araldite mixing put one drop of the mixing araldite into each mould at the tissues end (with a 2 ml. syringe). Place the drained tissues into the appropriate mould and line up as shown. After 20 minutes of mixing the araldite, syringe it into the moulds so that it covers the tissues adequately, but is flat when viewed from the side.

17. Place mould in 60^{o} oven for 48 hours.

Ultrathin sections (60-70nm) are cut with glass knives on Reichert OMU3 ultramicrotome.

(b) Grid Staining with Lead Citrate

Using a glass Petri dish and dental wax or coverslip and NaOH pellets. Place approximately 15 pellets around a piece of dental wax in the petri dish. Put one drop of lead stain per grid to be stained onto the dental wax. Grids are floated, section down on the lead drops. The Petri dish is covered while staining. Stain each for four minutes. Remove grid and wash by immersing in a beaker of distilled water for thirty seconds. Dry on a filter paper.

When staining more than one grid, the following formula must be used. One or two grids could be stained each time.

Grid No.	Grids into lead	Grids out lead for wash
1	0 min.	5
2	2 "	7
3	4 "	9
4	6 "	11
5	8 "	13
6	10 "	15

(c) Araldite Preparation

CY212	50	40	30	20	10	mls.
DDSA	50	40	30	20	10	mls.
Di-Butyl phthalate	5	4	3	2	1	mls.
DMP30	105	84	63	42	21	drops

Drops from a syringe with a green needle.

Above are the quantities for full strength araldite, depending on how many specimens are being processed.

Example for 6 specimen pots

50/50 E.P./araldite mixture

CY212	20 ml.	)	)
DDSA	20 ml.	)	)
D.B.P.	2 ml.	) *	) **
DMP30	21 drops	)	)
E.P.	42 ml.		)

Use glass measuring cylinder for E.P. and syringes for resins, green needles for DMP30. Prepare araldite. Mix (folding action) with glass rod. Place magnet in, cover with petri dish lid, heat at mark 15-20, spin at 4-6 for 20 minutes.

Araldite for ½ DMP stage

CY212	20 ml.	)	
DDSA	20 ml.	)	***
D.B.P.	2 ml.	)	
DMP30	21 drops	)	

Prepare as above

Araldite for 3 full strength changes

CY212	20 ml.	)	
DDSA	20 ml.	)	**** x 3
D.B.P.	2 ml.	)	
DMP30	42 drops	)	

Araldite for embedding

CY212	10 ml.	)	
DDSA	10 ml.	)	*****
D.B.P.	1 ml.	)	
DMP30	21 drops	)	

Prepare as above

VERY IMPORTANT - do not allow water near to any of the resins or tissue after starting dehydration.

(d) Solution Preparations

Glutaraldehyde Fixative

1. Prepare A + B buffer and store at +4°C (shelf life = several weeks).

   A buffer - $NaH_2PO_4 2H_2O$ = 1.56g/100 ml.

   B buffer - $Na_2HPO_4 12H_2O$ = 3.58g/100 ml.

2. Prepare PO_4 buffer by mixing 77 ml. B + 23 ml. A and store at +4°C.

3. Take 8.8 ml. PO_4 buffer and 1.2 ml. glutaraldehyde to make 10 ml. buffered glutaraldehyde. Make up fresh each time.

Lead Citrate Stain

1. Lead Nitrate = 2.66g
   Sodium Citrate = 3.52g
   100 ml. distilled water
2. Dissolve separately with approximately 30 ml. water.
3. Mix together in 100 ml. volumetric flask. The solution becomes white and cloudy.
4. Shake well for 1 minute.
5. Stand for 30 minutes with intermittent shaking.
6. Prepare 1N NaOH solution, i.e. 1g/25 ml. distilled water.
7. Add 16 ml. of NaOH (freshly prepared) and mix well. The solution should be clear and PH 12.0.
8. Centrifuge for 20 minutes at 2,000 r.p.m. and store at +4°C. Avoid contact with air.

Uranyl Acetate

This substance is radioactive and all instruments should be decontaminated with concentrated decon for 24 hours. Plastic gloves should be worn.

1. Prepare 0.33g Uranyl Acetate/11 ml. 50% alcohol.
2. Mix well on a magnetic stirrer (covered).
3. Centrifuge for 20 minutes at 2,000 r.p.m.
4. Prepare fresh each time.

Osmium Tetroxide (O_sO_4) Fixative

This substance is harmful and should always be handled in a fume cupboard. Plastic gloves should be worn.

1. O_sO_4 is distributed in lg glass capsules.
2. When preparing a 2% solution of Osmium - break open the capsule (using a wad of tissue), and quickly place the Osmium and the glass capsule into a brown stopped bottle.
3. Add 50 ml. distilled water and shake well.
4. Always return to the fridge immediately after use and store at this temperature.

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