MATERIALS AND METHODS

Chemicals and Antibodies

Anti-factor VIII-associated antigen serum was obtained from Calbiochem. An anti-keratin serum was prepared in this laboratory by immunisation of rabbits with a preparation of rat hair and foot pad callous keratin (Hedberg and Hunter 1988). An anti-type IV collagen serum prepared by Dr J. P. McArdle (McArdle *et al.* 1984) was supplied by Dr R. O'Grady. Freon 22 was obtained from Du Pont, while JB4 resin for embedding was supplied by Polysciences. All other chemicals and antibodies were supplied by Sigma.

Tissues

The same tissues were used in this study as were used in that described in Chapter 3. In addition to these, however, a further 7 gingival biopsies were obtained from patients with advanced periodontitis in the same manner as described in Chapter 3. Of the 7 additional periodontitis specimens, 6 were processed for electron microscopy, and one was used for frozen sections.

Tissue Processing for Light Microscopy

Identical blocks were used in the same way as described in Chapter 3. Fixed tissues were stained with toluidine blue, as well as with haematoxylin. 8 μm cryostat sections were prepared and fixed with cold acetone. Slides were stored desiccated at 4°C.

Quantification of PHyM

Since PHyM was only present in Fields 1 and 2 of gingival biopsies, only these fields were considered in this study. All vessels in close relation to the pocket or sulcular epithelium were assessed for the presence or absence of PHyM. The thickness of the material was also recorded as being either thick or thin for each vessel (Fig 4.1). Where several vessels were enclosed within a sheath of PHyM, each vessel was considered independent of the others. It is likely that this assessment provided a conservative estimate of the incidence of PHyM, as very thin deposits were considered too difficult to interpret and it was decided to record such vessels as being free of PHyM.

Histochemistry and Immuno-Histochemistry

A trichrome stain was performed on sections from six fixed specimens with extensive deposits of PHyM (Burns and Bretschneider 1981).

Sections of freeze-substituted tissues from six patients noted as having extensive PHyM deposits in the quantitative assessment, were stained using an anti-keratin serum, and an anti-factor VIII-associated antigen serum. Sections of freeze-substituted tissues were pre-treated in a 0.1% trypsin solution at 37°C for 10 minutes (Mepham *et al.* 1979). Cryostat sections were tested using an anti-type IV collagen serum. Primary antisera were incubated over sections for 45 minutes at 37°C at dilutions of 1/100. An alkaline phosphatase conjugated goat anti-rabbit serum at a dilution of 1/50 provided specific labelling of the sections when incubated with fast red violet 0.2mg/ml, α-naphthyl AS MX phosphate 0.1mg/ml and levamisole 6 mg/ml in 0.01M tris buffer at a pH of 8.1 at 37°C. Control sections were treated in an identical manner to test slides, but were not incubated with the primary antiserum.

Tissue Processing for Electron Microscopy

Tissues were fixed in Karnovsky's fixative, washed in 0.1M cacodylate buffer at pH 7.3, and post-fixed in 1% osmium tetroxide, before dehydration with graded alcohols and embedding in Spur's low viscosity resin. Thin sections were cut with an LKB ultramicrotome system 2128 ultratome and collected on copper grids with carbon coated nitrocellulose films. Grids were stained with uranyl acetate and lead citrate.

Grids were studied using both Phillips 400 and JEOL 100CX electron microscopes. The changes described were typical of all the specimens studied, however, it was decided that there was insufficient data to allow a quantitative analysis at the ultrastructural level.

Statistical Procedures

It was decided that a normal distribution of the data could not be assumed, and so non-parametric methods of statistical analysis were applied. Chi-Squared, Wilkoxons Rank Sign, and Man-Whitney-U tests were used as required (Champion 1970). All p values given were derived using these tests.

RESULTS

Appearance of PHyM in The Light Microscope

PHyM was found only in association with capillaries of the vascular plexus close to the pocket epithelium. The material was always thickest at the interface between epithelium and endothelium, whilst it was often either thin or absent where the vascular endothelium was not apposed to epithelium (Figure 4.1). Single vessels were often affected within the network. Occasionally, the epithelium appeared to have proliferated down and around vessels entombed in PHyM, isolating the vessels from the underlying connective tissues (Figure 4.2) On other occasions, however, all or most vessels were affected at multiple sites, fusing vessels together in a hyaline sheath (Fig. 4.1). Where large numbers of vessels were affected, the pocket epithelium was largely supported by PHyM. Occasional foci of ulceration were found over PHyM.

Quantitative Assessment of PHyM

As shown in Figure 4.3, Field 1 of periodontitis specimens had more extensive deposits of PHyM than periodontitis Field 2 and Field 1 of minimally inflamed gingiva ($p < 0.001$). In 18 out of 20 patients with periodontitis, relatively more vessels were affected with PHyM in Field 1 than in Field 2 ($p < 0.005$). Also gingivitis specimens had more extensive deposits of PHyM than were found in minimally inflamed gingiva ($p < 0.005$) and periodontitis Field 2 ($p < 0.01$).

There were more thick deposits of PHyM in Field 1 of periodontitis specimens than in Field 2 sites ($p < 0.001$). Similarly, gingivitis specimens had more thick PHyM than was found in Field 2 of periodontitis ($p < 0.001$) (Figure 4.4).

Electron Microscopic Observations

Although appearing to be amorphous with the light microscope, PHyM was found to be structured using the electron microscope. The bulk of the PHyM consisted of a flocculant material of moderate electron density. This material was often arranged in layers suggesting that it was derived from multiple basal lamina (MBL). However, the lamellar structure was disrupted in places suggesting either degradation of the MBL material, or inclusion of other connective tissue or serum proteins. Epithelial cells had a normal ultrastructure with a thick basal lamina (BL), as compared with the more delicate endothelial and PHyM BLs. Interspersed in this flocculant material were many collagen fibrils. More collagen bundles appeared to be present in the PHyM close to the epithelial cells, than in the region nearer to the

vascular endothelium. Collagen fibrils were occasionally orientated in a common direction, but were mostly disorientated in relation to each other. Fine fibrils of similar appearance to intracellular intermediate filaments were sparsely distributed throughout the PHyM, and occasionally appeared as tangled clumps. Membrane blebs were also an inconsistent observation so that some sites contained abundant quantities, while others contained only occasional fragments. Many of these blebs were small and circular, suggestive of cellular debris. These changes are illustrated in Figures 4.5 to 4.8.

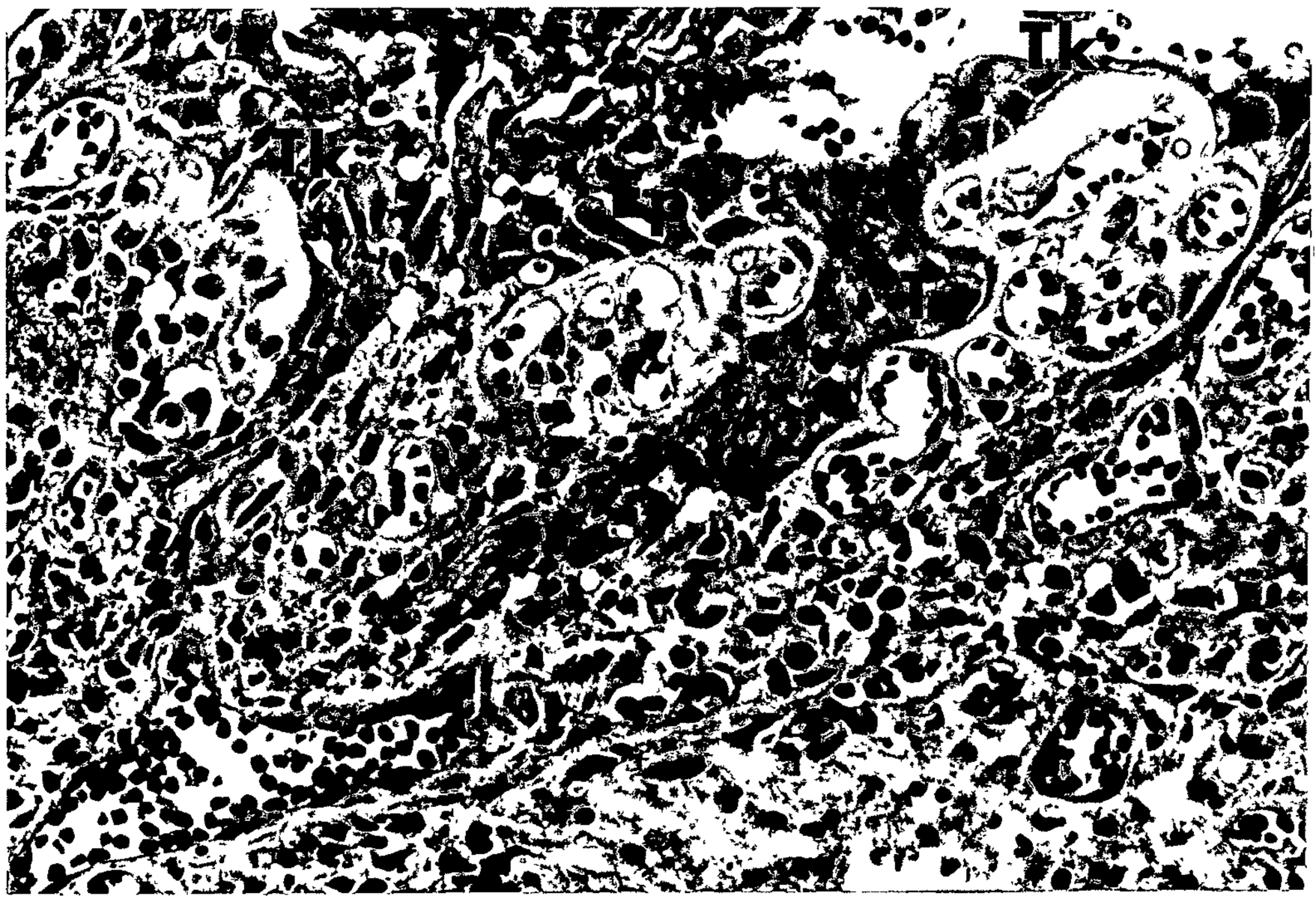
The vascular endothelium appeared to be normal in all respects, and pericytes were present at the inner border of the PHyM, sometimes separated from endothelial cells by several layers of MBL.

Histochemical and Immuno-Histochemical Findings

Collagen fibres and PHyM stained light blue with the Trichrome stain, suggesting that PHyM is of a collagenous nature.

The electron microscopic observation that the bulk of the PHyM consists of MBL material, was confirmed by immuno-histochemistry. An anti-type IV collagen serum gave strong and specific staining of the BLs of both epithelium and blood vessels, as well as labelling the PHyM (Figures 4.9 and 4.10).

Use of anti-keratin and anti-factor VIII-associated antigen serum did not result in convincing labelling of the PHyM, leaving the origin of the fine fibrils and cellular debris undefined. However, in one specimen, PHyM did appear to have small flecks of FVIII, suggestive of endothelial debris (Figures 4.11, 4.12, and 4.13).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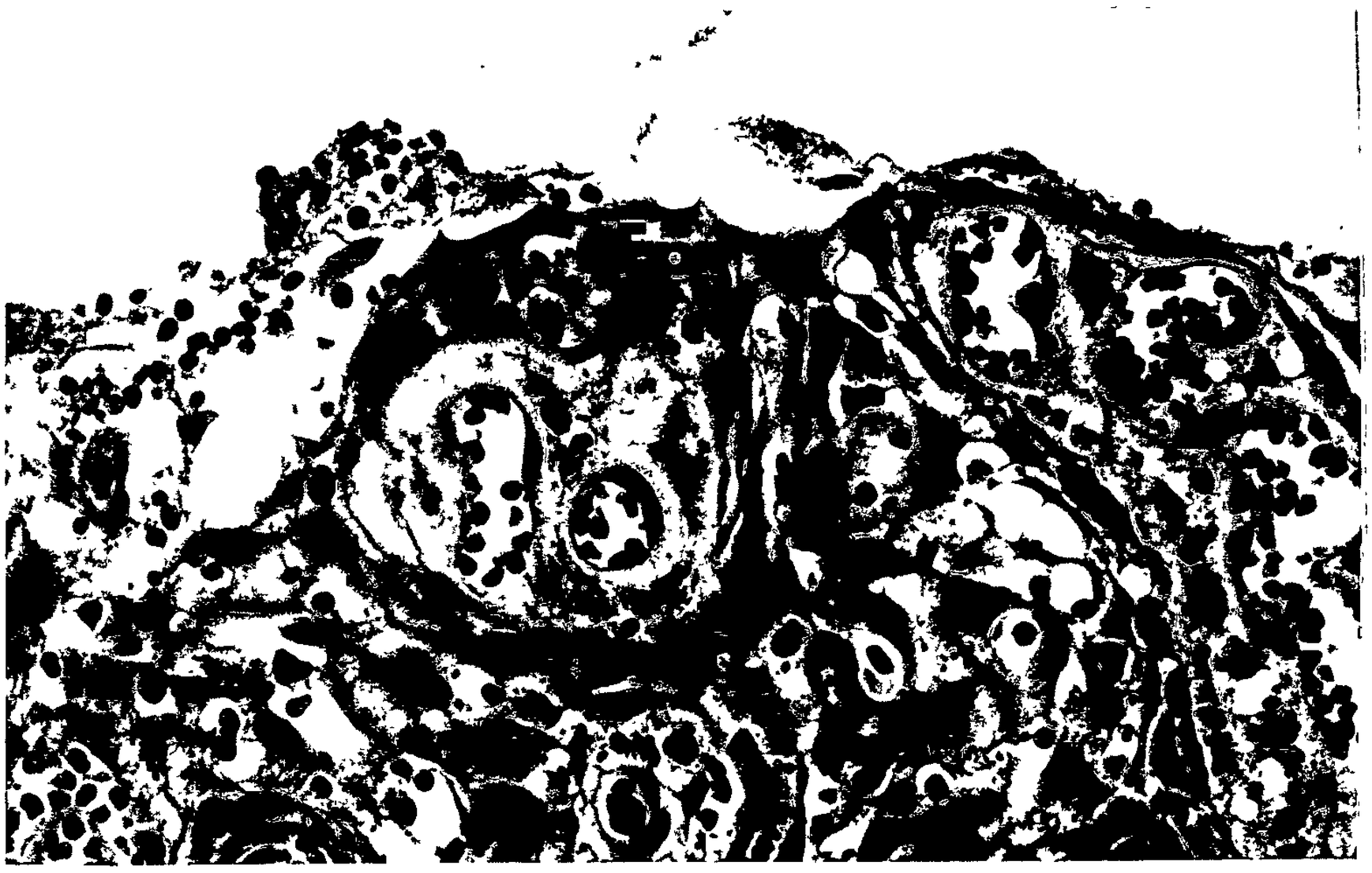


Figure 4.1

Photomicrograph of PHyM surrounding vessels in association with the pocket epithelium in a gingival biopsy from a patient with periodontitis.

PHyM underlies the pocket epithelium (Ep). The PHyM from several vessels has fused forming a plate of PHyM which supports the pocket epithelium apposed to the vessels. Deposits which were recorded as being thick (Tk) are compared with those which were scored as thin (Tn). Note that the material tends to be either thin or absent on the side of the vessel not apposing the epithelium. (Trichrome X 312)

Figure 4.2

Photomicrograph of PHyM surrounding vessels in association with the pocket epithelium (Ep) in a gingival biopsy from a patient with periodontitis.

The epithelium has proliferated around the material so that the vessels appear to be isolated from the underlying connective tissues. (Trichrome X 312)

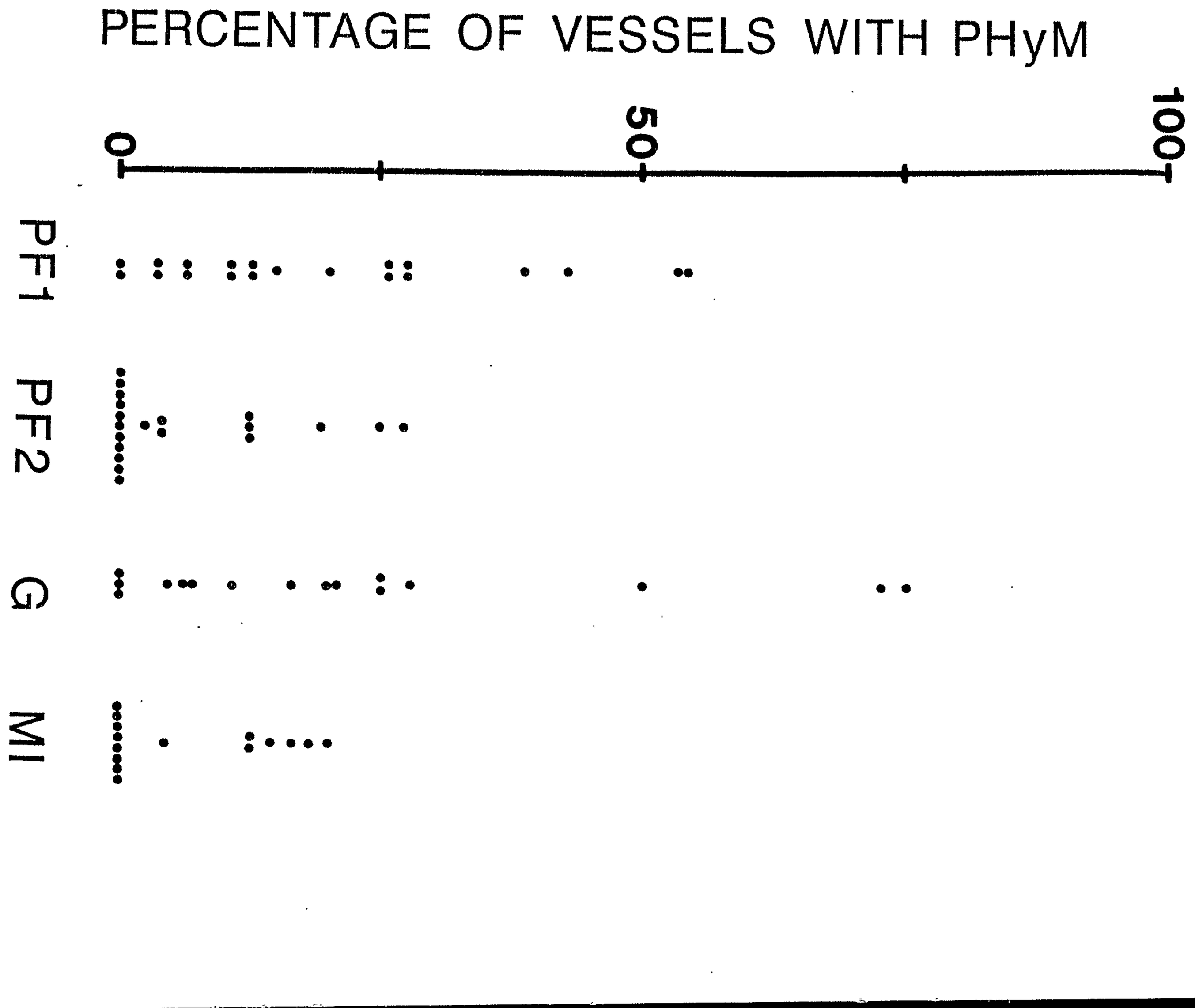

PERCENTAGE OF VESSELS WITH PHyM
0
50
100
PF1
PF2
G
MI

Figure 4.3

Scattergram in which each dot represents the relative percentage of vessel profiles in contact with the pocket epithelium affected with PHyM, in individual patients. These are aligned in columns representing periodontitis Field 1 (PF1), periodontitis Field 2 (PF2), gingivitis (G) and minimally inflamed tissues (MI).

Field 1 of periodontitis specimens had more extensive deposits of PHyM than periodontitis Field 2 and minimally inflamed gingiva ($p < 0.001$). In 18 out of 20 patients with periodontitis, relatively more vessels were affected with PHyM in Field 1 than in Field 2 ($p < 0.005$). Also gingivitis specimens had more extensive deposits of PHyM than were found in minimally inflamed gingiva ($p < 0.005$) and periodontitis Field 2 ($p < 0.01$).

% VESSELS WITH THICK PHyM

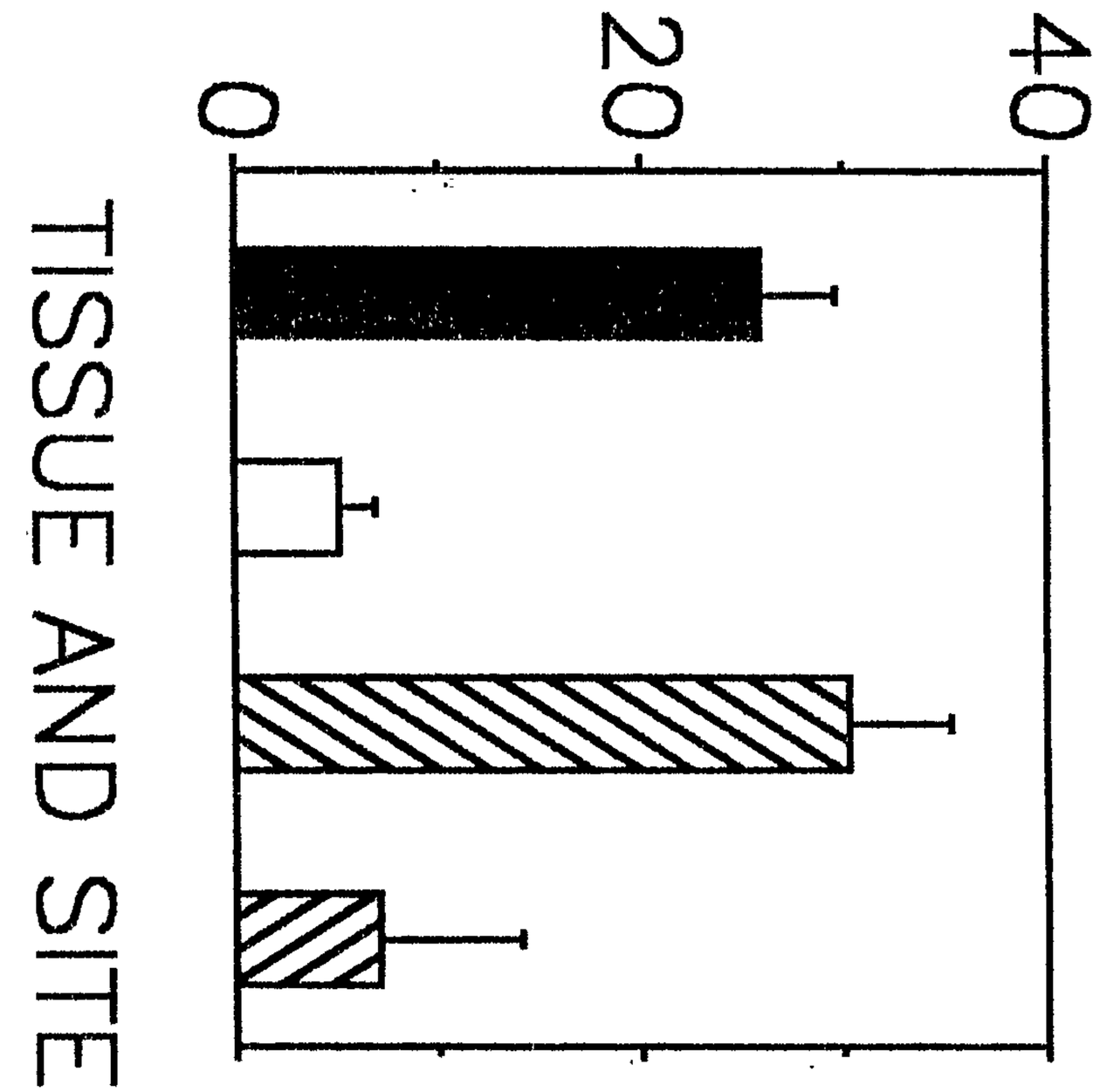
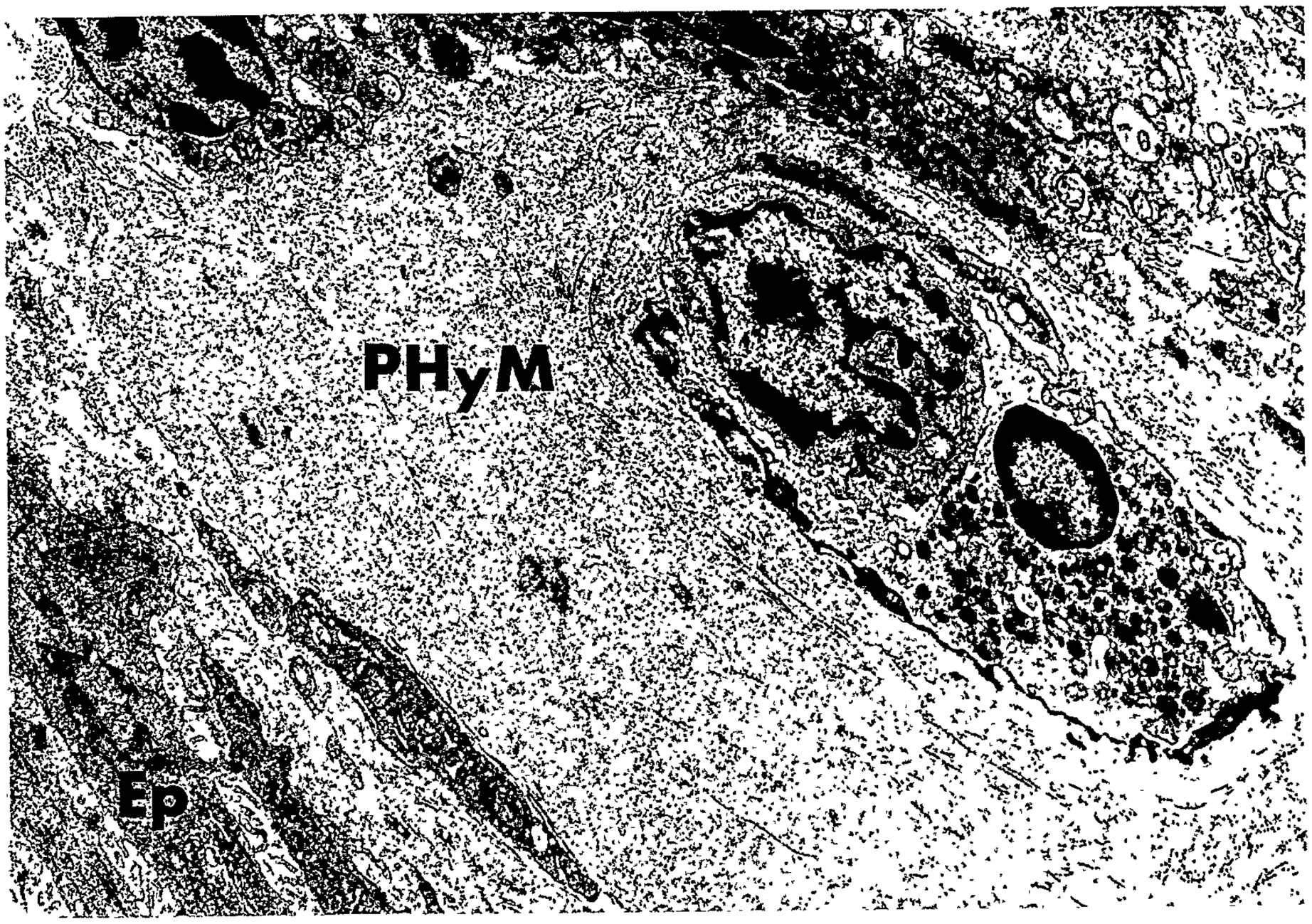


Figure 4.4

The relative percentage of vessels affected with thick PHyM deposits is compared for periodontitis Field 1 (Filled Bar), periodontitis Field 2 (Open Bar), gingivitis (Right diagonal), and minimally inflamed tissues (Left Diagonal).

There were more thick deposits of PHyM in Field 1 of periodontitis specimens than in Field 2 sites ($p < 0.001$). Similarly, gingivitis specimens had more thick PHyM than was found in Field 2 of periodontitis ($p < 0.001$). It was not possible to demonstrate statistically significant differences in the thickness of PHyM deposits, between minimally inflamed tissues and other tissues. This probably reflects the small number of vessels affected with PHyM in minimally inflamed tissues.


PHyM
Ep

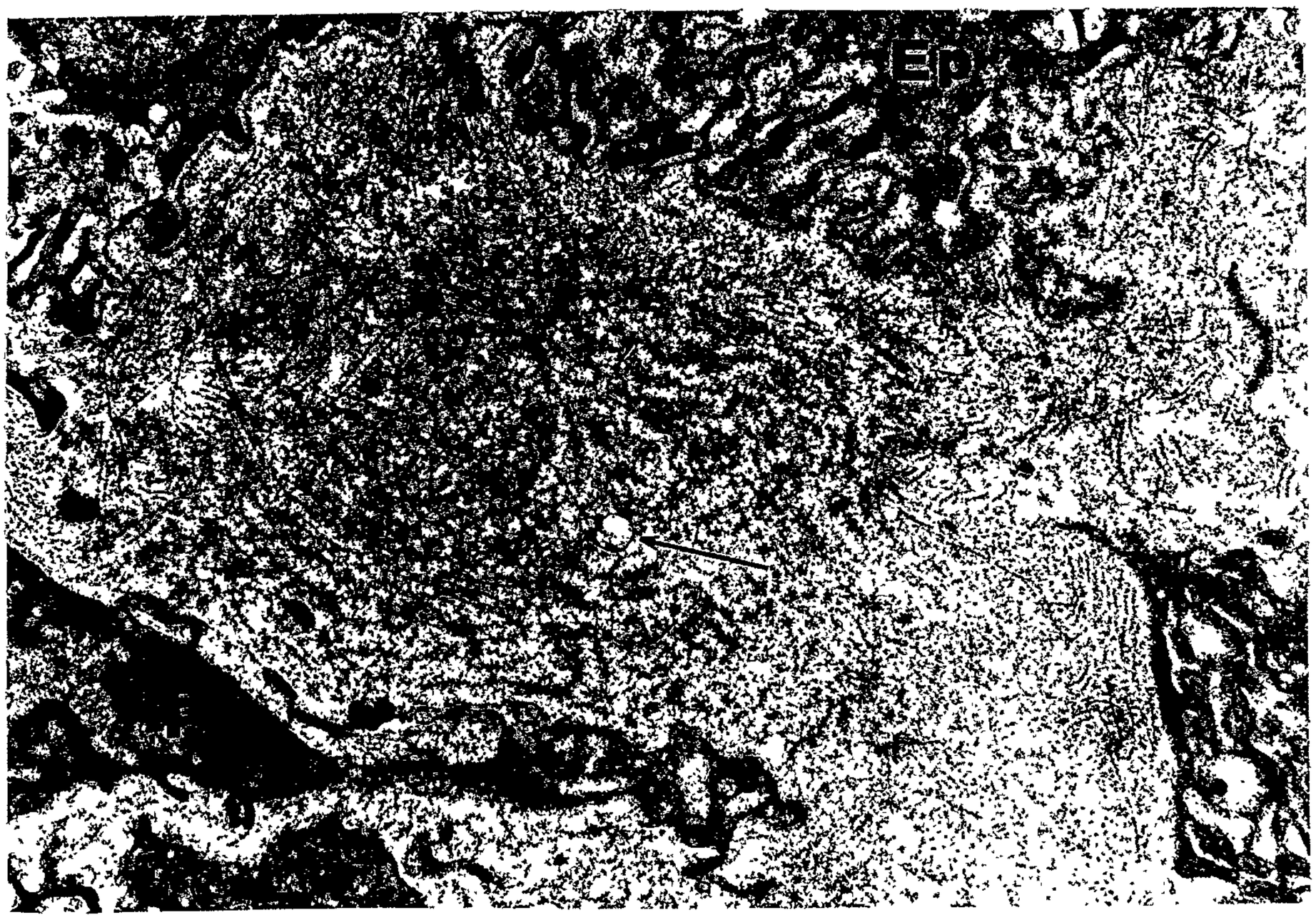

Ep

Figure 4.5

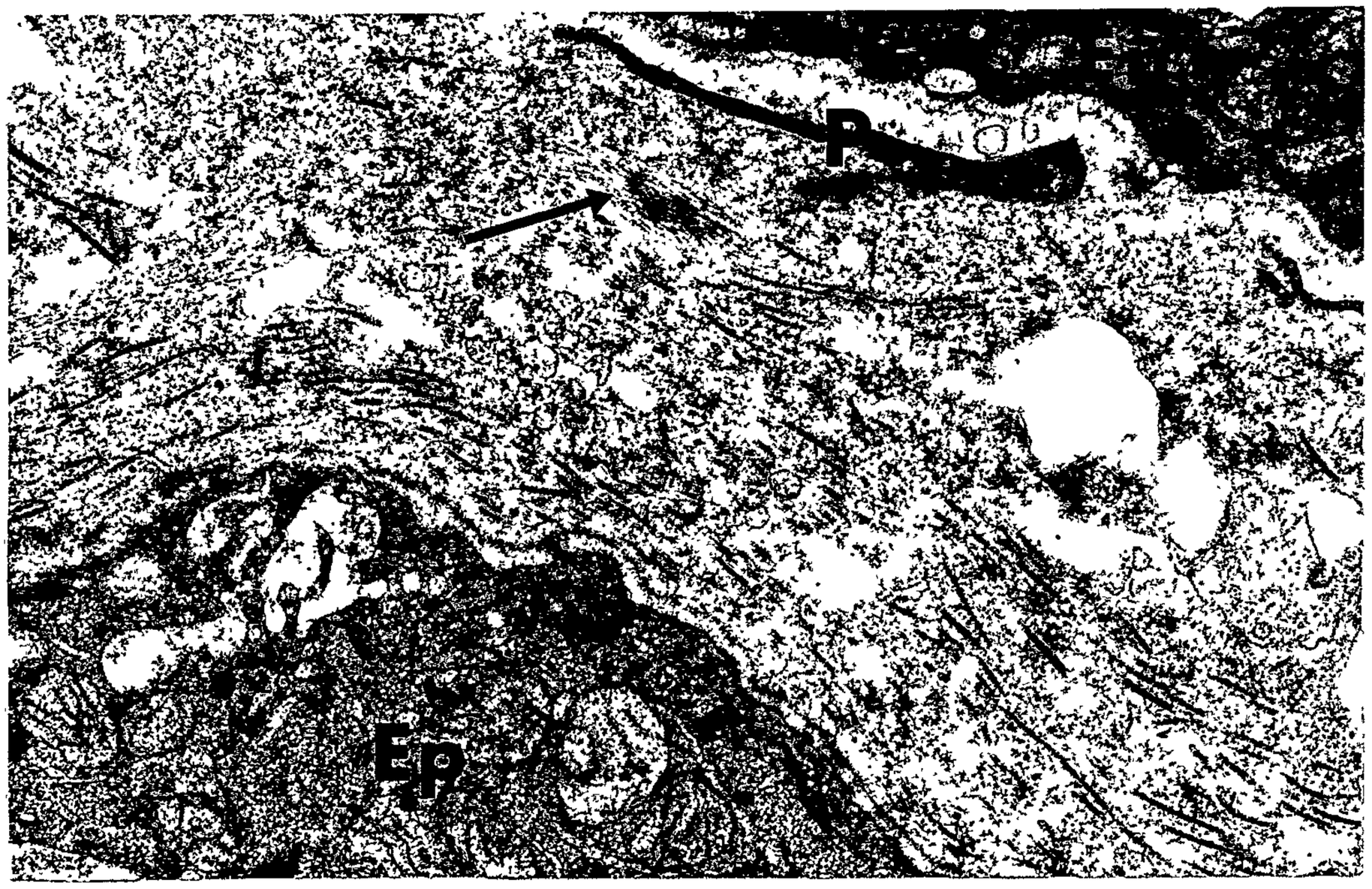
Low power electron micrograph of a vessel affected with PHyM adjacent to the pocket epithelium (Ep).

The MBL structure of PHyM is evident immediately adjacent to the endothelial cell, as is the apparent degradation and disruption of the lamellar structure towards the epithelium. (TEM X 6000)

Figure 4.6

Electron micrograph of a vessel affected with PHyM, showing a pericyte (P) and an epithelial cell (Ep).

The MBL structure of the material is clearly evident. However, the lamellation appears to lose definition close to the pocket epithelium. Note the comparatively thick epithelial basal lamina in comparison to the relatively thin endothelial basal lamina and MBL structures. Also, collagen fibrils appear to be more prevalent closer to the epithelial surface. Occasional membrane profiles are seen within the material (arrow). (TEM X 6200)


P
EP

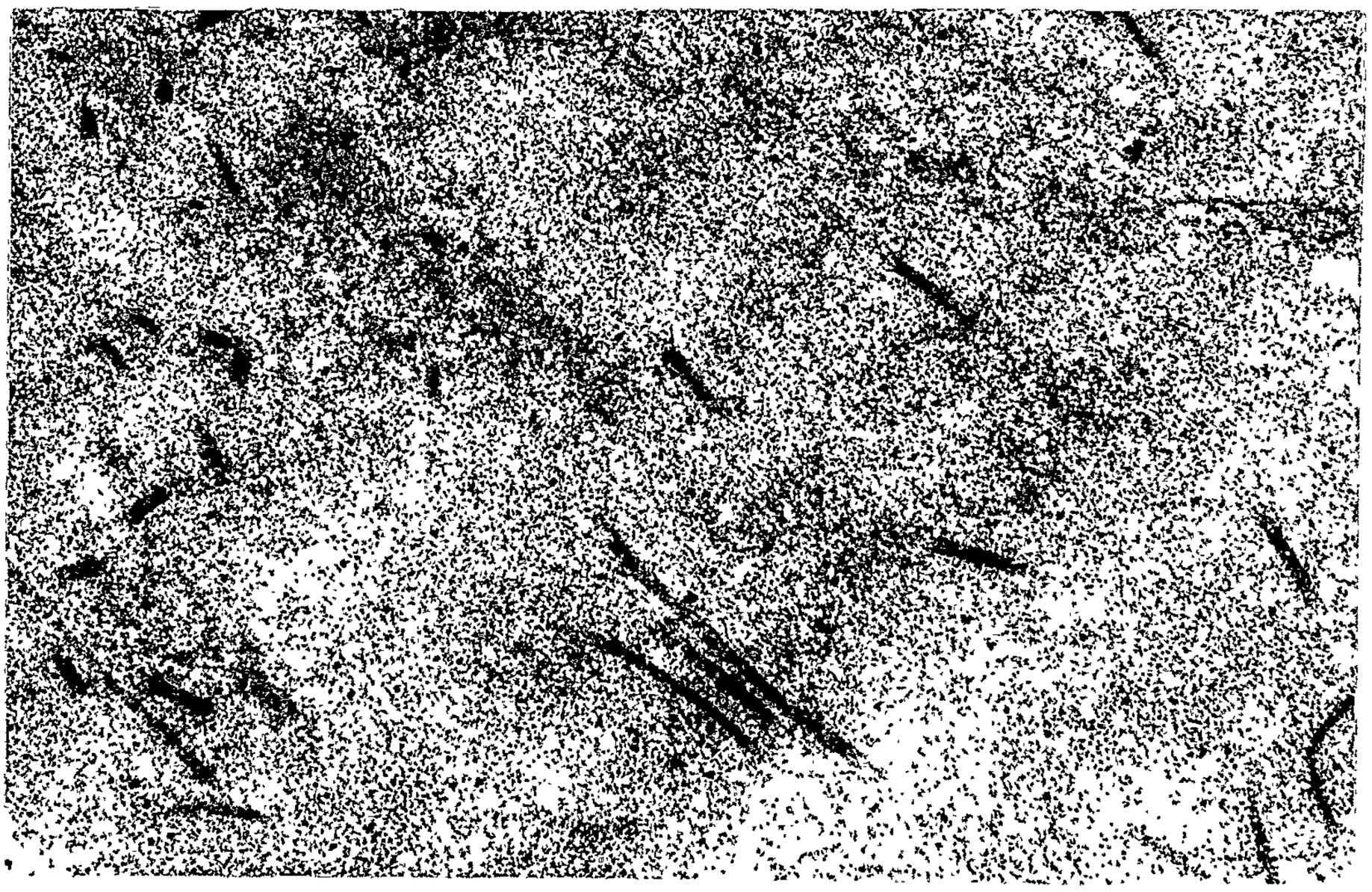


Figure 4.7

Electron micrograph of PHyM, separating an epithelial cell (Ep) from an endothelial cell (En) and pericyte (P).

Collagen fibrils, occasional fine fibrils (arrow) and membrane blebs can be seen embedded in flocculant basal lamina material. The lamellar structure of the PHyM is degraded so that the MBL nature of the material is difficult to detect. The epithelial cell BL is clearly thicker than the endothelial BL and PHyM MBL material. (TEM X 20,000)

Figure 4.8

Electron micrograph of PHyM.

No lamellation can be seen in the material. The only structure apparent is the presence of occasional collagen fibrils. The absence of lamellation could be the result of an unfavourable plane of section, or may indicate either extensive degradation of the material or the presence of large amounts of non-basal lamina material in the deposit. Such sites may be deposits of degraded serum proteins. (TEM X 40,000)

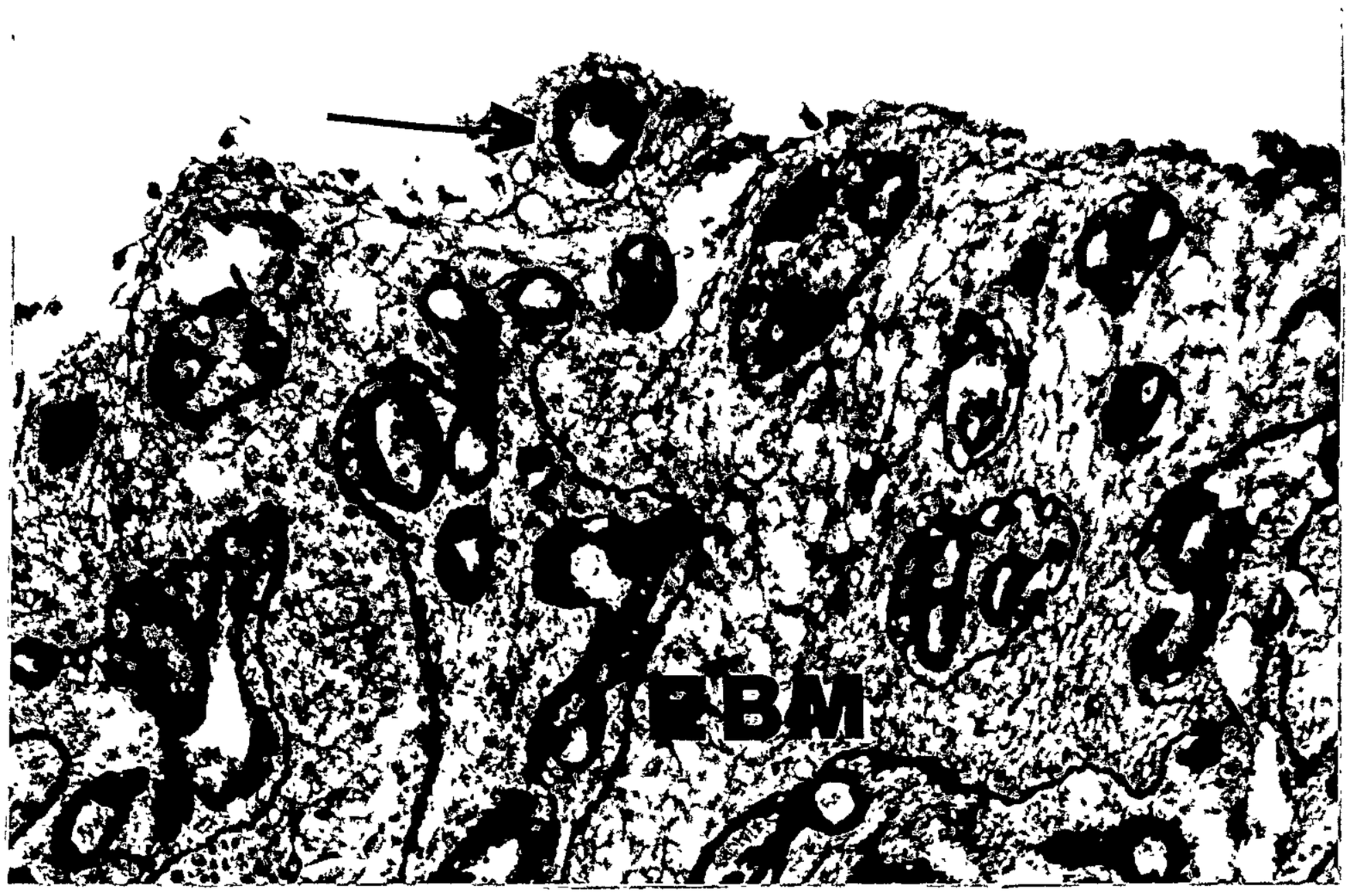

EBM

Figure 4.9

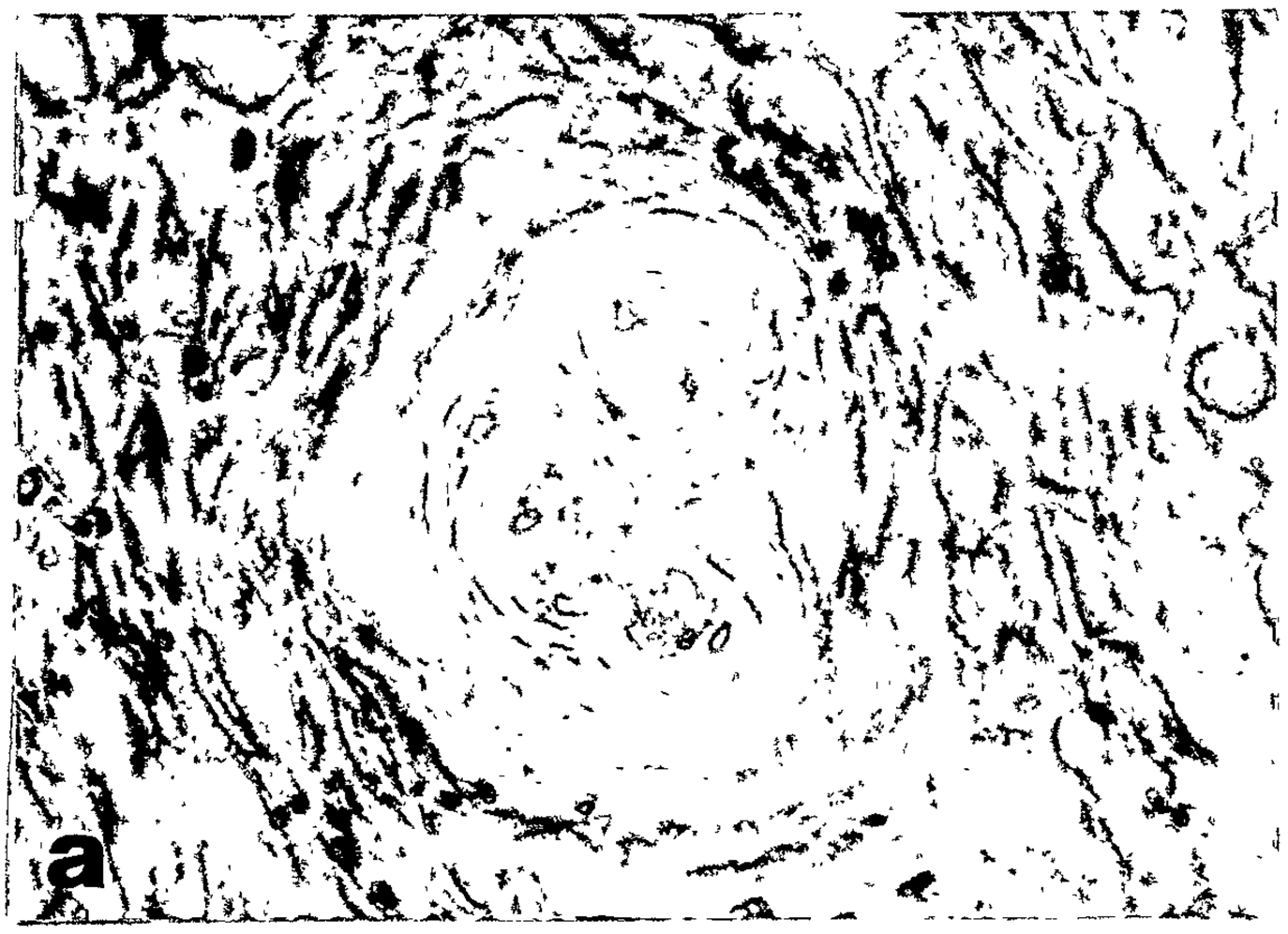
Photomicrograph of an anti-type IV collagen stain of a cryostat section of the periodontal pocket wall.

The epithelial basement membrane (EBM) can be seen, as can the basement membranes of the blood vessels, some of which share basement membrane material with the epithelium. A vessel seemingly isolated from the underlying connective tissues can be seen surrounded by basement membrane (arrow). This figure confirms that the bulk of the PHyM consists of MBL material. (Anti-type IV collagen + Haematoxylin X 125)

Figure 4.10

Photomicrograph of a separate cryostat section of the same biopsy shown above, which was processed for immuno-histochemistry for anti-type IV collagen, but without application of the primary antiserum.

No labelling was found, supporting the specificity of the label in Figure 9. (Control Immuno-histochemistry + Haematoxylin X 125)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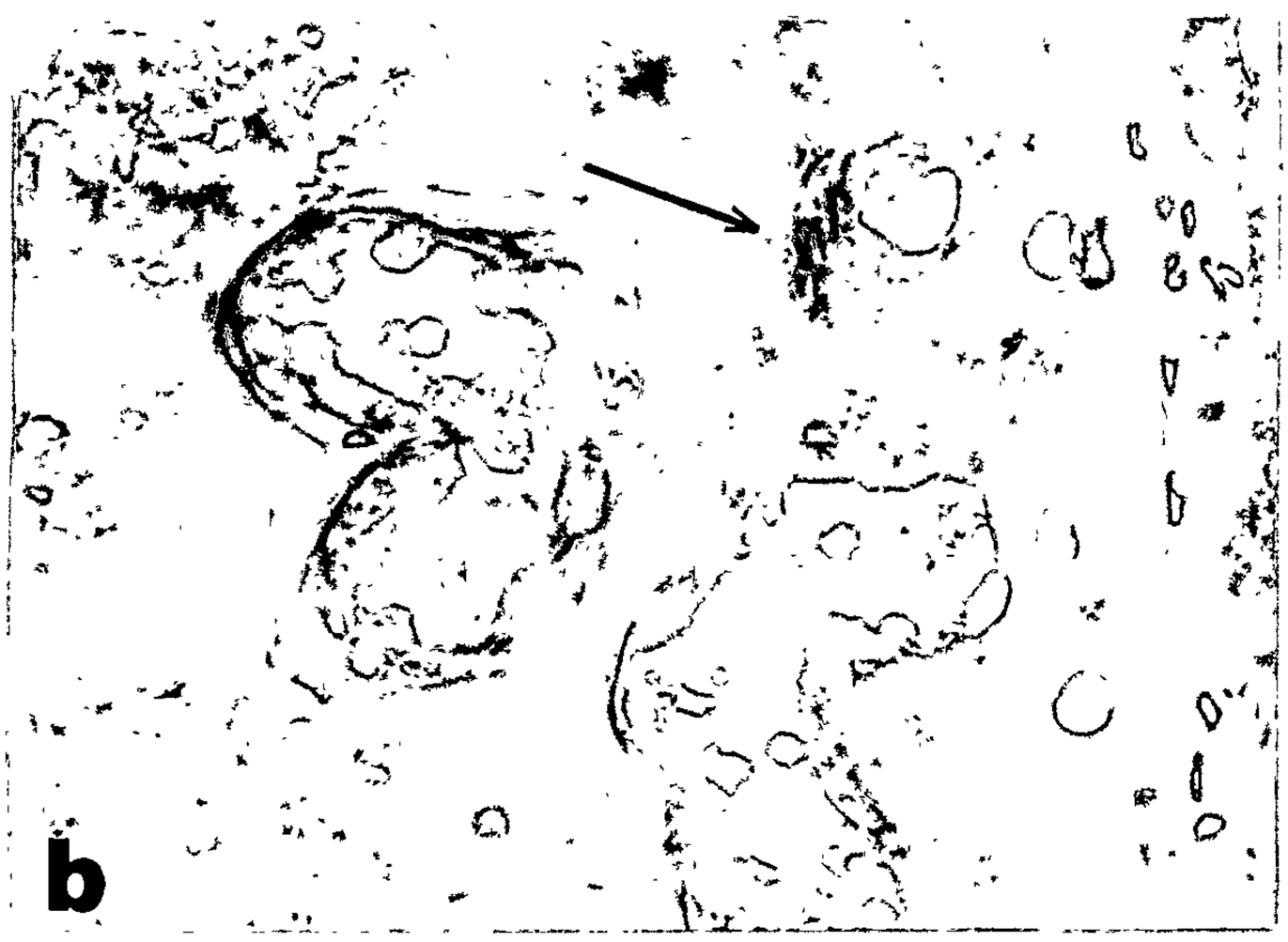
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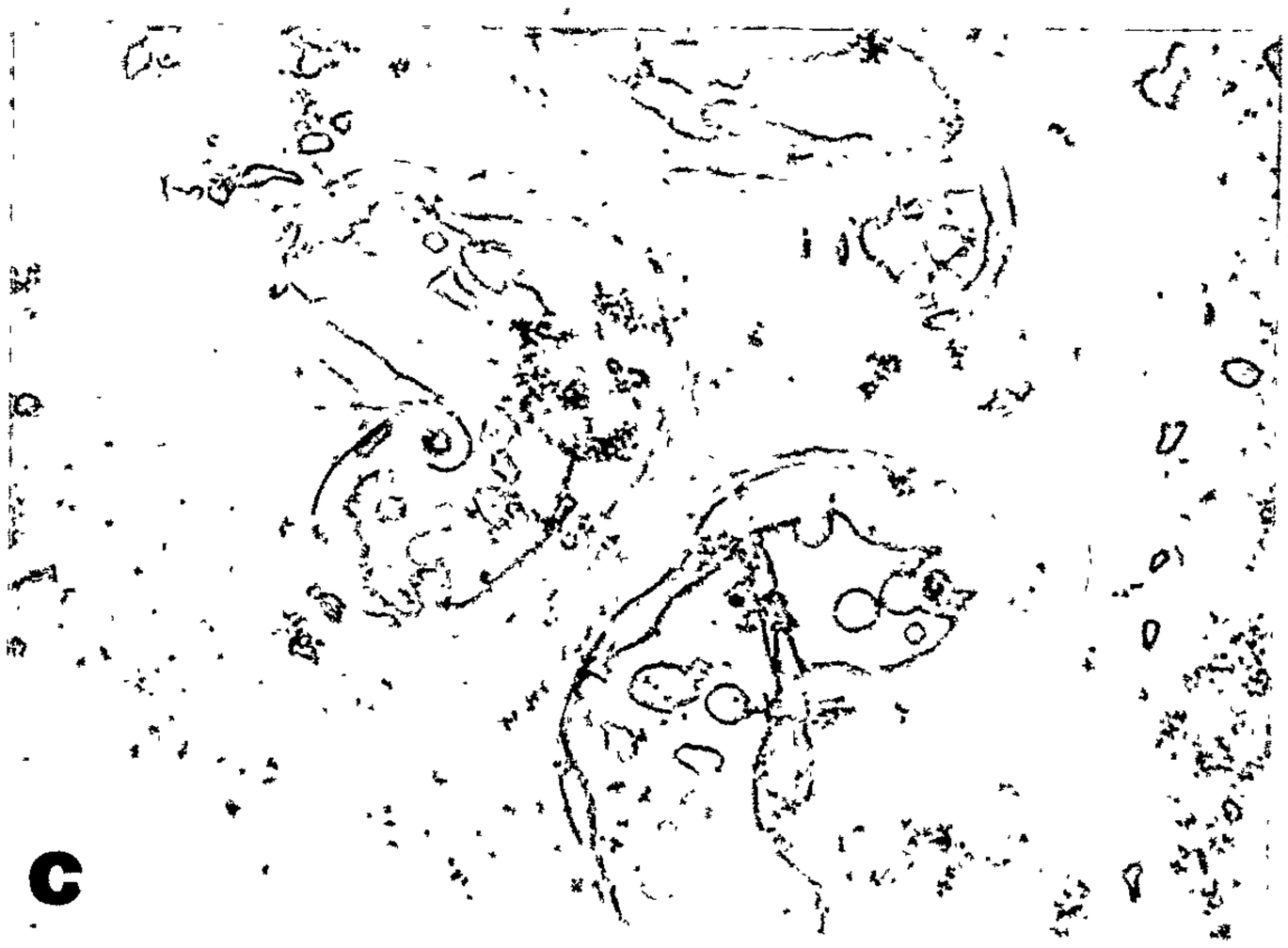
c

Figure 4.11

Anti-keratin stain of PHyM using a freeze-substituted gingival biopsy from a patient with periodontitis.

Keratin fibrils are seen in the epithelial cells as dark bundles of thread like material. The PHyM does not show any labelling for keratin, suggesting that this protein is not a major component of the material. (Anti-keratin No counterstain X 400)

Figure 4.12

Anti-FVIII stain of PHyM using a freeze-substituted gingival biopsy from a patient with periodontitis.

Vascular ECs show occasional flecks of activity, probably corresponding with the location of Weibel-Palade bodies. PHyM did not stain for this protein except in this specimen, where some limited labelling was found in the material (arrow). It is possible that this is due to a glancing section of a blood vessel. Alternatively, it could indicate the presence of EC debris within this deposit of PHyM. (Anti-FVIII No Counterstain X 400)

Figure 4.13

Control slide of the same site shown in Figure 4.12 in which the section was not treated with the primary antiserum.

No labelling is present, indicating that the AP conjugated antibody has recognised the primary antiserum. (Control Immuno-histochemistry No Counterstain X 400)

DISCUSSION

PHyM Appears to be Unique to Inflamed Gingival Tissues

Hyaline changes have been described in a number of disease processes (Hashimoto 1985) with a corresponding variety of histochemical and ultrastructural presentations. Amyloidosis (Hashimoto 1985), hyaline arteriosclerosis (Gamble 1986), and diabetes mellitus (Vrako *et al.* 1980) are conditions commonly associated with the appearance of hyaline changes around blood vessels. The significance of these deposits varies according to the particular site and type of change involved. There is one report of a sclerotic hyaline deposit surrounding vessels in inflamed periodontal tissues, but the relationship to the present findings is unknown, as the vessels described were located at the gingival crest and interdental papilla (Von Franke 1964). Also, there are several reports of MBLs surrounding vessels in inflamed periodontal tissues in humans (Freedman *et al.* 1968, Danilewskij and Kolesowa 1977) and rats (Garant 1976). These MBLs were also observed around some deeper vessels in this study. However, they had a different appearance to those found in PHyM, in that they were much less extensive, did not exhibit any sign of degradation, and had few if any collagen fibrils, fine fibrils or membrane blebs.

The heterogeneity of hyaline materials seen in many pathological states makes any comparison between the hyaline deposits found in different diseases difficult. However, the changes observed in periodontal tissues appear to bear some similarity to those described for diabetes mellitus (Vrako *et al.* 1980). In diabetes there is multiplication of BL material around the capillaries of muscles and skin. The material often has membranous cellular debris scattered between layers of MBL, and occasional collagen fibrils are described. The predominance of BL material as confirmed by immuno-histochemistry in human periodontal PHyM, as well as the presence of membrane blebs and fibrils suggests a common pathogenesis for both diabetic and periodontal MBLs.

The Possible Pathogenesis of PHyM

The MBL material observed in diabetes is thought to reflect an endothelial response to damage and the cellular debris is considered to consist of remnants of vascular ECs (Vrako *et al.* 1980). The location of PHyM in association with the pocket epithelium would suggest that the affected vessels are in frequent contact with bacterial products diffusing into the tissues from the bacterial plaque. This could damage ECs and generate MBL material. Deeper vessels could also be affected by bacterial products carried by the blood draining the superficial plexus of vessels.

Extracellular polysaccharides of glucose are a major component of dental plaque (Nolte 1977, Roitt and Lehner 1980), so that it is possible that high local concentrations of degraded polysaccharides could contribute to the formation of PHyM in a similar way to that thought to occur in diabetes. Also, TNF can cause EC damage (Goldblum *et al.* 1989, Schuger *et al.* 1989), so that the local production of this cytokine by LPS-stimulated macrophages could also contribute to EC injury.

Epithelial cells are an unlikely source of the MBL material in PHyM, as the BL of epithelial cells is considerably thicker than that of ECs and that found in the PHyM. Nonetheless, the bundles of fine fibrils of a similar appearance to keratin filaments, aligned in parallel with the basal surface of epithelial cells suggest that epithelial cells may degenerate and contribute to the formation of the PHyM in some circumstances. Similar apoptotic death of basal epithelial cells has been observed in some forms of cutaneous amyloidosis (Kabayashi and Hashimioto 1983). However, the failure to demonstrate keratin in PHyM does not support this suggestion. It is possible that with more sensitive immuno-gold techniques keratin might be demonstrated. The origin of the fine fibrils could also be explained as remnants of basal lamina anchoring fibrils. These fibrils form an extensive network beneath the basal lamina, and can be demonstrated specifically using anti-type VII collagen antibodies (Keene *et al.* 1987). This possibility could be investigated using immuno-gold techniques. The fine fibrils are unlikely to be amyloidic in origin, as parallel experiments by Short (1989) in this laboratory failed to reveal the presence of serum amyloid P protein in the PHyM.

Large numbers of collagen fibrils and degradation of MBL material are not seen in the MBL of deep periodontal vessels, or in diabetes. Other workers have observed dilatation and increased tortuosity of vessels in gingival tissues as a response to inflammation (Hock and Nuki 1971, Kasperk and Ewers 1968). This was confirmed in the work described in Chapter 3. Such vascular expansion must be accompanied by an equivalent loss of fibrous connective tissue elements. In relation to this, ECs are known to secrete collagenase and plasminogen activator in response to angiogenic stimuli (Gross *et al.* 1983). It is postulated that angiogenic stimuli which act on the vessels of inflamed gingiva, result in degradation of the surrounding connective tissue elements in addition to causing changes in the anatomy of the microcirculation. Proteinase secretion related to angiogenesis could explain the presence of irregular collagen fibrils and the degraded appearance of MBL in PHyM. In relation to this, the tendency for the MBL structure to be less distinct in those parts of the PHyM which are most distant from ECs could be interpreted as

representing older and hence more extensively degraded MBM deposits as compared with relatively new MBM structures closer to the affected vessel. In support of the idea that angiogenesis-related proteinase secretion could contribute to the formation of PHyM, is the report by Schroeder *et al.* (1975) of degradation of perivascular collagen close to the pocket wall.

It is possible that serum proteins are a component of PHyM, and that the amorphous appearance of the material in places is not due to the degradation of MBL structures. The anti-type IV collagen stain would suggest that this is not the case. It would seem that fibrin is not a major component of PHyM, since no typical fusiform cross striated fibrin fibrils were seen with the electron microscope. Also, an immuno-histological study performed in this laboratory, characterising the distribution of serum amyloid P protein in gingival biopsies using the same tissues as were collected for this study, failed to demonstrate this serum protein in PHyM deposits (Short 1989). The PHyM also failed to stain with the widely used amyloid marker, Congo-red. However, the possibility that serum proteins are a component of PHyM can not be excluded until an immuno-gold electron microscopic study of the material is performed testing for a panel of serum proteins.

PHyM May Contribute to Development of the Advancing Lesion

The distribution, incidence and thickness of the PHyM suggests that PHyM is associated both with the appearance of inflammation in gingival tissues, and with the advancing front of the periodontitis lesion. This association allows consideration of the possible role of this material in the development of the advancing periodontal lesion.

Changes in the bacterial composition of the plaque are thought to be related to attachment loss (Tanner *et al.* 1984). This implies that a specific bacterial flora activates pocket deepening. Many workers have suggested that the liberation of toxins and destructive enzymes, or the activation of inappropriate host defence mechanisms may contribute to pocket formation (Listgarten 1987). Defects in PMN function have been shown to predispose patients to some forms of rapidly advancing periodontal disease (Ciancola *et al.* 1977). PMN dysfunctions are the only defects in host resistance which have been clearly documented to predispose to periodontal diseases, yet it is clear that most patients with advanced periodontal disease are unlikely to suffer from significant PMN dysfunction. This is particularly significant with regard to CAP. It is possible that in these patients, PHyM is a significant local factor which in some way hinders normal PMN function, and thus predisposes to the development

of the advancing disease.

There are two possible mechanisms whereby PHyM could contribute to a localised defect in PMN function. Firstly, extensive deposits of PHyM could be expected to interfere with the diffusion of chemotactic bacterial products from the plaque into the underlying connective tissues, resulting in a loss of both stimuli that attract PMNs into the tissues, as well as in a diminished chemotactic gradient directing PMNs towards the plaque. Furthermore, it is possible that intravascular activation of PMNs by bacterial products contributes to the emigration of these leukocytes into the lesion, and extensive deposits of PHyM could interfere with the diffusion of bacterial products required to allow such activation. Secondly, it is known that during PMN emigration, the leukocytes are temporarily impeded in movement by the vascular BL (Marchesi 1964). A structure composed of MBL would present a significant barrier to PMN movement. PMNs emigrating from PCVs deeper in the tissues, may have difficulty penetrating thick sheets of PHyM in patients with extensive deposits.

In this way PHyM may compromise the local host defence response to plaque bacteria, and encourage pocket deepening. Such local changes could help to explain the sporadic nature of pocket deepening (Goodson *et al.* 1982). Vessels underlying the newly established apical pocket epithelium of recently deepened periodontal pockets would not be expected to have PHyM deposits, thus limiting further extension of the pocket. Such pockets may be stable until PHyM deposits have sufficiently compromised PMN emigration for a further burst of pocket deepening to occur. This hypothesis is outlined diagrammatically in Figure 4.14

This hypothesis does not address the nature of the actual pocket deepening event, so that the possible role of the epithelium as an active or passive participant in tissue destruction is not at issue. Neither is the nature of the proposed virulence factors appearing in plaque specified in this model of pocket progression. However, it is possible that an excessive rate of deposition of PHyM, or conversely, a failure to remove this deposit from inflamed gingival tissues, could account for the appearance of CAP in otherwise healthy patients. Also, PHyM could account for the cyclical pattern of highly localised tissue destruction which is typical of CAP.

Approaches to the study of this possibility are discussed in Chapter 8.

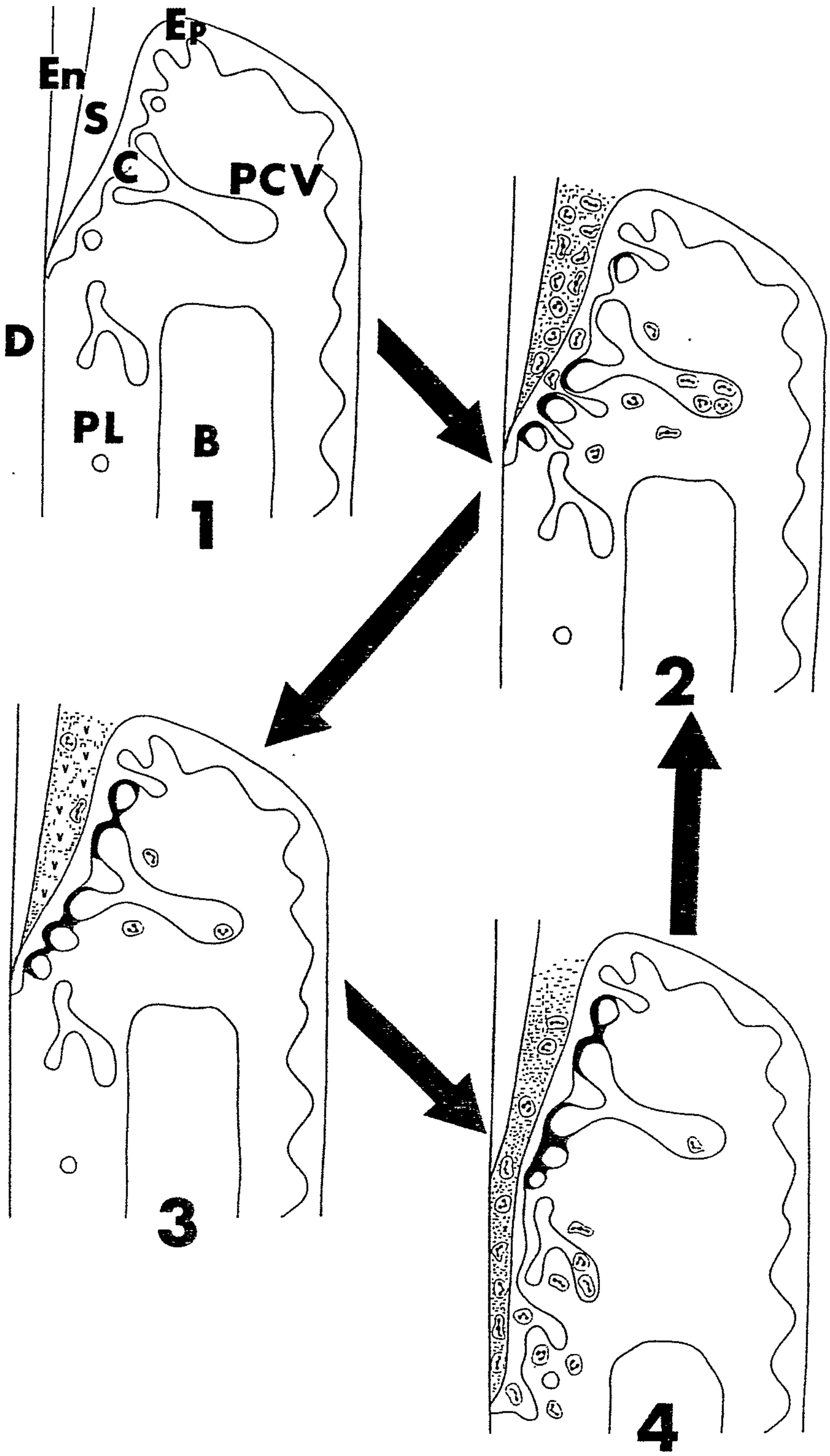
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Figure 4.14

Diagram outlining the hypothesis that PHyM contributes to pocket deepening by local compromise of PMN function.

1 Diagram of the sulcular region of a tooth with healthy gingiva.
The enamel (E) and dentine (D) of the tooth are indicated. The gingival sulcus (S) terminates at the cemento-enamel junction. The bone (B) and periodontal ligament (PL) supporting the tooth are shown, as is the epithelium (Ep) covering the soft tissues. Capillaries (C) forming the vascular network beneath the sulcular epithelium are shown draining into post-capillary venules (PCVs) deeper in the tissues.

2 The early gingivitis lesion.
Bacterial plaque has accumulated in the sulcus, establishing the lesion of gingivitis. There is expansion of the microcirculation (Chapter 3) as well as deposition of PHyM (Dark deposit). PMNs emigrate from PCVs into the bacterial plaque in defense of the host.

3 The advanced gingivitis lesion immediately prior to pocket deepening.
PHyM deposits have become sufficiently extensive to compromise PMN emigration into the plaque. This alters the plaque ecology with the appearance of virulence factors (V) capable of causing pocket deepening.

4 Formation of the periodontitis lesion.
Following a burst of pocket deepening, vessels in the most apical portion of the newly formed periodontal pocket do not have PHyM deposits. PMNs are now able to enter the plaque in normal numbers restoring the avirulent plaque ecology and limiting pocket progression to a burst of activity. The established pocket may now progress by cycling through repeated phases of PHyM deposition and pocket deepening.

This pattern of PHyM deposition and reduced defense could account for the cyclical nature of pocket deepening occurring independently in multiple sites within the mouth.

CHAPTER 5

HIGH ENDOTHELIAL LIKE VENULES IN GINGIVAL TISSUES EXCHANGE POLYMORPHS

INTRODUCTION

As described in Chapter 3, PHELVs were found in gingival biopsies, particularly in Fields 1 and 2 of periodontitis and gingivitis specimens. These vessels appeared to be responsible for the emigration of leukocytes from blood into the tissues. This chapter describes the histochemistry, ultrastructure and apparent function of PHELVs. PHELVs are compared with the well characterised HEVs found in rat lymph nodes. The possibility is discussed that HELVs have a wider role in inflammatory foci than has been previously supposed.

LITERATURE REVIEW

As described in Chapter 1, HEVs are PCVs found in lymphoid tissues, and are characterised by the presence of morphologically distinct HECs. HELVs occur in many chronic inflammatory sites, and share many of the characteristics of HEVs. HECs bulge prominently into the vessel lumen, have large vesicular nuclei and bind lymphocytes as the initial step in the migration of these leukocytes into lymphoid tissues and chronic inflammatory sites. This literature review will survey studies in which the morphology, ultrastructure, histochemistry, apparent function and phenotypic control of HECs has been investigated.

The Morphology of HEVs in Lymphoid Tissues

In early reports describing HEVs, investigators were clearly impressed by the "unusually high, almost cylindrical endothelial cells" which composed these vessels (Thomé[20] 1898, Schumacher 1899). These authors noted the morphological similarity of HEVs to the epithelium lining glandular ducts, and emphasised the contrast between this high EC morphotype, as compared with the normally flat ECs commonly found in tissues. HECs were seen as oval cells, with large vesicular nuclei containing scantly distributed chromatin and from one to two nucleoli. The cytoplasm

was described as consisting of two portions, with a peripheral area of non-homogenous material merging with a more central region containing many fine fibrils (Thome[2] 1898). HEVs were found in the lymph nodes of many species, including monkeys, dogs, cats, pigs, marmosets, rabbits, mice, rats, guinea pigs and humans (Thome[3] 1898, Schumacher 1899, Claesson *et al.* 1971). Little morphological difference is reported for HEVs amongst differing species, with slightly more flat ECs being found in rabbits and guinea pigs as compared with mice and rats (Claesson *et al.* 1971). The presence or absence of a cytoplasmic fibrillar network, as well as the identity of the inclusions found in HEV walls was debated by Thome[2] (1898) and Schumacher (1899). Even at this early stage of study it was generally thought that HEVs were unique amongst blood vessels. These vessels were variously thought to be either capillaries (Thome[2] 1898) or small venules (Schumacher 1899). The identity of HEVs as the smallest (post-capillary) venules was suggested by Schumacher in 1899, and was generally accepted by 1939 (Dabelow 1939). Reconstruction of the microcirculatory anatomy from tissue sections of lymph node tissues, has confirmed the supply of HEVs by capillaries, and thus their identity as post-capillary venules (Smith and Hennon 1959, Müller and Stempfel 1985). HEVs have been reported in lymphoid tissues other than lymph nodes, including Peyer's patches (Miller 1969, Hummel 1935, Anderson *et al.* 1976), tonsils (Miller 1969, Müller and Stempfel 1985) and the appendix (Miller 1969).

An integral component of HEV morphology has been the presence of large numbers of lymphocytes within the walls of the vessels (Schumacher 1899, Kraal *et al.* 1987). The morphology of HEVs appears to be essentially identical in all species having this type of vessel (Schumacher 1899, Claesson *et al.* 1971)

Ultrastructure of HEVs in Lymphoid Tissues

The ultrastructure of HEVs varies little amongst differing species (Claesson *et al.* 1971). One of the most consistently reported ultrastructural features of HECs is the presence of an extensive, well developed golgi apparatus (Anderson *et al.* 1976, Freemont and Jones 1983, Claesson *et al.* 1971, Sugimura *et al.* 1964). Other synthetic structures found in great abundance in HECs, are free and poly ribosomes (Freemont and Jones 1983, Anderson *et al.* 1976), and the rough endoplasmic reticulum (Freemont and Jones 1983, Claesson *et al.* 1971, Sugimura *et al.* 1964). HECs have multivesicular bodies (Freemont and Jones 1983, Sugimura *et al.* 1964), and lysosomal structures (Anderson *et al.* 1976, Freemont and Jones 1983, Claesson *et al.* 1971, Sugimura 1964).

The topography of HECs is complex, with many cells displaying a surface with multiple vesicular projections (Anderson *et al.* 1976, Sugimura *et al.* 1964). The intercellular junctions of HECs have a complex structure, with interdigitating foot processes extending beneath adjacent HECs, forming a network of intersecting plates (Anderson *et al.* 1976, Freemont and Jones 1983, Cho and De Bruyn 1986). This interdigitating structure is thought to be confined to the basal area of HECs in proximal portions of HEVs, but extended apically to involve the luminal surface of HECs in the distal part of the vessel (Cho and De Bruyn 1986). Maculae occludens have been observed between HECs in both apical and basal parts of the intercellular region (Anderson *et al.* 1976, Claesson *et al.* 1971, Sugimura *et al.* 1964). Intercellular junctions form clefts in the luminal surface of HEVs, with apical junctions being absent in HEVs in rat Peyer's patches, creating particularly deep intercellular clefts in these vessels (Anderson *et al.* 1976).

The electron-density of HECs is reported to vary within individual HEVs (Anderson *et al.* 1976, Freemont and Jones 1983). It is found that "light" electron lucent cells, with sparse endoplasmic reticulum and a comparatively smooth luminal plasma membrane, contrast with "dark" HECs with more endoplasmic reticulum and ribosomes. The dark HECs present a more convoluted luminal surface (Anderson *et al.* 1976, Freemont and Jones 1983).

The basement membrane of HEVs in lymph nodes is thicker than that of other vessels, by virtue of complex interdigitation of the surrounding pericytes (Anderson *et al.* 1976, Freemont and Jones 1983)

The means whereby lymphocytes emigrate through HEVs has been the subject of controversy, in that some authors contend that lymphocytes traverse HEVs via an intracellular route (Marchesi and Gowans 1964), while others maintain that lymphocytes migrate through intercellular junctions (Schoefl 1972, Van Ewijk and Rozing 1975). A cause for this disagreement lies in the very close apposition of the lymphocyte and HEC cytoplasmic membranes as seen in electron micrographs (Anderson *et al.* 1976, Freemont and Jones 1983, Claesson *et al.* 1971). Authors of a recent study employing both serial section techniques as well as scanning electron microscopy, have suggested that lymphocytes initially migrate intracellularly, but later assume an intercellular pathway (Cho and De Bruyn 1986).

Enzyme Histochemistry of HEVs in Lymphoid Tissues

HEVs share ATPase, acetyl-choline esterase, lactate dehydrogenase and isocitric dehydrogenase activities with ECs in other vessels (Müller and Stempfel 1985, Freemont and Jones 1983, Anderson *et al.* 1976). However, some enzyme histochemical activities are reported as specific markers for HEVs. The enzyme histochemistry of HEVs is similar for vessels from different sites and species.

Non-specific esterase activity has been described as a specific marker for HEVs amongst the lymph node blood vessels in several species, including mice, rats and humans (Müller and Stempfel 1985, Freemont and Jones 1983, Anderson *et al.* 1976). This is also the case for human tonsillar tissue (Müller and Stempfel 1985). Smith and Hennon (1959), however, found that non-specific esterase activity was also occasionally present in vessels other than HEVs, although this activity was much less intense than was found in HECs.

Other enzyme activities which have been demonstrated in HEVs are succinic dehydrogenase and N-acetyl-ß-glucosaminidase in human lymph nodes (Freemont and Jones 1983), as well as ß-glucuronidase in human and rat lymph node tissues (Freemont and Jones 1983, Anderson *et al.* 1976). There may be some differences in the enzyme histochemistry of FEVs amongst differing species, in that mouse lymph node FEVs are recorded as having weak succinic dehydrogenase activity (Smith and Hennon 1959), while those of human tissues are reported as being negative for this activity by Freemont and Jones (1983). Also, human lymph node FEVs are reportedly weekly positive for ß-glucuronidase activity (Freemont and Jones 1983), while in rats FEVs were negative for this enzyme (Anderson *et al.* 1976).

Acid phosphatase activity is also recorded as being present in the HEVs of rat and human lymph nodes (Anderson *et al.* 1976, Freemont and Jones 1983), with other vessels being negative in the rat, and weakly positive in human tissues. In the mouse, however, both HEVs and vessels with more flat ECs are recorded as not having this enzyme activity (Smith and Hennon 1959).

APA is absent in HEVs, but present in the capillary and arteriolar ECs of both human and rat lymph node tissues (Freemont and Jones 1983, Anderson *et al.* 1976).

The Function of HEVs

There has been much speculation regarding the significance of the plump endothelial morphology of HEVs.

Thome[10] (1898) recorded the presence of phagocytosed red blood cells within the cytoplasm of HECs, and suggested that phagocytosis of red blood cells was an important function of HECs. However, it is likely that this observation was an artifact of the staining mechanisms used to visualise the tissues, and that the cellular inclusions noted were lymphocyte nuclei. However, the more recent observation by Anderson *et al.* (1975), of microthrombi in HEVs draining skin allografts in rats, led these authors to suggest that HECs phagocytose microthrombi as one of their functions. Schumacher in 1899, was also interested by the large number of leukocytes found in the walls of HEVs, and suggested that lymphocytes migrated from the lymphoid interstitial tissues into the blood through the walls of these specialised vessels. This possibility was later excluded when Gowans and Knight (1964) found that HEVs were the site of lymphocyte recirculation from the blood into lymphoid tissues and lymph.

The consistent association between lymphocyte emigration and HEVs has led to the widely accepted conclusion, that HEVs are in some way specially adapted to assist in the prolonged emigration of lymphocytes from the blood into the tissues (Kraal *et al.* 1987). This view has been greatly reinforced by work investigating the mechanism of lymphocyte binding to HEVs. This work was reviewed in Chapter 1, and has exposed some of the molecular mechanisms involved in the selective binding of lymphocytes to HEVs in lymphoid tissues.

Although most authors agree that HECs are adapted to facilitate the exchange of lymphocytes from blood to tissues, there have been many suggestions regarding the specific advantage for the exchange of lymphocytes which is conferred upon HEVs and HELVs by their high ECs.

There is evidence, that by increasing the number of collisions between lymphocytes and the vascular wall, HECs aid in lymphocyte adhesion and hence emigration (Bjerknes *et al.* 1986). These authors found that the incidence of collision between lymphocytes and the luminal walls of HEVs was significantly greater than in vessels with flat ECs. The possibility that the intersecting plates of distal HEVs could generate turbulence which would increase the efficiency of such collision has been suggested by Cho and De Bruyn (1986).

It has been further suggested, that lymphocyte traffic through the vessel wall in HEVs may be intracellular, and it is inferred that the "high" morphology of HEVs

aids this mode of trans-vascular movement (Marchesi and Gowans 1964, Far and Di Bruyn 1975). This suggestion receives some support from the close adaptation of the HEC and lymphocyte plasma membranes seen with the electron microscope. Also, the tendency for lymphocytes to indent a single HEC during diapedesis, rather than sharing the burden of deformation equally between adjacent HECs is consistent with this interpretation of HEV function. This possibility acquires additional significance in light of the observation that ECs and lymphocytes exchange cytoplasmic material in-vitro (Guinan *et al.* 1988). Furthermore, analysis of serial sections with the electron microscope, has led to the conclusion that lymphocytes traversing the HEV wall are in an intercellular location (Schoefl 1972). This author has suggested that HECs may aid in lymphocyte emigration by limiting the loss of plasma around emigrating lymphocytes. Authors of a more recent study, employing both serial sections and scanning electron microscopy, have concluded that the path of lymphocytes through the HEV barrier is initially intracellular, and then adopts an intercellular path (Cho and De Bruyn 1986).

The extensive synthetic apparatus evident in electron micrographs of HECs, has suggested that a function of HECs may be to replace basement membrane material damaged during lymphocyte emigration. The presence of flocculant basal lamina-like material over lymphocytes penetrating the basal lamina of HEVs lends some support to this proposition (Anderson *et al.* 1976).

Rat lymph node HEVs are known to synthesise a sulphated glyco-lipid, which seems to be non-covalently associated with a glyco-protein (Andrews *et al.* 1982, Andrews *et al.* 1983). These authors suggest that this sulphated compound may play a role in controlling fluid balance during the secretion of compounds by the cell. The possible function of the proposed secreted HEC products was, however, not discussed. The further possibility that the sulphated compound is involved with lymphocyte recirculation is raised by the observation that sub-cutaneous injection of a preparation containing this compound, is followed by the accumulation of lymphocytes in the affected tissues (Andrews *et al.* 1980). However, the recent observation that lymphocyte adhesion to connective tissue elements is increased in inflammatory sites (Jack and Chapman 1989), raises the further possibility that the sulphated compound might elicit its effect upon lymphocyte trafficking by increasing the retention of lymphocytes in lymphoid tissues, rather than at the level of trans-endothelial cell migration.

High Endothelial Like Venules in Chronically Inflamed Tissues

Many chronic inflammatory lesions are noted for their dense lymphocytic infiltrates. Lymphocytes gain access to inflamed tissues via HELVs which often bear striking morphological similarity to the HEVs found in lymphoid tissues (Freemont and Ford 1985, Freemont 1988). Lymphocyte recirculation is known to occur through HELVs in a similar manner to that which occurs through lymphoid tissues (Smith *et al.* 1970). It is suggested that the similarity between HEVs and HELVs reflects the common demand for circulating lymphocytes by both lymphoid tissues and chronic inflammatory sites (Freemont 1983), and that HELVs, like HEVs, are particularly well suited to assist with the emigration of lymphocytes into tissues (Kraal *et al.* 1987). With respect to the work done towards this thesis, it must be noted that HELVs have been reported in inflamed gingival tissues (Wynne *et al.* 1988).

A difference in the morphology of HELVs as compared with their lymphoid counterparts, appears to be that HELVs have HECs which may vary considerably in size. The appearance of HELVs is thus more varied than HEVs, with parts of the PCV being lined by more cells with a low profile than are found in lymphoid tissues (Miller 1969). This heterogeneity amongst HECs in HELVs has been interpreted as reflecting the presence of vessels with properties intermediate to the fully active HELV (Freemont and Ford 1985). In their study of rat BCG granulomata, these authors noted the presence of vessels with moderately high ECs, and fewer synthetic organelles as compared with HEVs. Also, these vessels appeared to have an intermediate ability to bind lymphocytes. However, despite the less impressive appearance of these intermediate vessels as compared with fully developed HELVs and HEVs, Freemont and Ford (1985) emphasised that the histochemistry of these vessels was typical of HEVs.

Both the histochemistry, and ultrastructure of HELVs are recorded as being identical to that of HEVs in rats and humans (Freemont 1983, Freemont and Ford 1985). Also, in a model of peroxidase-induced arthritis in rabbits, Graham and Shannon (1972) noted the ultrastructural similarity of HELVs with the HEVs reported in the literature of the time. As in HEVs, HELVs appear to often have a thick basement membrane structure composed principally of collagen and pericytes (Freemont 1983).

Mab 325, recognises HEVs in mouse lymph nodes and Peyer's patches, as well as HELVs in chronic inflammatory sites (Duijvestijn *et al.* 1987). The authors concluded that the presence of this antigen in these vessels implied a role in lymphocyte emigration.

Human HELVs have been shown to incorporate radioactive sulphate in a similar manner to that described for rat lymph node HEVs (Freemont 1988). This was found to correlate well with the binding of lymphocytes to HELVs in the Stamper-Woodruff frozen section assay, providing some support for the idea that the sulphated compound is involved in lymphocyte emigration.

Of some interest, is the observation that inflammation of lymphoid tissues increases the extent of the vascular network, including HEVs, throughout the lymph node (Hillman *et al.* 1979, Kittas and Henry 1981, Anderson *et al.* 1975). Also, ultrastructural studies of such activated lymph nodes, have revealed an increase in the synthetic organellar structures of HECs, as well as an overall increase in HEC height (Kittas and Henry 1981). Proliferation of HECs occurs in HEVs of rat lymph node tissues which are stimulated by antigens (Anderson *et al.* 1975). This is consistent with the apparent increase in number of ECs seen in vessel profiles of PCVs with acquisition of the high endothelial phenotype.

Phenotypic Control of High Endothelial Cells

In light of the morphological, ultrastructural, histochemical and functional similarities between HEVs and HELVs, it seems likely that identical factors are responsible for induction and maintenance of the specialised HEC phenotype in both types of vessels. This is illustrated by the presence of a human HEC specific antigen, recognised by Mab HECA-452, on both HEVs and HELVs (Duijvestijn *et al.* 1988)

As a consequence of the association between HECs and lymphocyte emigration, the idea has been developed, that the extensive and prolonged emigration of these leukocytes through the post-capillary venular wall, induces the HEC phenotype (Freemont and Ford 1985, Goldschneider and McGregor 1968).

Alternatively, other workers have suggested that inflammatory cell products from lymphocytes (Duijvestijn *et al.* 1986) and or macrophage factors (Hendricks and Estermans 1983, Cavender *et al.* 1987b & 1989) could be responsible. G-Inf has been particularly implicated as a T cell product of potential importance in HEC phenotypic control, in that treatment of mouse ECs in-vitro with this cytokine induces the expression of the HEV marker Meca-325, while IL1, ß-Inf and EC growth factors fail to do so (Duijvestijn *et al.* 1986). Further circumstantial evidence for the possible contribution of g-Inf to HEC induction is provided by the observation that this T cell product induces the expression of the class II major histocompatibility antigen on cultured ECs (Pober *et al.* 1983, Collins *et al.* 1984), and that HEVs in humans have high levels of this antigen (Turner *et al.* 1987).

Cavender *et al.* (1989) have found that TNF and lymphotoxin induce some HEC-like characteristics in human umbilical vein EC cultures. These authors were able to demonstrate increased EC volume, lymphocyte adhesion, protein synthesis and mRNA levels in ECs treated with these cytokines. With regard to g-Inf and IL-1, however, a reduction in all parameters described, other than in lymphocyte binding, was observed, suggesting that control of HEC phenotype is dependent on at least two, and perhaps many cytokines.

Cultured ECs usually have a cobble-stone morphology, however, incubation of ECs with IL1, TNF and g-inf results in a change in EC cultures to a fibroblastic morphology (Montesano *et al.* 1985, Stolpen *et al.* 1986). As will be described in Chapter 7, ECs cultured from rat lymph node HEVs also have a fibroblastic morphology (Arger 1987), suggesting that the morphological changes in EC monolayers induced by these cytokines could be characteristic of HECs in culture.

Further evidence suggesting a role for T cell products in controlling HEC phenotype is found in the study of animals which have been rendered T cell deficient. Animals which are congenitally athymic have been reported to have either none or less developed lymph node HEVs as compared with their normal litter mates (Vos *et al.* 1980, Fossum *et al.* 1983). In contrast to these reports, however, are others in which such animals are described as having normal HEVs (Van Deurs and Röpke 1975, Sainte-Marie and Peng 1983, Müller and Stempfel 1985). Still further papers report a variable expression of the HEV phenotype within nodes and amongst animals (Jorgensen and Claesson 1972, Fossum *et al.* 1980). The retention of some T cell function by athymic animals has been reported (Chen *et al.* 1984), and it is possible that this could help to explain the contradictory observations described above.

Antigenic stimulation of lymph nodes is essential for the induction and maintenance of HECs. If the supply of antigen is denied to lymph nodes, by de-afferentization of the node, HEVs rapidly lose their specialised phenotype, and present as flat endothelial vessels. This is, however, reversed by direct antigenic stimulation of the de-afferentized lymph node, with the reappearance of HEVs (Hendriks and Estermans 1983, Drayson and Ford 1984). The dependence of HECs on antigenic stimulation is further illustrated by the extension of HEVs which occurs in lymph nodes draining antigenically stimulated tissues (Anderson *et al.* 1975). While it is possible that the effect of antigens upon HEVs is direct, it seems more probable that these changes are mediated by inflammatory cells, especially in light of the inability of LPS to restore HEVs in de-afferentized lymph nodes (Hendriks and Estermans 1983).

Questions Arising From the Literature

The morphological, ultrastructural and histochemical characteristics of HEVs and HELVs have been described, as has the distribution of these vessels in many lymphoid tissues and chronic inflammatory sites (Claesson *et al.* 1971, Anderson *et al.* 1976, Miller 1969). However, despite the evidence that the specialised phenotype of these vessels in lymph nodes is dependent upon the supply of antigens and inflammatory cells (Hendriks and Estermans 1983, Duijvestijn *et al.* 1986), the specific signals responsible for the induction and maintenance of HECs have not been determined. Nor is it known why these cells only appear in the PCVs of appropriately stimulated tissues.

The role of these vessels in exchanging lymphocytes has been investigated (Gowans and Knight 1964, Smith *et al.* 1970). However, a clear functional role for the unusual size of HECs in aiding lymphocyte emigration remains to be determined.

The synthesis of the sulphated-glyco-lipo-protein by HECs has been studied and the properties of this compound investigated (Andrews *et al.* 1980, 1982, 1983). The biological function of this compound, as well as the further synthetic capacity of these cells have, however, not been defined.

It is clear that an understanding of the role of HECs in inflammation and immunity will require further research. In the remainder of this chapter, work characterising PHELVs will be described, and the relation of the data to some of these issues discussed.

MATERIALS AND METHODS

Chemicals

Freon 22 was obtained from Du Pont, while JB4 resin for embedding was supplied by Polysciences. $Na^{35}SO_4$ was obtained from New England Nuclear. Ilford radiographic emulsion was used for autoradiography. Dulbecco's phosphate buffered saline (DPB) was purchased from Oxoid. Monensin was supplied by Calbiochem. All other chemicals were supplied by Sigma.

Tissues

A total of fifty eight gingival biopsies were examined in this study. Fifty seven of these were the same as those described in the previous three chapters. An additional gingival biopsy from a patient with CAP was obtained for use in autoradiography.

Submandibular lymph nodes were removed from six inbred Wistar-Furth rats (Blackburn Animal House, University of Sydney). The nodes from five animals were processed by freeze-substitution for light microscopy, while the nodes from the remaining rat were used for autoradiography.

Tissue Processing for Light Microscopy

The same blocks were used for this study as were used in those described in the previous three chapters, so that tissue processing was identical to that described earlier.

Histochemical Staining

Enzyme histochemical stains were performed for the activities that have been reported as specific for rat lymph node HEVs amongst ECs (Anderson *et al.* 1976). Sections of five freeze-substituted human periodontal tissue specimens known to be rich in PHELVs and of five rat lymph nodes were stained for alkaline phosphatase (APA), acid phosphatase (Higuchi *et al.* 1979), 5'nucleotidase (Klaushofer and Von Mayerbach 1979), Burstone's esterase (Higuchi *et al.* 1979), naphthol ASD acetate esterase and AS acetate esterase (Tsuda *et al.* 1979) activities before counterstaining with haematoxylin. ß-D-glucuronidase activity was detected by a method modified from that used for establishing acid phosphatase activity, by substituting naphthyl ß-D-glucuronide as the substrate.

The morphology of the freeze-substituted tissues was often damaged in patches by ice crystal formation, making an effective random assessment of morphology and leukocyte exchange impossible. To overcome this, fixed tissues were stained for APA

for use in the quantitative assessment of leukocyte margination.

Autoradiography

A single periodontitis specimen, and one lymph node were sliced into 1 mm cubes and then incubated concurrently in a solution of DPB supplemented with 0.1% bovine serum albumin, 0.11% glucose and 10^{-6}M monensin at 37° C for 1 hour. Test tissues were incubated in the presence of 40 μCi of $^{35}SO_4$. After 1 hour of incubation the tissues were fixed by immersion in 10% acrolein in DPB for 1 hour, and then washed four times in DPB. Dehydration was carried out with graded alcohols, and the tissues were infiltrated and embedded in JB4 resin. Sections were cut at 1.5 μm and dipped in radiographic emulsion. After exposure for 4 days, sections were developed and counterstained with haematoxylin. Control tissues were treated in the same manner as the test samples, but without the use of $^{35}SO_4$. Periodontitis test slides were assessed by counting grains of activity immediately overlying ECs throughout the section.

Quantitative Assessment of Leukocyte Exchange

As indicated in Chapter 3, vessels with HECs were most predominant in Fields 1 and 2. For this reason only these fields were considered in this study. A Leitz Dialux 20 EB microscope at a magnification of 312 was used to assess the tissues. The characteristics of all vessels seen in randomly selected zones in each field were recorded in tabular form, a zone being defined as the area enclosed by a photographic graticule. In almost all cases it was possible to record the desired minimum of 20 vessels per field, sometimes necessitating the use of several zones in a field. Vessels were classified on the same basis as that described in Chapter 3. Leukocytes were classified on the basis of morphological characteristics as being PMNs, lymphocytes or monocytes, and their intravascular numbers were recorded.

Tissue Processing for Electron Microscopy

The same tissues were used to study PHELVs at the ultrastructural level as were used in the study of PHyM, so that details of tissue processing can be found in Chapter 4.

Again, because of the comparatively small number of PHELVs available for study, it was decided that it was not possible to perform a meaningful quantitative analysis at the ultrastructural level.

Statistical Procedures

It was decided that a normal distribution of the data could not be assumed, and so non-parametric methods of statistical analysis were applied. Chi-Squared, Chi-Squared Goodness of fit, Wilcoxon's Rank Sign, and Mann-Whitney-U tests were used as required (Champion 1970). All p values given were derived using these tests; 95% confidence intervals are shown in figures.

RESULTS

Vascular Morphology

PHELVs (Figure 5.1) and rat lymph node HEVs were recognised as having ECs which bulged conspicuously into vessel lumens, with large vesicular nuclei and prominent nucleoli. The height of ECs in PHELVs varied within and between vessels such that within a single vessel some moderately high and flat ECs could be recognised. PHELVs were found to have a branching structure, arising from a network of FEVs underlying the pocket epithelium. The diameter of PHELVs varied according to their location in this branching complex. PHELVs and rat lymph node HEVs both contained large numbers of intravascular leukocytes.

MEVs were identified as having ECs which were not as cuboidal as those in PHELVs, and had more dense elongated nuclei than the high cells.

As shown in Chapter 3, the relative proportion of PHELVs to other vessels varied between the fields and lesion types studied, there being fewer PHELVs in minimally inflamed tissues than in gingivitis ($p<0.01$) and the long standing chronic periodontitis specimens ($p<0.001$) (Table 5.1).

Histochemical Findings

The histochemistry of PHELVs, as summarised in Table 5.2, was essentially similar to that of HEVs with the following exceptions. ß-D-Glucuronidase activity was not observed in PHELVs, whilst it was present in HEVs. 5'Nucleotidase activity on the other hand was absent in all HEVs, whilst it was present in 50% of the PHELVs. Also, variation in the intensity of labelling was noted for some enzymes. As was indicated in Chapter 3, APA was consistently absent in PHELVs and HEVs (Figures 3.2a and 3.2c). HEVs and PHELVs were esterase negative.

As indicated in Chapter 3, since APA was absent from PHELVs, it was decided that the absence of APA could be used to help distinguish functionally active PHELVs from the more prevalent MEVs in fixed human tissues.

Autoradiographic Findings

Lymph node HEVs and PHELVs both selectively incorporated $^{35}SO_4$, as seen in the autoradiographs shown in Figure 5.2. Other vessel types did not incorporate this label. In the gingival biopsy, some perivascular and interstitial cells incorporated $^{35}SO_4$. Due to lateral tracking of high energy ß emissions, this extravascular labelling resulted in the presence of some grains over vessels other than PHELVs. 4.8 grains of activity were observed per EC in PHELVs, as compared with 1.1 grains per EC in their flat counterparts. PHELVs had more moderate labelling of ECs as compared

with lymph node HEVs. There was negligible background activity in control slides (Figures 5.2b and 5.2d). This is reflected by the absence of grains over tissue surrounding rat lymph node HEVs in Figure 5.2a.

Leukocyte margination and migration

Results of the quantitative assessment of intravascular leukocyte numbers showed that the apparent adhesiveness of PHELVs for leukocytes was comparable in all periodontal tissues studied (Table 1). FEVs and MEVs did not differ significantly in the number of their intravascular leukocytes so that no clear difference in function could be defined. However PHELVs appeared to be responsible for leukocyte exchange (Figure 5.3), as were lymph node HEVs.

Neutrophils were seen in large numbers in the tissues, apparently streaming towards the pocket epithelial surface in response to chemotactic stimuli. They were not more prevalent surrounding PHELVs as compared with sites closer to the pocket wall, suggesting a rapid rate of movement through the tissues relative to the rate of emigration from PHELVs.

Leukocyte Types found in PHELVs

PMNs far exceeded other leukocyte types found in PHELV lumens ($p < 0.001$), as seen in Figure 5.4.

The predominance of PMNs in PHELVs was a consistent finding in all specimens and sites studied, but there was some variation in the extent of lymphocyte binding such that in gingivitis 21 % of intravascular leukocytes were lymphocytes, whilst in both periodontitis Fields 1 and 2, 4% were lymphocytes ($p<0.001$). However, despite the larger proportion of lymphocytes in gingivitis as compared to periodontitis PHELVs, 69% of leukocytes found in gingivitis PHELVs were PMNs, significantly exceeding mononuclear leukocytes in number ($p<0.01$),(Table 5.1).

This data suggests that PHELVs in chronic periodontitis lesions exchange PMNs almost exclusively, whilst in gingivitis specimens, some lymphocyte margination occurs in conjunction with PMN exchange.

Electron Microscopic Findings

Many PMNs were seen surrounding and in the process of emigrating from PHELVs between periodontal high ECs (PHECs). PHELVs were enveloped by pericytes and their processes, and PMNs were often seen squeezing past these structures (Figure 5.5). PHELVs were surrounded by a multiple basal lamina.

PHECs varied considerably in ultrastructure, there often being several

morphological types apparent in one vessel. PHECs with both electron lucent cytoplasm, and electron dense, ribosome rich cytoplasm, were found and are referred to as light and dark cells respectively. Prominent smooth and rough endoplasmic reticulum, golgi and large numbers of cytoplasmic ribosomes were present. In the basal area of intercellular junctions, cells were closely apposed with interdigitating foot processes, and were connected by macula occludens. Intercellular junctions usually had a long unattached apical and mid-cellular region, although this was not a constant feature. Luminal surfaces were typically ruffled with many pseudopodia. These features are shown in Figures 5.5 and 5.6.

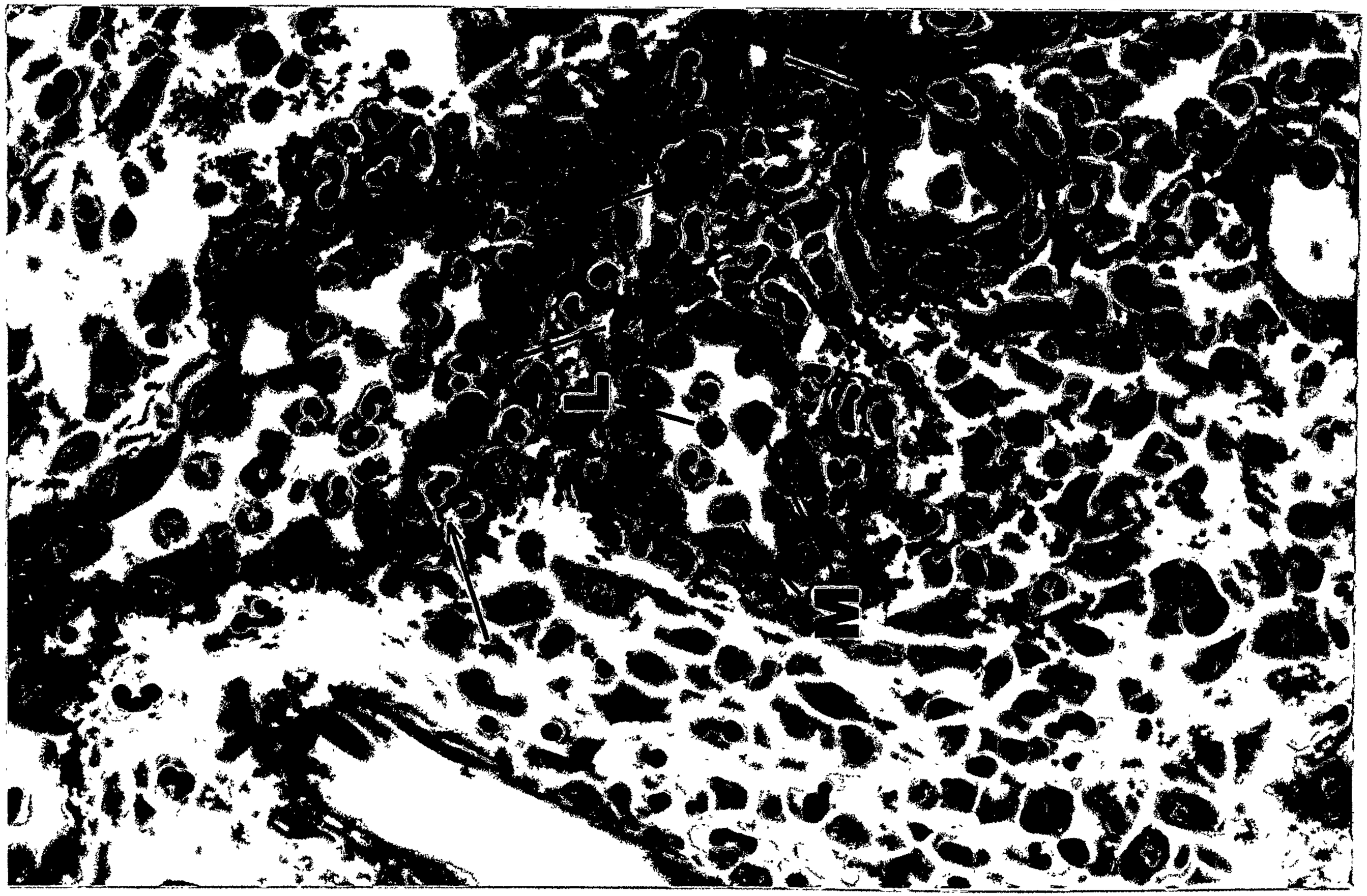
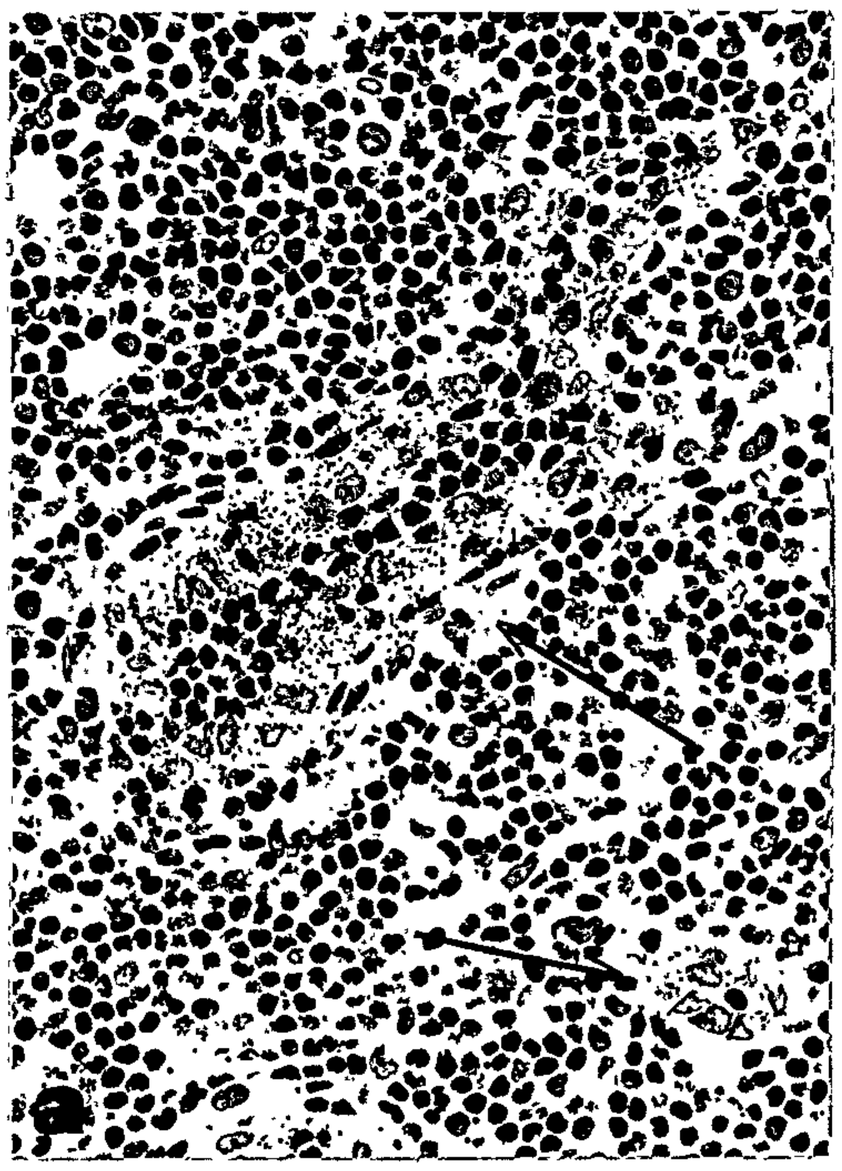
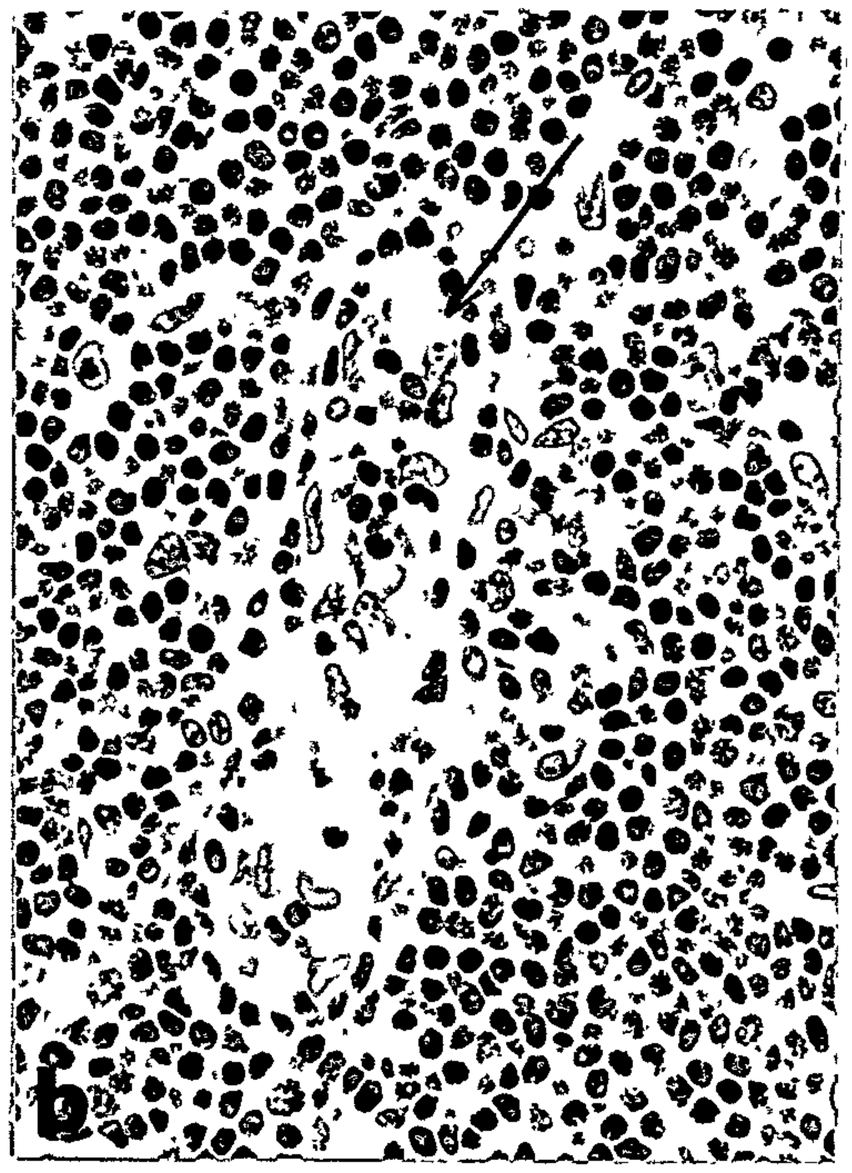


Figure 5.1

Photomicrograph of typical PHELVs.

PHELVs have ECs which are large and bulge prominently into the vessel lumen. Nuclei are large and vesicular with prominent nucleoli. PMNs constitute the majority of leukocytes in PHELVs, although occasional monocytes (M) and lymphocytes (L) are seen. Many PMNs are seen in the process of migrating through the vessel wall (arrows). (Toluidine Blue X 500)





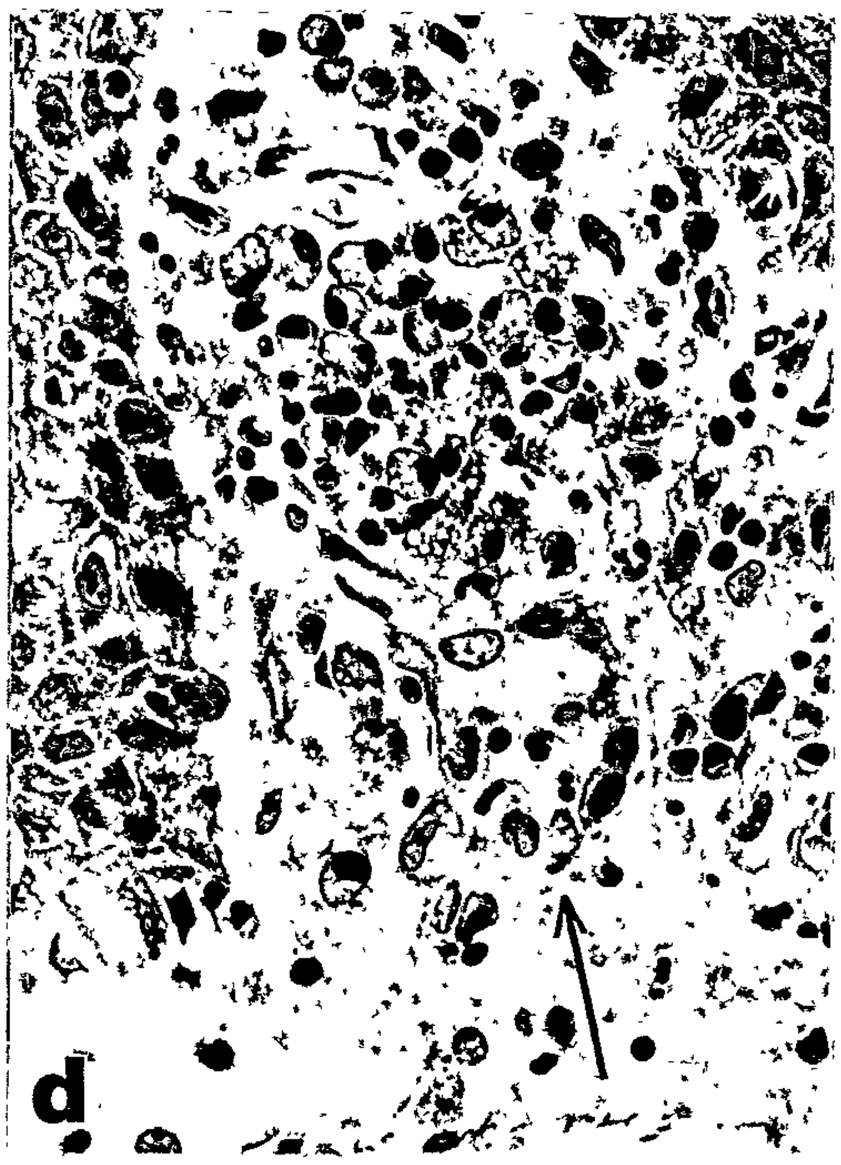


Figure 5.2

Autoradiographs of rat lymph node tissue (a) and a periodontitis specimen (c), which have been pulsed with $^{35}SO_4$. Control tissues, not treated with radioactive label, but processed for autoradiography at the same time as the test tissues are also shown (b and d).

(a) HEVs have incorporated the radioactive label. No other cells or type of vessel in this tissue exhibited this metabolic property. (Haematoxylin X 312)

(b) Control autoradiograph of lymph node tissue. HEVs (arrow) did not have grains of activity, establishing that labelling reflected true uptake of radioactivity, and was not artifactual. (Haematoxylin X 500)

(c) Grains of activity are concentrated over PHELVs. However, the uptake of the label by other cells in the tissue has resulted in some minor labelling of FEVs. This is explained by lateral tracking of ß emissions from labelled mast cells in close relation to these vessels. Labelling is not as intense as that in lymph node HEVs, however, the presence of ^{35}S in these cells suggests that PHELVs and rat lymph node HEVs are functionally similar. (Haematoxylin X 500)

(d) Control autoradiograph of gingival tissue. PHELVs (arrow) did not have grains of activity, indicating that labelling reflected true uptake of radioactivity, and was not artifactual. (Haematoxylin X 500)

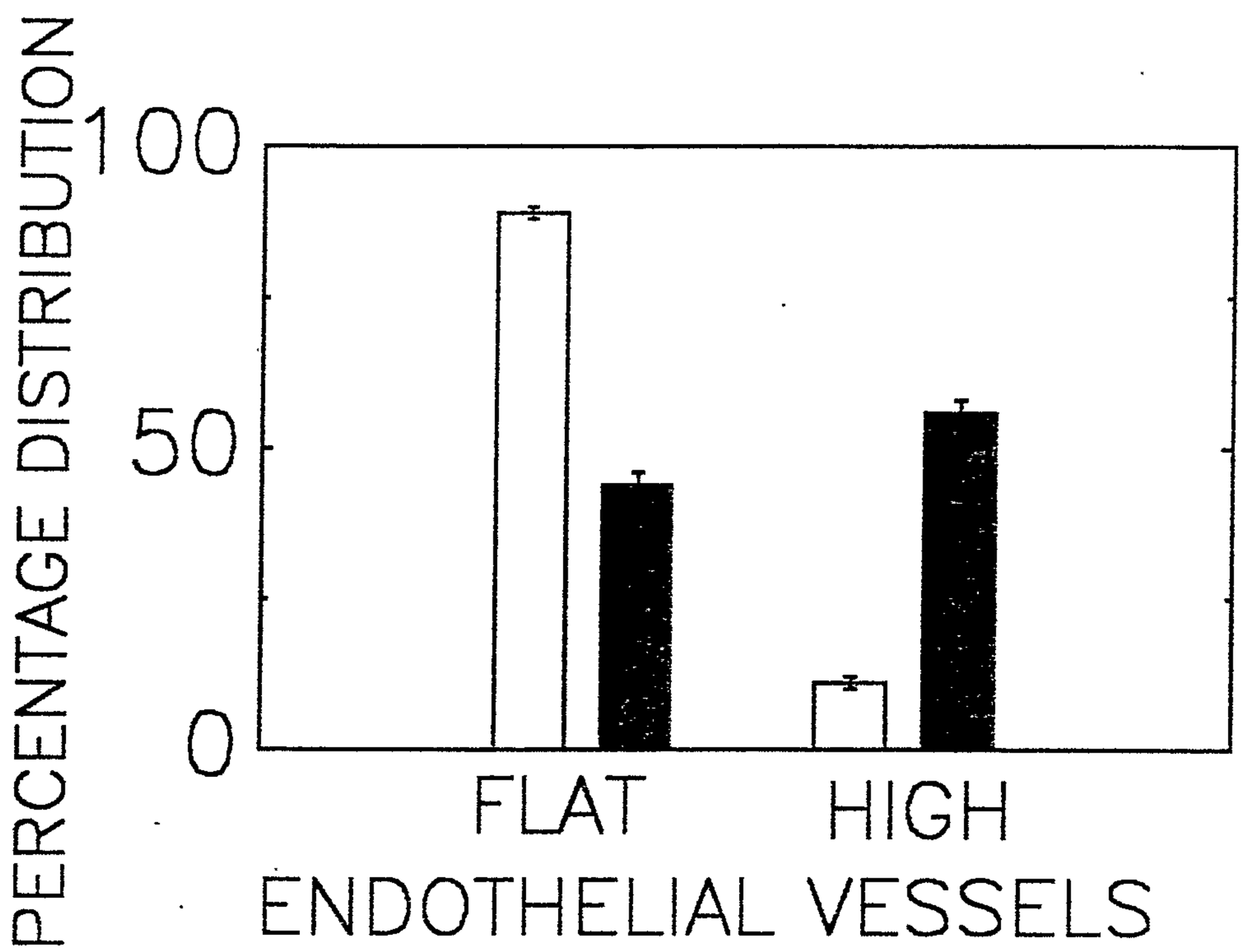

PERCENTAGE DISTRIBUTION
100
50
0
FLAT
HIGH
ENDOTHELIAL VESSELS

Figure 5.3

Percentage distribution according to endothelial height of the number of vessels (open bars) and the number of intravascular leukocytes (filled bars). FEVs and MEVs are considered together (Flat Endothelial Vessels), and compared with PHELVs (High Endothelial Vessels).

PHELVs accounted for 56% of the intravascular leukocytes in tissues studied. PHELVs constitute only 11% of the vessels studied, but they account for the majority of intravascular leukocytes suggesting that like HEVs described by others, PHELVs are the preferred site of leukocyte exchange. An index of leukocyte adhesion was calculated by dividing the percentage of intravascular leukocytes by the percentage of vessel type. From this, PHELVs are at least 10 times more adhesive for leukocytes than other vessels ($p < 0.003$).

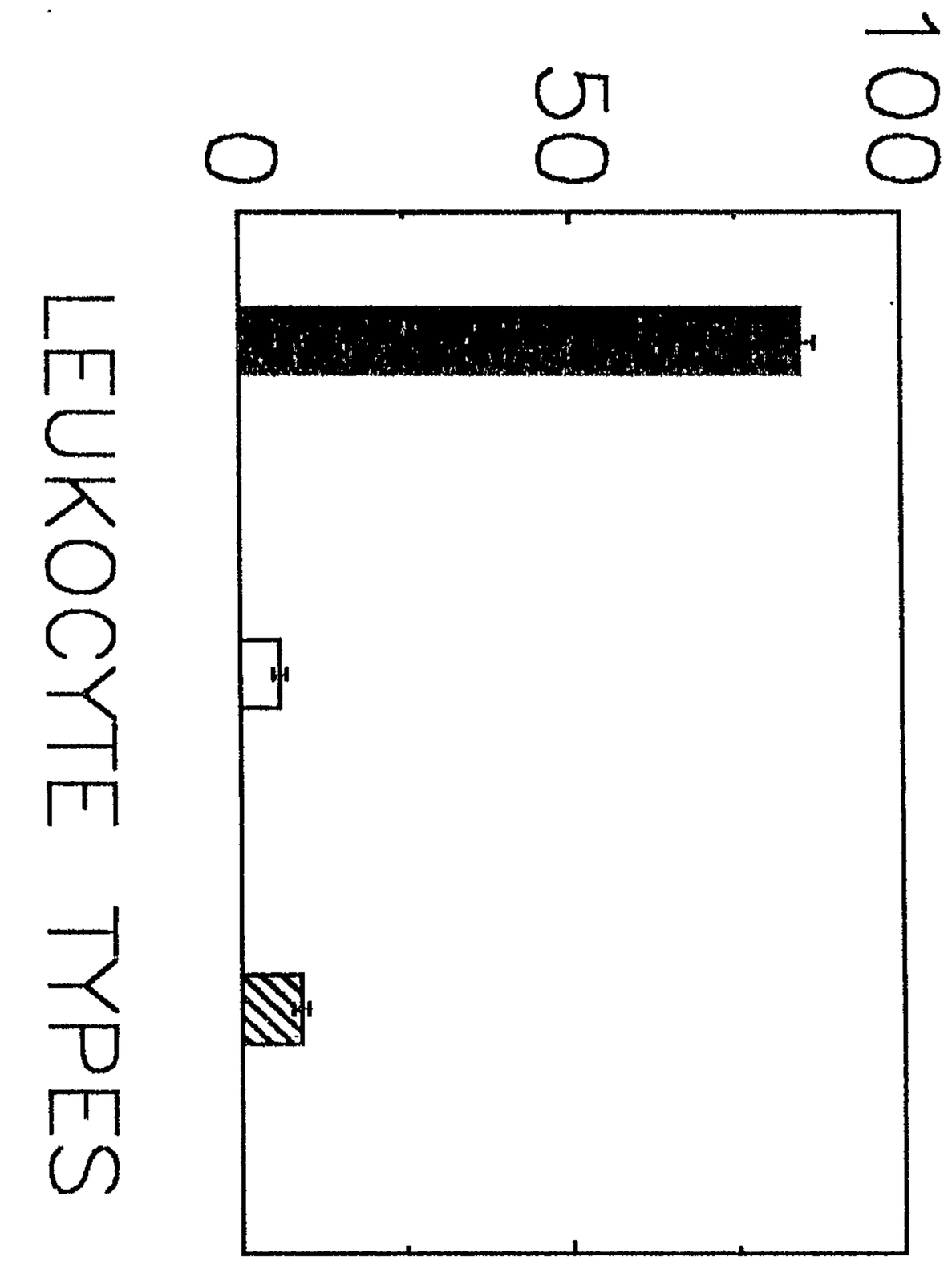
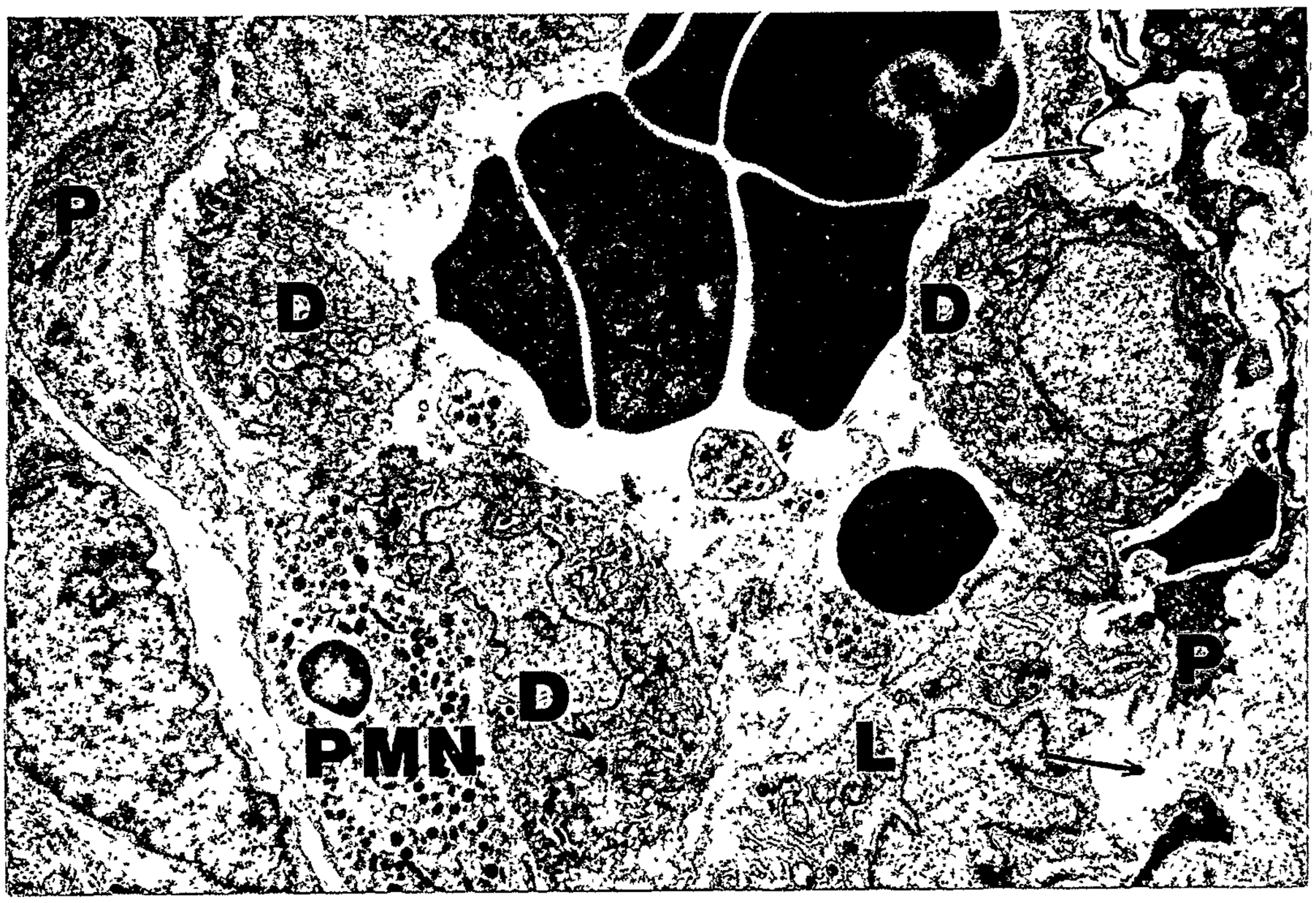

% LEUKOCYTES IN PHELVs
0
50
100
LEUKOCYTE TYPES

Figure 5.4

Histogram showing the relative percentages of PMNs (filled bars), lymphocytes (open bars), and monocytes (right diagonal bars), found in PHELVs.

PMNs exceeded lymphocytes and monocytes in number whether considered separately or together as mononuclear cells ($p < 0.001$). No significant difference was found between the percentage of lymphocytes and monocytes in PHELVs.


P
D
D
PMN
D
L
P

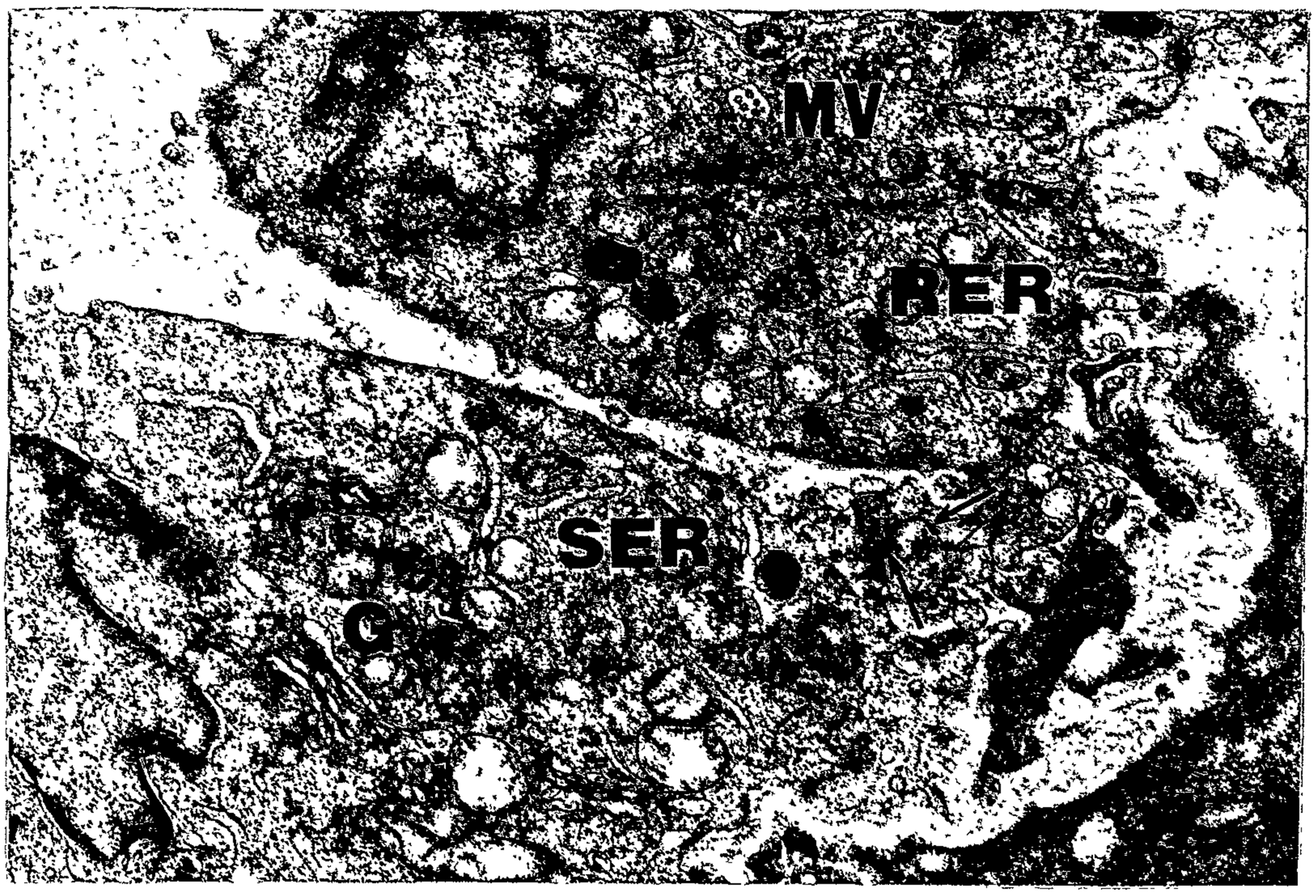

MV
RER
SER
G

Figure 5.5

Electron micrograph showing a PHELV with PMNs emigrating through the vessel wall.

A PMN is seen between a pericyte (P) and a PHEC. PHECs with both dark (D) and light (L) cytoplasm can be recognised, as can large clefts separating the apical portions of adjacent PHECs. MBL are seen surrounding the PHELV. (TEM X 4000)

Figure 5.6

Electron micrograph of the intercellular region of two adjacent PHECs.

The cytoplasm of PHECs is rich in synthetic structures such as golgi apparatus (G), rough endoplasmic reticulum (RER), and smooth endoplasmic reticulum (SER). Occasional multi-vesicular bodies (MV) are found. The intercellular junctions are open in the apical region, but are tight in the basal region with macula occludens (arrow) binding PHECs in this area. PHECs have complex foot processes in this basal area. (TEM X 13000)

Table 5.1 Summary of the data derived from the quantitative assessment.

The number of each type of vessel (Ves. No.), numbers of intravascular PMNs, monocytes and lymphocytes found in all sites and lesions studied including Field 1 (F1) and Field 2 (F2) of periodontitis, are shown. Relative percentages are indicated in brackets.

	Periodontitis F1 20 Specimens			Periodontitis F2 20 Specimens			Gingivitis 16 Specimens			Minimally Inflamed 15 Specimens		
	PHELVs	MEVs	FEVs	PHELVs	MEVs	FEVs	PHELVs	MEVs	FEVs	PHELVs	MEVs	FEVs
Ves. No.	85 (15)	149 (27)	320 (58)	48 (11)	135 (31)	251 (58)	32 (9)	129 (34)	212 (57)	21 (7)	83 (28)	194 (65)
PMNs	346 (89)	48 (82)	90 (78)	106 (80)	12 (63)	47 (72)	42 (69)	4 (57)	40 (64)	22 (88)	37 (77)	74 (68)
Monocytes	28 (7)	5 (9)	13 (11)	21 (16)	2 (11)	11 (17)	6 (10)	_	11 (18)	1 (4)	2 (4)	14 (13)
Lymphocytes	17 (4)	5 (9)	13 (11)	6 (4)	5 (26)	7 (11)	13 (21)	3 (43)	11 (18)	2 (8)	9 (19)	21 (19)

Table 5.2 Results of histochemical and autoradiographic studies comparing PHELVs with rat lymph node HEVs.

Some differences were found in the histochemical and sulphate uptake properties of PHELVs as compared with the HEVs of rat lymph nodes. However, some degree of similarity was apparent between these vessels, implying functional similarity between these vessels.

Histochemical Marker	Human Periodontal Tissues			Rat Lymph Node Tissues		
	PHELVs	FEVS	Comments	HEVs	FEVs	Comments
Alkaline Phosphatase	-	+++	Cell Surface Staining	-	+++	Cell Surface Staining
Acid Phosphatase	++	++	Patchy Staining	+	+	Patchy Staining
5-Prime Nucleotidase	50% ++	+++	Cell Surface Staining	-	++	Cell Surface Staining
Beta-D-Glucuronidase	-	-	Some Plasma Cells +	++	-	Discrete Granules
Non Specific Esterase	_	_	Macrophages ++	-	-	Macrophages ++
Radioactive Sulphate	+	-	Non Endothelial Cells +	+++	-	No Other Cells

DISCUSSION

PHELVs are Similar to HEVs

The histological appearance of PHELVs in this study was typical of that described for HELVs in chronic inflammatory lesions. This applies especially with regard to the irregular endothelial height and vessel diameter of PHELVs (Freemont 1988, Miller 1969, Freemont 1983). Large numbers of intravascular leukocytes in PHELVs further reinforce the morphological similarity between these vessels and HELVs as reported by others.

HECs are reported to have a similar histochemistry in different species and regardless of their location in HEVs or HELVs (Freemont 1985, Anderson *et al.* 1976, Freemont 1983). This similarity in histochemistry was exploited to compare HEVs from rat lymph nodes with PHELVs. The likeness in the histochemical profile of these vessels suggests some degree of biochemical similarity between HEVs and PHELVs. The differences that were noted, could be a reflection of the preferred exchange of PMNs by PHELVs.

Esterase activity is recorded as a reliable marker for both HELVs and HEVs (Anderson *et al.* 1976, Freemont 1983), and the absence of this activity in the vessels studied is difficult to reconcile with the positive controls provided by macrophages within the sections. Acid phosphatase activity is reported as a specific marker for HECs as against flat ECs, which are recorded as being negative for this marker (Anderson *et al.* 1976). However, in the present study FEVs and MEVs did not differ from HEVs with regard to acid phosphatase activity. The use of freeze-substituted tissues may account for departures from the histochemistry reported in the literature.

Electron microscopy revealed many similarities between PHELVs and the HELVs and HEVs reported elsewhere (Anderson *et al.* 1976, Graham and Shannon 1972, Nightingale and Hurley 1978). The presence of light and dark cells, the nature of the intercellular junctional complexes with foot processes and maculae occludens, and the presence of extensive synthetic structures, are ultrastructural features of HECs shared by ECs in PHELVs. Multiple basal lamina have been reported in inflamed gingiva (Freedman *et al.* 1968, Garant 1976), and could be a sign of intermittent damage to PHELVs (Vrako *et al.* 1980), by bacterial products. This has particular relevance in relation to the formation of PHyM described in Chapter 4.

The functional property of $^{35}SO_4$ incorporation is known to be peculiar to HECs, their flat endothelial counterparts failing to take up the label (Freemont 1988, Andrews *et al.* 1980). The specificity of sulphate uptake by lymph node HEVs was confirmed in this study. PHELVs are seen to have a similar albeit less pronounced uptake of this label. Apart from leukocyte exchange, $^{35}SO_4$ uptake is the only

functional marker of HECs which is currently available for testing.

From these observations it seems that PHELVs are essentially similar to the HELVs described by others, but most significantly exchange PMNs rather than lymphocytes in the long standing chronic periodontitis lesions, and to a lesser extent in gingivitis specimens.

PMN Emigration is Inconsistent with the Putative Role of HEVs in Lymphocyte Exchange

Electron microscopy of PHELVs showed that PMNs emigrated through the endothelial barrier, ensuring that the large number of intravascular PMNs were not a reflection of non-specific adherence to the luminal wall. PMNs and monocytes are known to emigrate from pre-existing HEVs and HELVs in some experimental lesions (Graham and Shannon 1972, Marchesi and Gowans 1964), as well as from HEVs in the lymph nodes of nude mice (Van Deurs and Röpke 1975). However, PMNs were not reported as the major leukocyte type to emigrate from these vessels. There is an inversion of the classical pattern of leukocyte emigration in this study. PMNs are usually associated with the early acute inflammatory response, and with the exception of chronic abscesses, are replaced by a mononuclear infiltrate in persistent lesions. In periodontitis, however, chronic abscesses are not seen, yet PMNs appear to emigrate to the exclusion of lymphocytes, whilst in earlier gingivitis lesions more lymphocytes are found migrating into the tissues.

It could be argued that PHELVs are derived from pre-existing HELVs present in the early gingivitis lesion. Recent work describing HELVs in experimental gingivitis and treated periodontitis lesions would support this view (Wynne *et al.* 1988). However, the extensive remodelling of tissue which accompanies the development of the advanced chronic periodontal lesion makes this unlikely. Also, the time course of chronic periodontitis, which can often be measured in decades, is such as to allow ample time for loss of the specialised HELV phenotype. PHELVs thus seem to be established as PMN exchange vessels, and are not lymphocyte exchange vessels which have adopted additional functions.

Maintenance of HELVs in the absence of prolonged lymphocyte emigration is not consistent with the current view of HEV induction and function. Two models of HEV and HELV induction have been proposed. In one model the prolonged emigration of lymphocytes is thought to induce the high endothelial phenotype (Freemont and Ford 1985, Goldschneider and McGregor 1968), whilst in the second, lymphocyte (Duijvestijn *et al.* 1986, Cavender *et al.* 1987) and or macrophage factors (Hendricks and Estermans 1983) are suggested as being responsible.

Prolonged and extensive lymphocyte emigration are not required for the generation and maintenance of PHELVs, supporting the view that locally produced factors stimulate the expression of the high endothelial phenotype in PCVs.

Lymphocyte recirculation occurs in the small intestinal mucosa and liver (Gowans and Steer 1980, Morris 1980a) despite the absence of HEVs in these sites. Also HEVs have not been described in sheep lymph nodes, which have been widely used in the study of lymphocyte recirculation (Morris 1980b). Further, flat endothelial vessels in athymic nude rats are as efficient at binding lymphocytes as are HEVs in euthymic littermates, although an apparent reduction in the efficiency of diapedesis is recorded (Fossum *et al.* 1983). HEVs do not occur in experimental macrophage granulomas and abscesses where there is prolonged non-lymphocytic infiltration of tissues (Nightingale and Hurley 1978). From these observations, HECs are clearly not an absolute requirement for prolonged leukocyte, and particularly lymphocyte exchange.

HECs May be Phenotypically Specialised for the Production of Cytokines

Many explanations have been proposed to justify the cellular hypertrophy in HEVs and HELVs based on lymphocyte emigration. For instance, there is evidence that by increasing the rate of lymphocyte collision with the vascular wall, HECs aid in lymphocyte adhesion and hence emigration (Bjerknes *et al.* 1986). However, the equally effective emigration of leukocytes across flat endothelial venules does not lend substance to this argument. Intracellular lymphocyte trafficking (Marchesi and Gowans 1964, Farr and Di Bruyn 1975), has also been suggested as the primary function of HECs. In this study PMNs emigrated between PHECs. Intracellular trafficking is thus an unlikely justification for the formation of PHECs, and it is suggested that this is also the case for HECs in other sites. The minimisation of plasma loss during leukocyte emigration has also been proposed as a special function of HEVs (Schoefl 1972), however, the absence of HEVs in sheep lymph nodes does not seem to result in disturbances of fluid dynamics in these animals.

ECs are known to be capable of producing potent cytokines including IL-1 (Miosec *et al.* 1986), interleukin-6 (Sironi *et al.* 1989) and colony stimulating factors (Segal *et al.* 1987). These factors may contribute to both local and systemic aspects of inflammatory and immune lesions. However, the quantitative impact of such synthesis by ECs in most sites must be questioned. It is possible that HECs are particularly well suited for the synthesis of cytokines, and that these cells are hypertrophied as a reflection of their increased synthetic capacity. The extensive synthetic apparatus observed in ultrastructural studies supports this view, as does the

location of HECs in inflammatory foci and lymphoid tissues.

Since lymphoid tissues are strategically placed to collect and process antigens, it could be argued that such tissues are in a site of physiologically maintained chronic inflammation, and consequently that HEVs in these organs are essentially similar to those seen in other chronic inflammatory sites. The dependence of HEVs upon afferent lymph (Hendricks and Estermans 1983), and the extension of these vessels following antigenic stimulation (Anderson *et al.* 1975) are observations consistent with this interpretation of lymph node function.

CHAPTER 6

IDENTIFICATION OF THE ENDOTHELIAL ISOENZYME OF ALKALINE PHOSPHATASE

INTRODUCTION

As discussed in Chapters 3 and 5, PHELVs as well as HEVs are AP negative. The negative association between these vessels, and hence leukocyte exchange, with APA suggested a possible role for this enzyme in the control of leukocyte emigration. Literature relating to this suggestion will be discussed following this introduction.

In order to further investigate the question of the possible role of AP in the control of leukocyte emigration, a source of endothelial AP is required for experimentation. A barrier to this work is the relatively small quantity of AP that can be obtained from EC cultures. Also, there is some risk that AP from contaminating cells in cultures and non ECs in tissue homogenates could provide misleading data regarding the endothelial isoenzyme.

An alternative strategy is to identify the endothelial isoenzyme, and hence a rich source of the protein for experimentation. There is evidence that there is only one gene coding for each isoenzyme of AP (Moss and Whitaker 1985), so that characterisation of the enzyme in-situ using a panel of inhibitors for AP should allow the correct identification of the endothelial isoenzyme, and therefore the determination of a credible, abundant source of AP with which to study the role of this enzyme in vitro. In order to do this it is necessary to utilise reproducible high resolution histochemical techniques, which allow application of quantitative methods of assessment.

Frozen section techniques do not provide the resolution and uniformity of section thickness required to perform a meaningful quantitative assessment of enzyme inhibition. Also, formaldehyde fixation is known to inhibit APA (Lev and Griffiths 1982). This has been confirmed using plastic embedded tissues as described in Chapter 3, and casts doubt on the applicability of standard fixation and embedding techniques to this work. The use of freeze-substitution and high resolution plastic embedding techniques to overcome these problems and to positively identify the

endothelial isoenzyme of AP as being the LBK form is described in this chapter.

LITERATURE REVIEW

General Characteristics of Alkaline Phosphatase

Alkaline phosphatase (AP) is defined by its ability to cleave mono-phosphate ester bonds at alkaline pH optima, and is found in all vertebrates, many invertebrates and in some fungi, protozoans, algae and bacteria, but is absent in plants with the exception of some parasitic forms (McComb *et al.* 1979). AP is a zinc metaloenzyme, and seems to consist of subunits assembled into dimers or tetramers. In eukaryotes, the enzyme is a membrane bound ecto-enzyme anchored by phosphatidylinositol (Ikezawa and Taguchi 1981) (Webb and Todd 1988). In bacteria, however, AP is found in the periplasmic space.

Isoenzymes of Alkaline Phosphatase

Alkaline phosphatase activity (APA) has been found in many tissues using both biochemical and histochemical techniques (McComb *et al.* 1979). AP from mammalian tissues has been extensively studied, and three major isoenzymes are described for human tissues. These are the placental, intestinal and liver/bone/kidney (LBK) forms, which are encoded by different genes (Kam *et al.* 1985, Henthorn *et al.* 1987, Weis *et al.* 1988a) and are distinguishable on the basis of their electrophoretic mobility, heat lability, sensitivity to inhibitors and antigenic properties. A fourth isoenzyme with similar antigenic properties to the placental form, but which has differing sensitivity to specific inhibitors of the placental isoenzyme has been identified in human thymic and testicular tissues (Harris 1982). This thymic/testicular isoenzyme has not been as extensively studied as the major forms but seems to be encoded by a separate gene to the placental form (Millan and Stigebrand 1983).

The related intestinal, placental and thymic/testicular isoenzymes are encoded by genes localised to the second chromosome (Martin *et al.* 1987, Griffin *et al.* 1987), whilst the LBK isoenzyme is encoded by a gene on chromosome number 1 (Swallow *et al.* 1986). It is generally thought that the differing isoenzymes of AP arose as a result of gene duplication, with the intestinal isoenzyme having 87% amino acid homology with the placental form (Henthorn *et al.* 1987), and only 57% amino acid homology with the LBK isoenzyme (Weis *et al.* 1986, Harris 1982). The placental isoenzyme has slightly less identity with the LBK isoenzyme with 52% homology at the amino acid level (Weis *et al.* 1986). Human foetal intestinal AP

appears to be distinct from that of the adult, in that small differences in electrophoretic mobility, as well as in peptide maps derived by cyanogen bromide (Vockley *et al.* 1984a) and trypsin digestion (Mueller *et al.* 1985) have been found. Differences in the sialation states of intestinal AP from the two sources do not seem to be the cause of observed differences between the foetal and adult forms (Vockley *et al.* 1984a). However, antigenic differences between the adult and foetal forms have been described (Vockely *et al.* 1984b). Despite this, no differences in the 40 amino terminal amino acids have been found (Hua *et al.* 1986), leaving the question of a further foetal intestinal isoenzyme unresolved.

Histochemically, the placental isoenzyme is localised to the surface of the syncitiotrophoblast, whilst the intestinal form is bound to the intestinal brush border. In contrast to these, the LBK isoenzyme has a wide distribution and is found in many tissues. In the liver this isoenzyme is localised to the bile canaliculi, and is reported to be present in the sinusoidal endothelium of some species (Manns 1969). In the kidney, however, APA is consistently localised to the kidney tubular brush border, whilst in bone, osteoblasts are AP positive (McComb *et al.* 1979).

Isoenzymes of AP are in general more similar amongst differing species, than they are when compared with other isoenzymes within species. The placental isoenzyme, however, provides the exception to the rule, in that it appears to be unique to humans and other higher primates. Other species have the LBK isoenzyme in placental tissues. The placental isoenzyme seems to be closely related to both the intestinal and thymic/testicular forms, and is thought to have appeared relatively late in evolution (Harris 1982, Henthorn *et al.* 1987, Millan & Stigebrand 1983, Knol *et al.* 1987). The placental isoenzyme is also unique in that it is polymorphic, having three common alleles at an autosomal locus, with less common additional forms occurring throughout the population (Harris 1982).

The Biological Role of AP

The generally strict conservation of AP enzymes within tissues of differing species suggests that the function of AP is important, demanding tight conservation of isoenzymes. Yet despite the abundant literature describing this family of enzymes, and the widespread use of APA as a clinical diagnostic tool in the identification of many diseases, the role of AP is unknown (McComb *et al.* 1979). Many authors have speculated upon possible functions of AP and have been guided in their thinking by the distribution of the enzyme in nature.

Trans-Cellular Transport

The association of the enzyme with surfaces at which the transport of materials occurs, has suggested to many workers that AP may play a role in the transport of substances across cell barriers. Examples of such surfaces in mammals are the small intestinal mucosa, the brush border of kidney tubules, bile canaliculi and vascular ECs. In some parasitic invertebrates and plants, the interface between host and parasite is AP positive (McComb 1979 *et al.*), giving further support to the idea that transport may be an important role for AP.

There is some evidence that the transport of inorganic phosphate, and of phosphorylated compounds across the cell membrane is a function of AP. The most convincing of this evidence has come from the study of microorganisms, where the expression of AP by many bacteria (Horiuchi *et al.* 1959, Hassan and Pratt 1977), fungi (Nyc *et al.* 1966) and algae (Walther and Fries 1976) is inversely related to the concentration of inorganic phosphate in the culture media. In studies using rings of small intestinal tissue, uptake of inorganic phosphate is reduced by inhibitors of intestinal AP (Moog and Glazier 1972) implying generality for the role of AP in the transport of phosphate.

It has been suggested that by binding inorganic phosphate and shuttling between the internal and external surface of the cell membrane, AP could effect transport of orthophosphate groups (McComb *et al.* 1979 p868). One difficulty associated with the idea that AP plays a purely mechanical role in the transport of phosphate, is that many cells are capable of obtaining inorganic phosphate from their environment, yet lack APA. Also, the anchorage of AP to the plasma membrane by phosphatidylinositol would seem to make this possibility energetically unfavourable, as the enzyme would need to repeatedly plunge into and through the hydrophobic lipid bilayer in order to perform this task.

Another possibility is afforded by the observation that phosphate can be obtained by AP positive cells from phosphorylated compounds. For example, experiments using ^{32}P labelled glucose-6-phosphate have shown that AP positive Escherichia coli (E. coli) selectively incorporate phosphate from the phosphorylated glucose compound, even in the presence of excess inorganic phosphate (Malamy and Horecker 1961). Also, phosphate derived from ß glycerophosphate is as effectively incorporated by small intestinal rings as is inorganic phosphate (Moog and Glazier 1972). It is therefore possible that a role for AP in the transport of phosphate and phosphorylated molecules, is the release of orthophosphate groups from phosphorylated organic compounds. This could allow other transport mechanisms to

direct the movement of the resulting free orthophosphate and remaining organic residue across the cell membrane. This suggestion was first made when it was found that yeast cells were unable to use the sugar moiety of glucose phosphates in the presence of inhibitors of AP (Rothstein and Meier 1949). In light of this, the induction of APA in microorganisms grown in media with low concentrations of inorganic phosphate can thus be interpreted as a strategy for obtaining phosphate from organic compounds in the media. Small intestinal AP could reasonably be expected to have a similar function in the gut.

The transport of substances other than phosphate has also been suggested as a role for AP. Water (Wilkins and Thompson 1974), lipid (Linscheer *et al.* 1971), and thiamine (Schaller and Höller 1975) transport have all been associated with APA.

Mineralisation of Tissues

Histochemical studies have revealed that at sites of calcification, osteoblasts and chondroblasts are strongly AP positive (McComb *et al.* 1979). It has previously been proposed that a phosphatase in bone and cartilage could hydrolyse organic phosphates, to effect mineralisation of these tissues (Robison 1923). This hypothesis has not been widely accepted, partly because peak levels of APA and calcification do not coincide (Siffert 1951). Also, the absence of calcification in tissues such as the gut and kidney which are rich in AP does not lend support to this argument. A further suggestion which has been made in relation to the role of AP in calcification is that by hydrolysing pyrophosphate, AP removes a potential physiological inhibitor to normal mineralisation (Russell 1965, Fleisch *et al.* 1966). The use of matrix vesicle preparations to study the role of AP in mineralisation is now common (Genge *et al.* 1988), however, the precise role of AP in mineralisation is still not clear. Nonetheless, in the rare congenital disease, hypophosphatasia, the LBK isoenzyme is known to be either absent or defective, supporting the suggestion that AP plays a critical role in the normal mineralisation of tissues (Weis *et al.* 1988b).

Cell Differentiation

It has been suggested that AP could play a role in cell proliferation and differentiation (Prakash 1961). In support of this is the observation that, while many embryonic tissues express APA, this activity is lost in the developed animal (McComb *et al.* 1979). Similarly, APA appears in the cells of regenerating limbs in amphibians (Karczmar and Berg 1951). Also proliferating fibroblasts in scar tissues often express APA (Kopf 1957). AP, particularly the placental isoenzyme has been described as an onco-developmental enzyme. The association with many neoplasms

implies a possible role in deregulated cell function (Fishman 1974).

Protein Phosphatase Activity

Proteins are often phosphorylated at serine, threonine and tyrosine residues, with serine residues being most commonly involved. There is considerable evidence that the phosphorylation of proteins is critical in determining their activity, and it has been suggested that AP could play a role in controlling the activation of some surface receptors (Lau *et al.* 1989).

Specific classes of protein phosphatases seem to be responsible for the de-phosphorylation of either phosphoserine and phosphothreonine proteins, or phosphotyrosyl protein (Shenolikar 1988). Because phosphotyrosyl proteins comprise only a small fraction of the phosphorylated proteins found in cells, yet seem to play a key role in the regulation of many cellular processes, a great deal of work has been done investigating phosphotyrosyl protein phosphatases (Lau, *et al.* 1989). Both the phosphotyrosyl protein phosphatases and the phospho-serine/threonine protein phosphatases have been sub-grouped into specific classes (Shenolikar 1988). The possibility that AP could be a representative of one of the phosphotyrosyl protein phosphatases was suggested by Swarp *et al.* (1981). These authors reasoned that the similarity of the widely used substrate for AP, para-nitrophenol phosphate, with phosphotyrosyl implied that phosphotyrosyl residues in phosphoproteins would be likely substrates for AP. This was further supported by the identification of phosphotyrosyl proteins that were sensitive to de-phosphorylation by AP (Witte *et al.* 1980). Swarp *et al.* (1981) demonstrated that AP from calf intestine, bovine liver and E. coli had phosphotyrosyl protein phosphatase activity at physiological pH, and that this activity was 5 to 10 times greater than the respective phophoserine protein phosphatase activity. Further, they found that membrane proteins from A-431 cells phosphorylated at tyrosine were more effective substrates for dephosphorylation by AP than serine/threonine phospho-proteins. These authors concluded that AP could be a membrane bound tyrosyl protein phosphatase with some serine/threonine protein phosphatase activity.

However, Lau *et al.* (1985) have found that the optimal pH for the phosphotyrosyl protein phosphatase activity of AP is alkaline, casting doubt upon the potential role of AP as a neutral phosphotyrosyl protein phosphatase. Also, levamisole and EDTA, which are well known inhibitors of AP, failed to inhibit the activity of phsphotyrosyl protein phosphatase from kidney membrane fractions (Rotenberg and Brautigan 1987). Chernoff and Li (1983) have also found that the major phosphotyrosyl protein phosphatase activities isolated from cardiac muscle are

physically separable from AP, and are not activated by Mg^{2+} as is AP. Nonetheless, some phosphotyrosyl protein phosphatase activity was associated with APA. The relative affinity of AP for phosphotyrosyl protein phosphates has been shown to be comparatively low by Lau *et al.* (1985), who suggested that acid phosphatase was responsible for phosphotyrosyl protein phosphatase in skeletal tissues.

Although the identity of the major phosphotyrosyl protein phosphatases is clearly separate from AP, the possibility that this enzyme could play an important role in dephosphorylating membrane-bound proteins can not be discounted. It is possible that only a subset of such proteins are good substrates for AP, and that these are under the control of this enzyme. Tissue homogenates, and crude whole cell membrane preparations would be expected to yield a large number of phosphoproteins and phosphoprotein phosphatases irrelevant to plasma membrane function. Many receptors are known to be inactivated by phosphorylation (Shenolikar 1988), and many cellular transport proteins seem to be phosphorylated (Taborsky 1974). The possible role of AP in the control of these plasma membrane-associated phosphoproteins has yet to be investigated.

Alkaline Phosphatase in the Microcirculation

Capillary and arteriolar ECs are reported to be AP positive (McCombe *et al.* 1979). Differing reports as to the distribution of AP in the microcirculation appear in the literature. For example, Higgins and Eady (1981) reported that endothelial APA was only present in cells lining terminal arterioles and forming the tips of capillary loops. The activity was localised to the luminal surface of ECs and was noted to be associated with vesicles close to this surface, while activity was absent in these structures on the abluminal surface (Higgins and Eady 1981). Other authors, however, report that APA is present throughout the capillary network (Gomori 1941) (Takamatsu H 1939) and a further report illustrates increased endothelial APA at arteriolar junctions (Romanul *et al.* 1962). These authors speculate that increased APA at vessel junctions might reflect a role in transport for AP in these cells, with the purpose of sampling the chemical content of blood to assist in the regulation of blood flow by arterioles. A general observation made in these papers is that the venular ECs are AP negative. However, as noted in Chapter 3, in periodontal tissues, venules and venular ECs were also AP positive (Figure 3.2a), although this activity was not consistently present and is often less intense than throughout the rest of the microcirculatory network. Also, in contrast to the observations of Higgins and Eady (1980), APA was found on both the luminal and abluminal EC surface (Figure 6.1).

Since APA is so readily observed in capillary ECs, this activity has been used to identify microvascular ECs in cell culture (Mulkins and Allison 1987).

Microvascular ECs are recorded as having a levamisole sensitive, phenylalanine resistant APA (Lev and Griffiths 1982), suggesting that endothelial AP is the LBK isoenzyme. There is, however, no further information on the characteristics of AP from vascular ECs.

Because the microcirculation is clearly a site of active metabolic exchange, the presence of APA in microvascular ECs has been attributed to the role of these cells in the transport of materials across the vascular barrier. However, the negative association of APA with HECs and thus with leukocyte emigration (described in Chapter 5) compels the question that AP may play a role in controlling leukocyte emigration. This possibility is supported by the location of AP at the cell surface as an ecto-enzyme, giving AP the potential to interact with leukocyte docking molecules both on passing leukocytes and on the EC surface itself.

Support for the suggestion that AP might play a role in the control of leukocyte docking molecules is found in the literature where it is reported that APA is reduced in EC cultures exposed to IL1 (Mulkins and Allison 1987), and that IL1 increases the binding of leukocytes to EC cultures (Cavender *et al.* 1986). In view of this hypothesis, the comparatively low levels of APA seen in venular ECs might reflect the routine trafficking of lymphocytes through tissues in immune surveillance.

As discussed in Chapter 1, phospho-sugars, particularly mannose-6 phosphate, are implicated as potential leukocyte docking molecules. It has been further suggested that the extensively studied Mel-14 antigen, could act through mannose-6 phosphate binding (Yednock *et al.* 1987b). Both the Mel-14 antigen, and the endothelial leukocyte binding molecule ELAM-1, have lectin like sites (Siegelman *et al.* 1989). Since mannose-6 phosphate is an efficient substrate for E. coli AP (McComb *et al.* 1979), the possible control of Mel-14 antigen, and ELAM-1 dependent leukocyte binding by AP should be considered. Such control could be exerted by de-phosphorylation of the carbohydrate ligands recognized by the lectin binding component of these molecules. As discussed in Chapter 1, Goldstein *et al.* (1989) have evidence that the Hermes antigen is controlled by post-translational events, setting a precedent for the post-translational control of leukocyte binding molecules.

Questions Arising from the Literature

AP has been most intensively studied, so that the distribution in nature, sensitivity to inhibitors, substrate specificity, kinetics and genetics of many isoenzymes of AP have been described (McComb *et al.* 1979, Kam *et al.* 1985, Henthorn *et al.* 1987, Weis *et al.* 1988). An impetus for much of this research has been the use of this enzyme as a clinical marker for many disease processes in both medicine and veterinary practice (McComb *et al.*). However, despite the large literature describing AP, the physiological role of this enzyme is not known. Recent studies of the possible role of AP in the de-phosphorylation of phospho-proteins have not provided strong evidence for this function. The possibility remains, however, that further study will define a sub-set of phospho-proteins which are good substrates for this enzyme. The further possibility that phospho-sugars in glycosylated proteins may be natural substrates for AP, has not been addressed.

MATERIALS AND METHODS

Chemicals

Freon 22 was obtained from Du Pont, while JB4 resin for embedding was supplied by Polysciences. All other chemicals used were obtained from Sigma.

Tissues

Fourteen gingival biopsies were selected at random from the tissues prepared for the work described in Chapters 3 to 5.

Six inbred Wistar-Furth rats (Blackburn Animal House, University of Sydney) were sacrificed to obtain separate samples of a range of tissues. These were: submandibular lymph node, small intestine, kidney, liver, abdominal muscle and skin. Samples were used to prepare composite blocks ensuring a meaningful comparison of tissues from each animal.

Tissue Processing

All tissues were processed using freeze-substitution as described in Chapter 3. Sections were cut to a thickness of 2 μm, using glass knives in a Sorval JB4 Porter-Blum microtome. Slides were stored desiccated at 4°C.

Staining Procedure

Sections were incubated at 37°C for 1 hour in an 80% solution of a filtered alkaline phosphatase reaction mixture to which an inhibitor solution was added. Slides were then counterstained with haematoxylin. The reaction mixture consisted of a 0.01M tris buffer at pH 8.1 to which Fast Red Violet 0.2mg/ml, and α-naphthol AS MX phosphate had been added (Higuchi *et al.* 1979). Inhibitor solutions were prepared by dissolving inhibitors in distilled water at five times the concentration required for the final solution. All inhibitors dissolved without difficulty except for EDTA which required heating.

The inhibitors and the concentrations at which they were present during co-incubation with sections were: mercuric chloride 2mM (Breslow and Katz 1968), L-leucyl-glycyl-glycine 5mM (Mulivor *et al.* 1978), beryllium sulphate 0.001mM (Thomas and Aldridge 1966), EDTA 1mM (Conyers *et al.* 1967), L-phenylalanine-glycyl-glycine 1mM (Mulivor *et al.* 1978), lysine HCL 0.2M (Bodansky 1948), sodium arsenate 0.3mM (Eaton and Moss 1967) and levamisole 0.5mM (Borgers 1973).

For each biopsy specimen studied, two control slides were prepared by substituting distilled water for the inhibitor. This allowed direct comparison of sections tested for APA with and without the presence of inhibitors.

Quantification of APA in Human Gingival Blood Vessels

Slides were coded and zones selected at random for study at a magnification of 312. As described in Chapter 3, the blood vessels in Field 4 were almost exclusively capillary loops and displayed intense, reliable labelling for APA, and so it was decided to use only vessels in this field for this aspect of the study. Zones were defined by the border of a photographic graticule aligned against the basal surface of the oral epithelium. With frequent use of a reference slide, the intensity of APA was assessed for all vessels present in the zone regardless of the angle of sectioning. This activity was graded from 0, no activity present, to 4 (Figure 6.2). The APA of twenty vessels from each slide was recorded, and compared with that of the controls. This was done by firstly establishing an average control APA score for the specimen in question by dividing the sum of APA scores for the two control slides by two. The sum of the APA scores for each test slide was then multiplied by 100, and divided by the average control score. This value was then subtracted from 100 to provide a percentage index of inhibition for each test slide where 100% represented a complete lack of APA, and 0% indicated no inhibition.

Quantification of APA in Rat Lymph Node Blood Vessels

Blood vessels in rat lymph nodes were generally found to have more intense APA when compared with other tissues. Consequently, it was decided to use lymph node tissues to perform the quantitative assessment of rat endothelial APA inhibition. APA inhibition of these vessels was assessed in a similar manner to the blood vessels of human gingival tissues. Zones were selected at random in tissue immediately underlying the lymph node capsule. HEVs were recognised by their large ECs, as well as by the presence of large numbers of lymphocytes in their walls, and were excluded from this study.

Quantification of APA in Rat Kidney and Small Intestinal Tissues

The intensity and distribution of APA throughout sections of kidney tissue was such as to make it possible to clearly distinguish three grades of APA, ranging from 0, no activity present, to 3 (Figure 6.3). A zone was selected which could be identified in all sections made from the tissues of individual animals, in which only

kidney tubular APA was clearly visible. With frequent use of reference slides, an APA score was assigned for this zone for slides treated with inhibitors as well as the two control slides. A percentage inhibition score for each slide was calculated in a similar way to that described for gingival blood vessels, where the individual kidney APA score of each inhibitor treated slide was substituted for total blood vessel APA, and the average control kidney APA score substituted for blood vessel control APA. This provided percentage inhibition scores for kidney tissues comparable to those derived for blood vessels.

The small intestinal mucosa was assessed in the same way as were kidney tissues (Figure 6.3).

APA in Liver Canaliculi and Other Tissues

The intensity of staining of liver canaliculi, and capillaries in muscle and skin as well as in the fibrous connective tissue elements of the liver, kidney and small intestinal tissues was insufficient to allow a reliable quantitative assessment of APA. However, the presence or absence of APA in these sites was noted.

Statistical Procedures

It was decided that a normal distribution of the data could not be assumed, and so non-parametric methods of statistical analysis were applied. Wilkoxons rank sign, and Man-Whitney-U tests were used as required. All p values given were derived using these as two tailed tests (Champion 1970). In Figures 6.4 to 6.6, the average percentage inhibition for each tissue and inhibitor studied is shown. Standard deviations are shown on histograms to give a visual impression of the range and distribution of the final APA percentage inhibition scores observed between specimens. Where no bars are indicated, no inhibition was observed. The absence of standard deviations implies that the percentage inhibition scores for all specimens was the same.

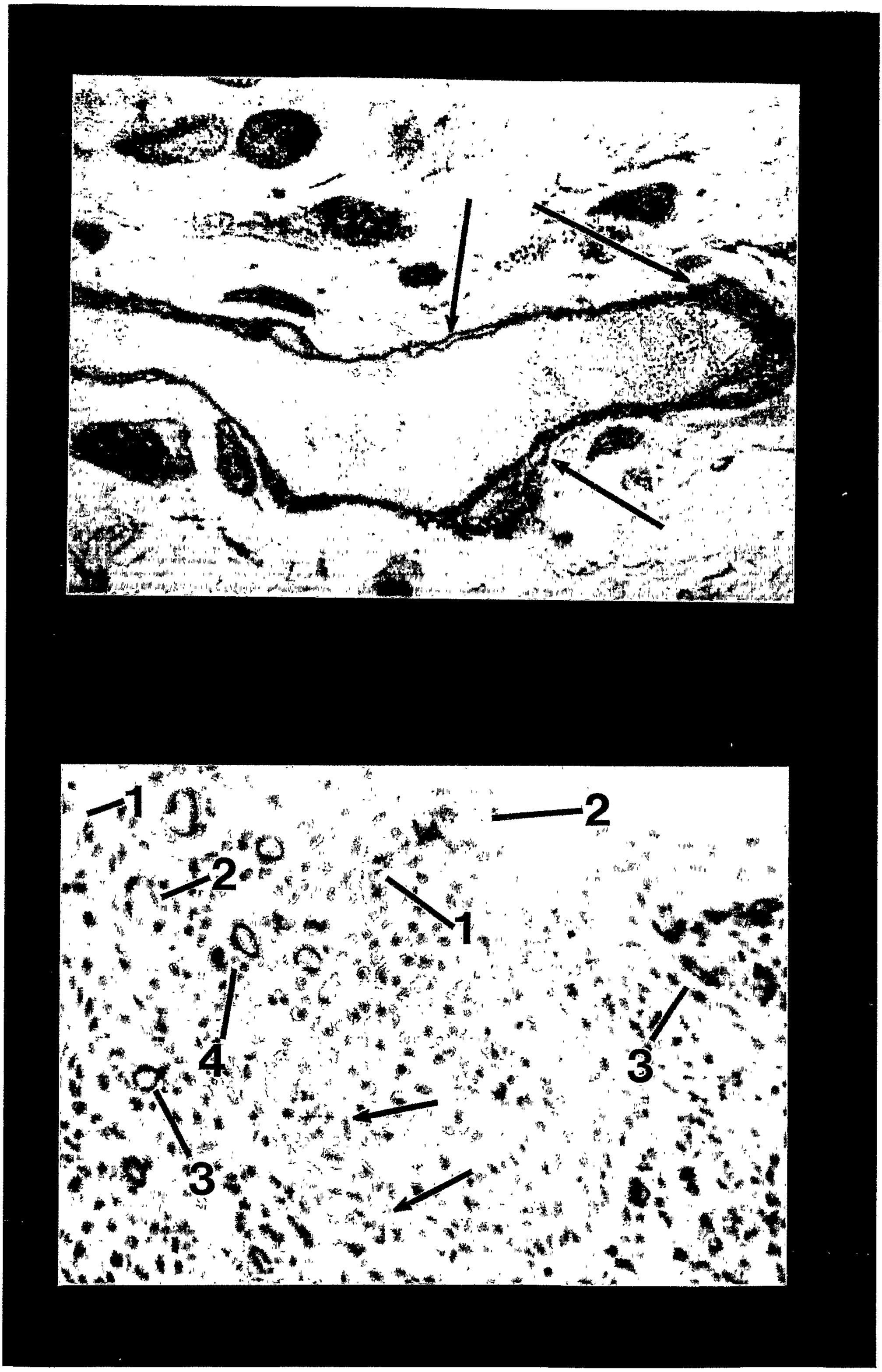

1
2
3
4

Figure 6.1

Photomicrograph of a small blood vessel stained for APA in a gingival biopsy which has been processed by freeze-substitution.

The vascular EC APA is clearly associated with both the luminal and abluminal plasma membranes (arrows). (Alkaline Phosphatase + Haematoxylin X 400)

Figure 6.2

Photomicrograph of gingival blood vessels which have been scored for their APA, in a biopsy which has been processed by freeze-substitution.

Four grades of EC APA could be identified, with the most intense activity scored as grade 4 and the least active vessels recorded as grade 1. Vessels not having APA were recorded as having a score of 0. (Alkaline Phosphatase + Haematoxylin X 312)

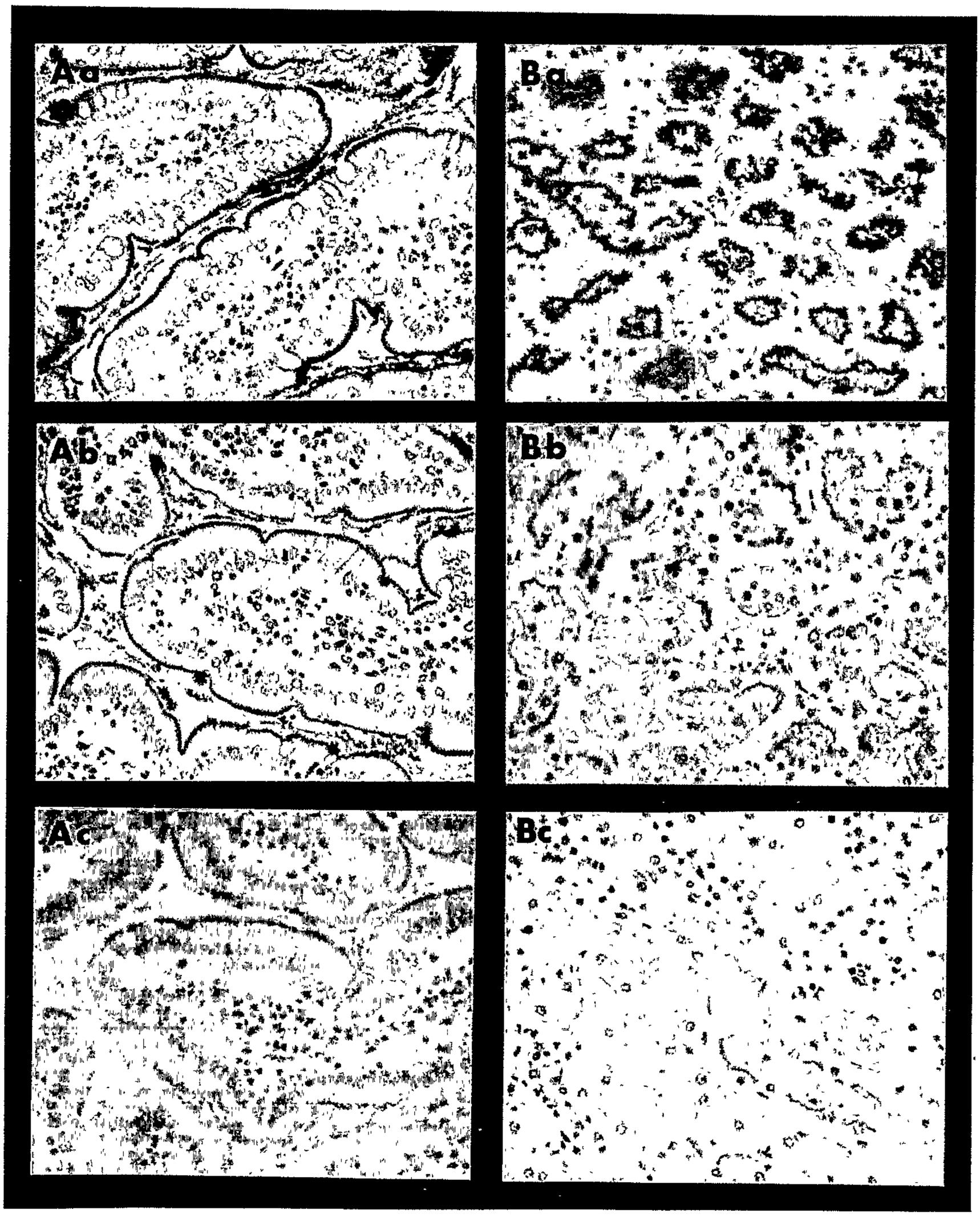

Aa
Ba
Ab
Bb
Ac
Bc

Figure 6.3

Composite photomicrograph of rat small intestinal tissues (A) and rat kidney tissues (B) which have been processed by freeze-substitution and stained for APA both without and in the presence of inhibitors of APA. (Alkaline Phosphatase + Haematoxylin X 200)

It was possible to distinguish 3 grades of APA in sections of these tissues.

Grade 3 (a) was the most intense and was seen in control sections as well as in test samples which were not significantly affected by inhibitors.

Grade 2 (b) and grade 1 (c) were evidence of clearly diminished APA. The absence of APA was recorded as grade 0.

RESULTS

Human Gingival Blood Vessels

Considerable variation in the APA score of individual vessels was seen in both control and inhibited slides. However, despite this variability, when the summated APA scores of different patients were compared, inhibited slides showed a consistent relative inhibition in relation to controls.

As summarised in Figure 6.4, considerable differences in the percentage of inhibition were observed between different inhibitors. Statistically significant differences were found between all inhibitors which varied by more than 10 percentage units of inhibition ($p < 0.03$) except for between EDTA and sodium arsenate for which no significant difference was demonstrated.

Rat Lymph Node Blood Vessels

The inhibitor profile of rat lymph node blood vessels paralleled that of human gingival blood vessels (Figure 6.4), with significant differences being observed between inhibitors varying by more than 20 percentage points of inhibition ($p < 0.05$). No statistically significant differences were found between human gingival and rat lymph node endothelial APA percentage inhibition scores for the same inhibitors (Table 6.1).

Blood Vessels of Other Rat Tissues and Bile Canaliculi

Where inhibition of kidney tubular and lymph node endothelial APA was strong or complete, APA activity was either weak or absent in the blood vessels of skin, muscle, smooth muscle and of the connective tissue elements of the liver, kidney and small intestine. Also APA in the bile canaliculi was seen to follow an identical pattern of inhibition to these structures. The liver sinusoidal and kidney glomerular endothelial vessels were consistently negative for APA, with the exception of occasional short segments of glomerular capillaries.

Rat Kidney Tubular AP

The inhibitor profile of rat kidney tubular APA was similar to that of rat lymph node blood vessels (Figure 6.5). Significant differences were found between inhibitors varying by more than 30 percentage points of inhibition ($p < 0.05$), with the exception of lysine hydrochloride and EDTA, where no significant difference could be shown.

No significant differences were found between the inhibitor profiles of kidney tubular AP and that of lymph node blood vessels, with the exception of levamisole ($p < 0.05$) (Table 6.1).

Rat Small Intestinal AP

For inhibitors of small intestinal AP, significant differences were found between agents varying by more than 33 percentage points of inhibition ($p < 0.05$) (Figure 6.6).

The inhibitor profile of small intestinal AP differs markedly from that of lymph node blood vessels, inhibition being significantly different for: mercuric chloride, L-leucyl-glycyl-glycine, beryllium sulphate, EDTA, lysine hydrochloride, sodium arsenate and levamisole ($p < 0.05$). This suggests that small intestinal AP is different from the kidney and vascular forms of the enzyme (Table 6.1).

In Figure 6.7, the pattern of inhibition of APA in human gingival and rat lymph node ECs is clearly seen to parallel that of the kidney isoenzyme. In contrast to this is the clear difference in the pattern of inhibition of the small intestinal enzyme (Figures 6.7D1 and 6.7D2).

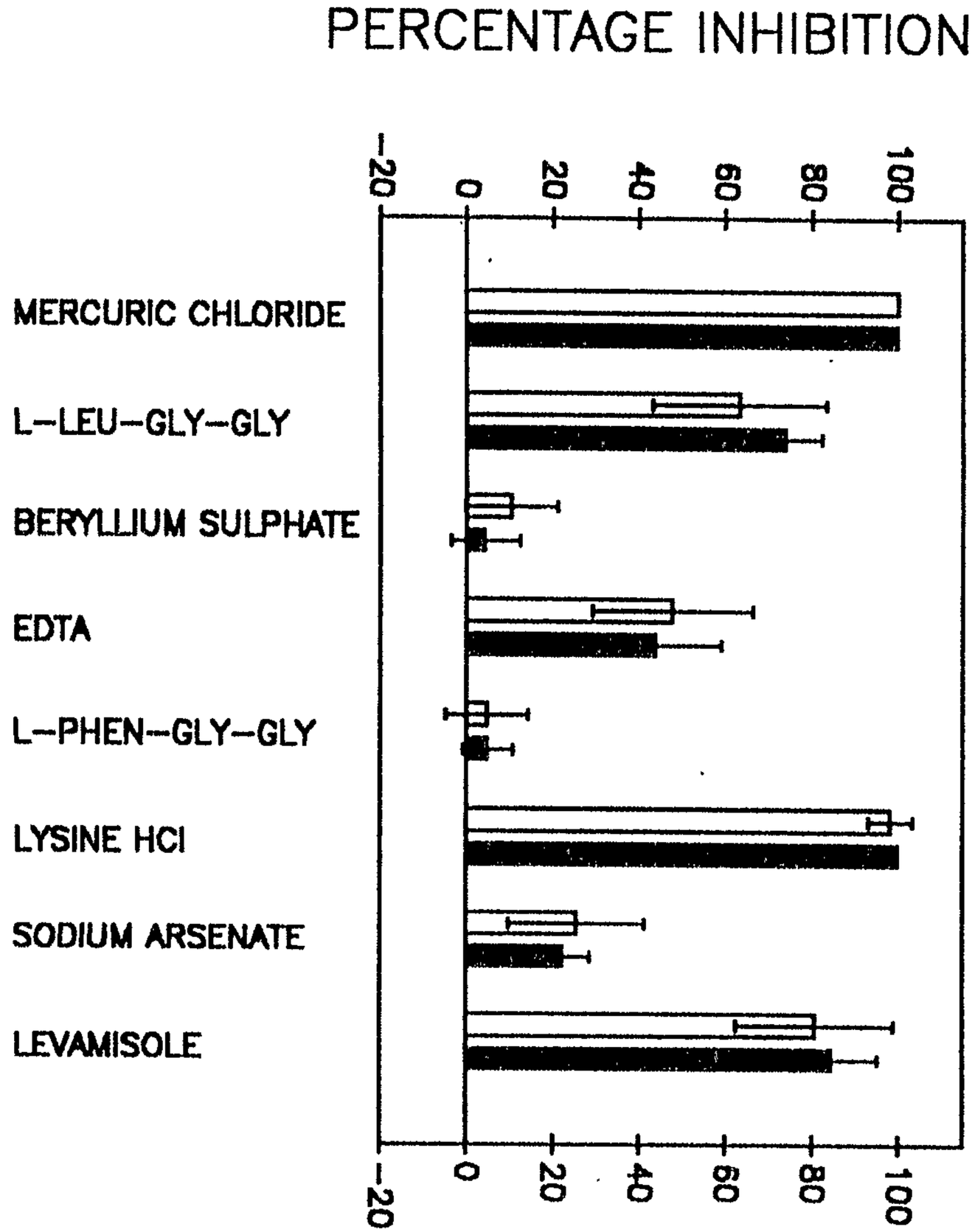

PERCENTAGE INHIBITION
-20
0
20
40
60
80
100
MERCURIC CHLORIDE
L-LEU-GLY-GLY
BERYLLIUM SULPHATE
EDTA
L-PHEN-GLY-GLY
LYSINE HCl
SODIUM ARSENATE
LEVAMISOLE

Figure 6.4

Percentage inhibition of human gingival endothelial AP (open bars), as compared with that of rat lymph node endothelial AP (filled bars) for all inhibitors studied.

Amongst gingival blood vessels, statistically significant differences were found between all inhibitors which varied by more than 10 percentage units of inhibition ($p < 0.03$), except for between EDTA and sodium arsenate for which no significant difference was demonstrated.

For rat lymph node blood vessels significant differences were observed between inhibitors varying by more than 20 percentage points of inhibition ($p < 0.05$).

No statistically significant differences were found between human gingival and rat lymph node endothelial APA percentage inhibition scores for the same inhibitors (Table 6.1).

ECs from both species and sites have the same pattern of inhibition.

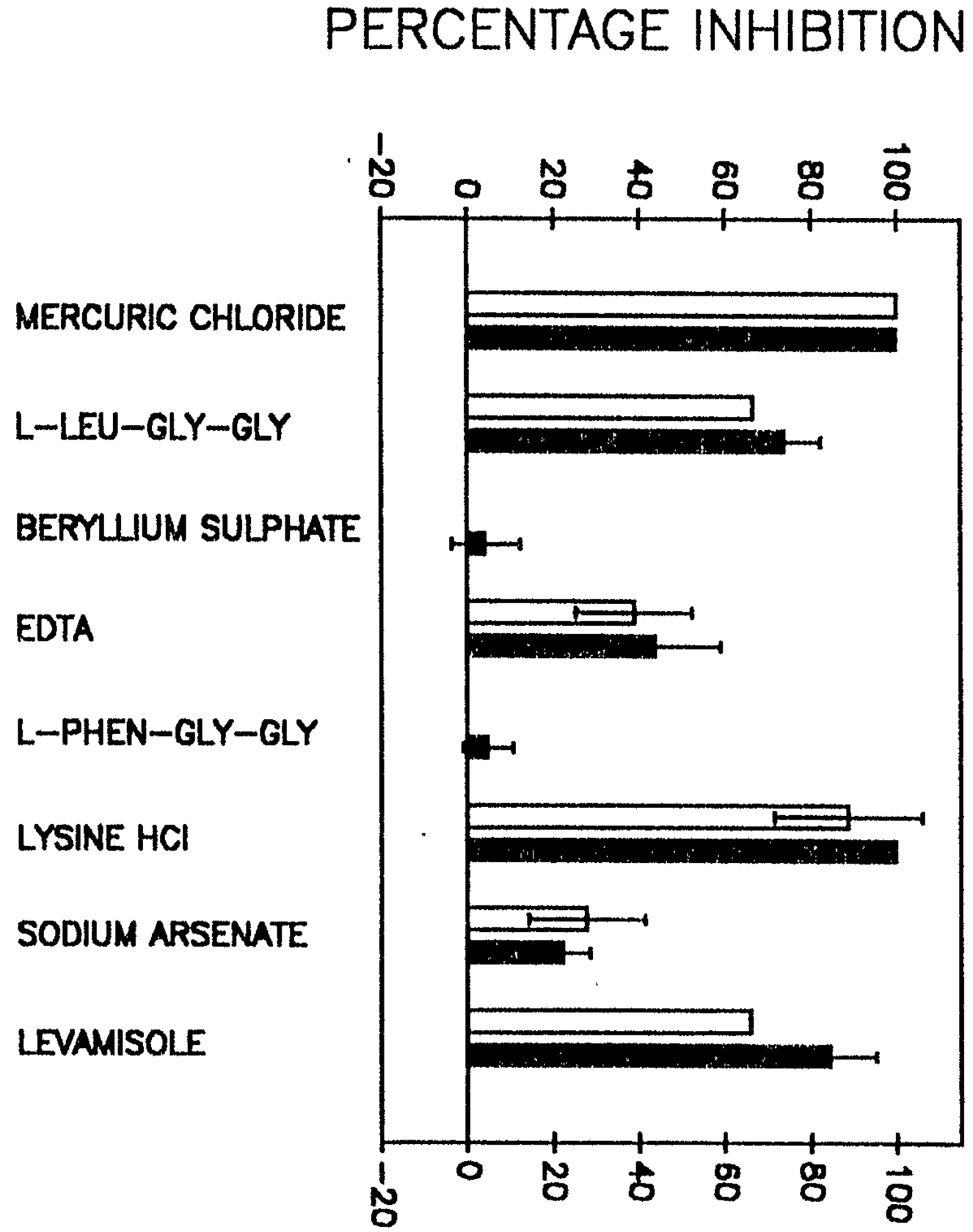

PERCENTAGE INHIBITION
-20
0
20
40
60
80
100
MERCURIC CHLORIDE
L-LEU-GLY-GLY
BERYLLIUM SULPHATE
EDTA
L-PHEN-GLY-GLY
LYSINE HCl
SODIUM ARSENATE
LEVAMISOLE

Figure 6.5

Percentage inhibition of rat kidney tubule AP (open bars), as compared with that of rat lymph node endothelial AP (filled bars) for all inhibitors studied.

The inhibitor profile of rat kidney tubular APA was similar to that of rat lymph node blood vessels. Significant differences were found between inhibitors varying by more than 30 percentage points of inhibition ($p < 0.05$), with the exception of lysine hydrochloride and EDTA, between which no significant difference could be shown.

No significant differences were found between the inhibitor profiles of kidney tubular AP, and that of lymph node blood vessels, with the exception of levamisole ($p < 0.05$) (Table 6.1).

Endothelial and kidney tubular inhibition patterns are similar.

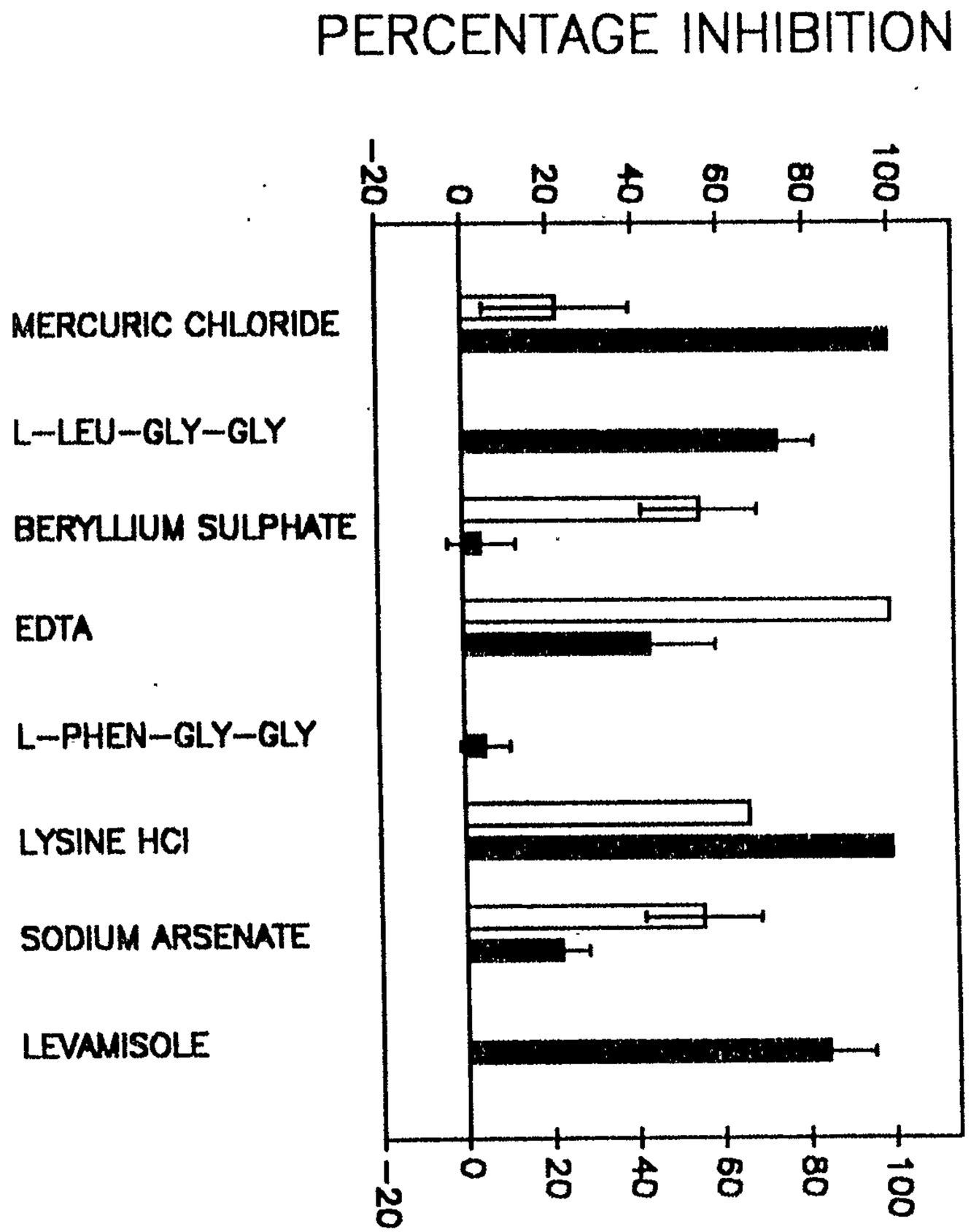

PERCENTAGE INHIBITION
-20
0
20
40
60
80
100
MERCURIC CHLORIDE
L--LEU--GLY--GLY
BERYLLIUM SULPHATE
EDTA
L--PHEN--GLY--GLY
LYSINE HCl
SODIUM ARSENATE
LEVAMISOLE
-20
0
20
40
60
80
100

Figure 6.6

Percentage inhibition of rat small intestinal AP (open bars), as compared with that of rat lymph node endothelial AP (filled bars) for all inhibitors studied.

For inhibitors of small intestinal AP, significant differences were found between agents varying by more than 33 percentage points of inhibition ($p < 0.05$) (Figure 6.4).

The inhibitor profile of small intestinal AP differs markedly from that of lymph node blood vessels, inhibition being significantly different for: mercuric chloride, L-leucyl-glycyl-glycine, beryllium sulphate, EDTA, lysine hydrochloride, sodium arsenate and levamisole ($p < 0.05$). This suggests that small intestinal AP is different from the kidney and vascular forms of the enzyme (Table 6.1).

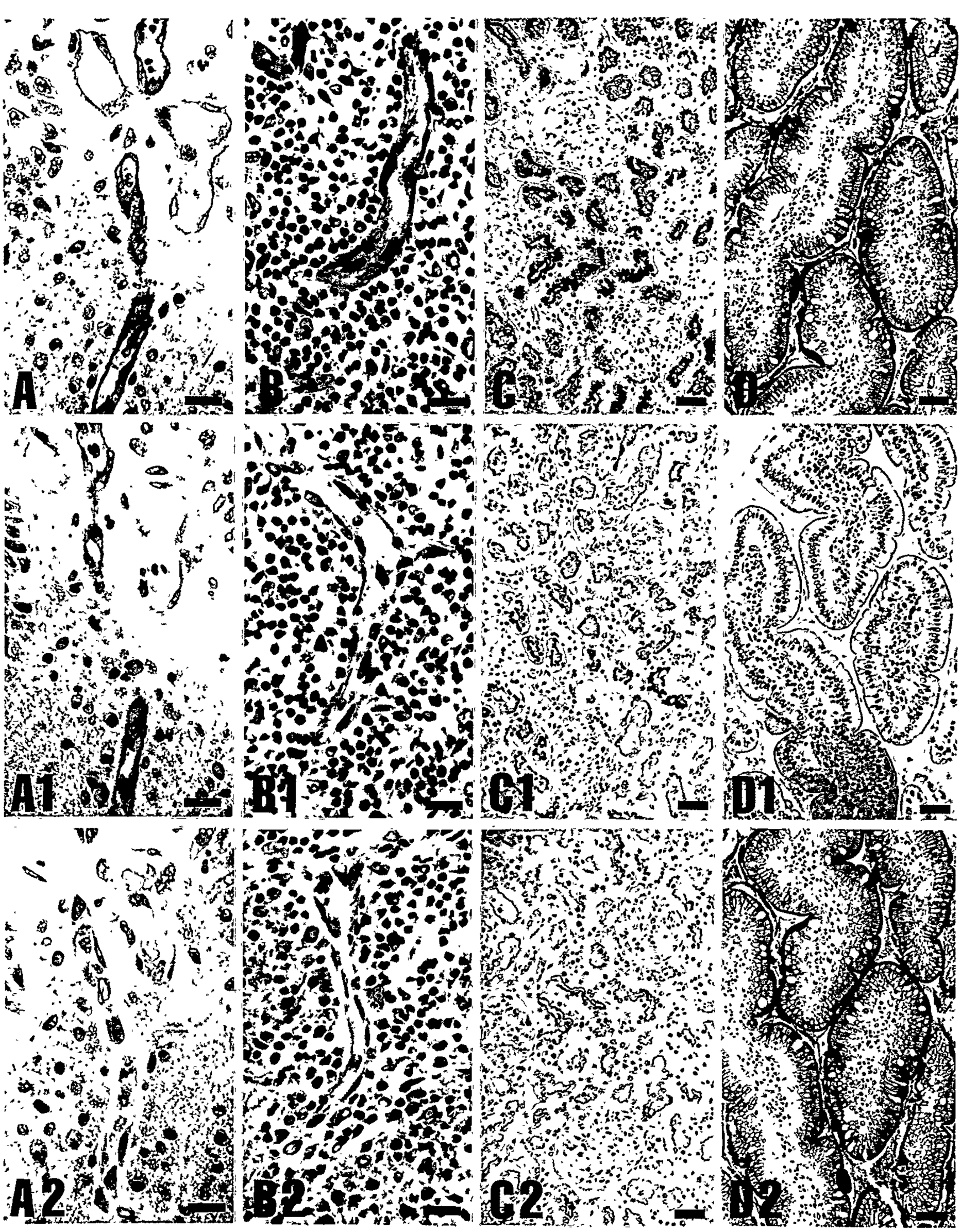

A
B
C
D
A1
B1
C1
D1
A2
B2
C2
D2

Figure 6.7

Photomicrographs comparing control sections of human gingival blood vessels (A), rat lymph node blood vessels (B), rat kidney tubules (C) and rat small intestine (D), with the same tissues when co-incubated with EDTA (A1), (B1), (C1), (D1) and levamisole (A2) (B2) (C2) (D2).

Note the sensitivity to EDTA and resistance to levamisole of small intestinal APA (D1,D2), as compared with APA in other tissues. From this set of photomicrographs, the endothelial isoenzyme of AP appears to be related to that seen in the kidney. Bar = 20 μm for A&B; 30 μm for C&D

Table 6.1

A table comparing the significance of relative differences in percentage inhibition of APA for human gingival blood vessels, kidney tubular epithelium and small intestinal epithelium, relative to lymph node blood vessel inhibition for each inhibitor tested. N signifies that no significant difference was found, whilst Y represents that significance of $P < 0.05$ was demonstrable. * implies a probable type one statistical error.

	Mercuric Chloride	L-Leu-Gly-Gl	Beryllium Sulphate	EDTA Gly	L-Phen-Gly-HCl	Lysine Arsenate	Sodium	Levamisole
Human Gingival Vessels	N	N	N	N	N	N	N	N
Rat Kidney Tubules	N	N	N	N	N	N	N	Y*
Rat Small Intestine	Y	Y	Y	Y	N	Y	Y	Y

DISCUSSION

Identification of the LBK Isoenzyme of AP in Endothelial Cells

The method described in this chapter allows the quantitative comparison of enzyme inhibition without the use of complex photometric or image analysis equipment. Statistical tests can be applied to the data providing a measurable level of confidence in the conclusions drawn from such studies. Clear discrimination between the LBK isoenzyme, and that of small intestine was made in this study. This is only possible because of the reproducible section thickness and good enzyme preservation resulting from the use of freeze-substitution and plastic embedding techniques. This approach is unlikely to be successful using cryostat or standard paraffin embedding techniques, as there is less control of section thickness, with the result that direct comparisons of enzyme activities are difficult to interpret. Also, considerable damage to enzyme activity is a likely consequence of standard fixation regimes. The degree of inhibition of APA in freeze-substituted sections was often different from that expected from the literature. Levamisole for example, at the concentration employed in this study has been reported to cause complete inhibition of the LBK isoenzyme in formalin and glutaraldehyde fixed tissues (Borgers 1973), yet gave incomplete inhibition of APA in freeze-substituted tissues. This suggests that freeze-substitution gives good preservation of APA when compared with fixed tissues. The identification of statistically significant differences in the percentage inhibition scores for different inhibitors in the same tissues, illustrates the sensitivity of the technique.

The statistical evidence grouping endothelial APA in both man and rat with that of the rat kidney suggests that endothelial AP is the same family in both species studied, and that it is the LBK isoenzyme. The statistically significant difference in the result for levamisole between kidney and rat endothelial APA is interpreted as a type 1 statistical error, resulting from the large number of tests performed (Champion 1970). It is most likely that a larger sample size would show this difference to be artifactual. The conclusion that the endothelial isoenzyme might represent a variant of the LBK isoenzyme as defined on the basis of its inhibition by levamisole is remote in light of the identification of a single genetic locus for this isoenzyme (Weis *et al.* 1988a). Consequently, it seems reasonable to discount this apparent difference between the kidney and endothelial isoenzymes as being artifactual. A levamisole resistant APA associated with intracellular granules, has been reported in rat liver sinusoidal ECs (Borgers 1973). The absence of APA in liver sinusoidal endothelium

in this study may be the result of the use of a different substrate for detection of APA.

Since rats do not have the placental isoenzyme, and because human placental tissue was unavailable for this study, the possibility that the endothelial isoenzyme is the placental form could not be directly investigated. However, to counter this potential problem, the placental AP inhibitors L-Phen-Gly-Gly, and L-Leu-Gly-Gly were included in the study. These two inhibitors are reported to strongly inhibit the placental isoenzyme, while L-Leu-Gly-Gly is reported to give moderate inhibition, and L-Phen-Gly-Gly no inhibition of the LBK isoenzyme. Also, the intestinal form is resistant to these inhibitors (Mulivor *et al.* 1978). The pattern of inhibition of endothelial and kidney tubular APA for these inhibitors is identical to that predicted in the literature for the LBK isoenzyme. The response of intestinal APA to these inhibitors is also that which would be expected from the literature. These observations give confidence in the conclusion that the endothelial isoenzyme is not the placental form.

Minor variations in thermostability and molecular weight reported for the LBK isoenzyme obtained from different tissues are thought to result from differences in glycosylation, rather than to the presence of differing amino acid sequences (Moss and Whitaker 1985). This is supported by genetic evidence identifying the LBK isoenzyme as a single copy gene (Weis *et al.* 1988a). The identical inhibitor profiles of endothelial and kidney APA indicate that the same protein is responsible for APA in both sites.

Microvascular ECs in many tissues have been described as having APA (McCombe *et al.* 1979). Yet despite the broad distribution of this enzyme in contact with blood, the microvasculature has not been proposed as a potential source of serum AP. In light of the central role played by blood vessels in disease processes, it is possible that some of the alterations in serum APA used in clinical diagnosis could reflect vascular changes in pathology. This has special relevance for the diagnosis of Paget's disease, where there is a concomitant increase in the size of the bony vasculature and in serum levels of the LBK isoenzyme, in association with bone formation and destruction.

Alkaline Phosphatase as a Potential Regulatory Molecule

The negative association for APA and leukocyte trafficking in the HEVs of lymphoid tissues and chronic inflammatory sites (discussed in Chapter 5), suggests that AP may play a role in the down regulation of specific receptors responsible for

leukocyte docking.

In support of this contention, endothelial APA is reduced in vitro in response to IL-1 (Mulkins and Allison 1987), where this cytokine is known to increase the binding of lymphocytes to endothelial monolayers (Cavender *et al.* 1986).

In relation to the putative mechanism by which AP could alter receptor function, it is accepted that phosphorylation has an important role in the allosteric control of enzymes and receptors (Sprang *et al.* 1988, Shenolikar 1988). While the function of AP has not yet been determined, there is the potential for allosteric control of receptors by the addition and removal of phosphate groups. AP is known to be capable of cleaving phosphate groups from a wide range of substrates including phosphoproteins, so that the activation or inactivation of phosphorylated cell surface receptors would seem possible by the action of AP. It is of interest to note that the mouse L cell glucocorticoid receptor is phosphorylated, and that alkaline phosphatase is capable of cleaving this receptor phosphate (Dalman *et al.* 1988). Although this is not likely to be of any significance in vivo, since the glucocorticoid receptor is intracellular, it is possible that this event is typical of the role of alkaline phosphatase on the surface of cells.

The potential inactivation of leukocyte docking molecules by AP could be an example of a mode of receptor control which has not been investigated. Where phosphorylation has been shown to affect the activity of receptor proteins, it has generally resulted in receptor inactivation (Shenolikar 1988), whereas it is suggested that leukocyte adhesion molecules may be activated by phosphorylation. In this sense, leukocyte docking molecules might be more representative of the intracellular signal transduction proteins, which seem to be activated by phosphorylation (Shenolikar 1988).

It is possible that leukocyte docking proteins are related to the surface proteins responsible for intercellular adhesion in other cells. The association between the intercellular adhesion protein ICAM 1 and lymphocyte adhesion to EC cultures suggests that this might be the case (Dustin and Springer 1988, Boyd *et al.* 1988). From this perspective, if AP plays a role in the control of such adhesion proteins, the presence of AP in cells of embryonic, healing and malignant tissues could be partially explained on the basis of the control of these adhesion molecules, which are known to be important in development (Edelman 1988). In support of the suggestion that normal adhesion molecules may be modulated by AP, is the observation that normal cells have phosphotyrosyl proteins concentrated at intercellular junctions (Maher *et al.* 1985), and that a major fibroblast collagen binding protein is

phosphorylated (Nataga and Yamada 1986).

It could be postulated that the efficiency of receptor regulatory molecules, is enhanced by mechanisms which increase access of the regulatory molecules to their cell surface associated ligands. High lateral mobility in the cell membrane may be an example of such a mechanism. In view of this, the observation that AP has a very high lateral mobility in the cell membrane (Noda *et al.* 1987) supports the idea that AP may play a role in the control of cell surface receptors. This high rate of lateral mobility seems to result from the anchorage of AP by phosphatidylinositol to the membrane surface.

As discussed earlier, the primordial role of AP may be in the acquisition of phosphate by the organism. From an evolutionary standpoint, the role of AP could have readily expanded from one of simply harvesting phosphate from the environment by non-specifically cleaving phosphate groups from phosphorylated compounds, to one of altering phosphorylated proteins on the cell surface.

The appearance of new cell surface proteins would have accompanied the emergence of multicellular organisms, and the complex molecular apparatus required to control cellular interactions. Evolution of phosphorylated molecules, regulated by AP, towards new functional roles would not necessitate changes in AP. It is conceivable that the evolution of new roles for AP by the evolution of phosphorylated ligands, without alteration of the enzyme itself, could help to explain the wide distribution of AP throughout tissues with apparently unrelated functions. The ubiquitous distribution of AP amongst cells undergoing critical differentiation steps in embryonic tissues, wound healing and tissue calcification provides circumstantial evidence in support of a basic regulatory role for AP. This applies particularly to the LBK isoenzyme which is found in such diverse sites as bone, kidney tubular epithelial cells, ECs, liver canalicular cells, proliferating fibroblasts and PMNs, as well as in the placenta of all mammals other than the higher primates.

Phosphorylated surface proteins are known to be important in the transport of many ions and compounds across cell membranes (Taborsky 1974) and the possible role of AP in controlling such transport proteins as well as proteins involved with aspects of cell proliferation and differentiation should be considered.

Endothelial Cell Cultures as a Model to Investigate the Role of AP

The vascular endothelium provides an ideal model for elucidating the physiological role of AP, since it is possible to modify the expression of AP on ECs in vitro (Mulkins and Allison 1987), thereby allowing ready examination of changes in both the leukocyte binding properties of these cells and the transport of substances across cultured cell monolayers. The positive identification of the LBK isoenzyme in ECs is essential information for the further investigation of the role of this enzyme. This has particular relevance to the question of identifying a convenient yet credible source of the enzyme for use in experimentation. The kidney would seem to be a rich source of the appropriate isoenzyme for such studies. An approach to this work is discussed in Chapter 8.

CHAPTER 7

ISOLATION AND CULTURE OF HIGH ENDOTHELIAL CELLS

INTRODUCTION AND LITERATURE REVIEW

Two principle questions requiring further investigation, are raised by the observations described in Chapters 3, 5 and 6. Firstly, the synthetic potential of HECs in the production of inflammatory cytokines must be addressed. Also, the possibility that AP may regulate the activity of some leukocyte adhesion molecules, demands further study. In order to investigate both of these questions in-vitro, a culture model of HECs must be established.

Microvascular ECs have been cultured from many tissues, including: human foreskin, spleen, adipose tissue and adrenal glands; bovine adrenal glands and retinal tissues; rat heart, lung, brain, kidney, and adrenal and adipose tissues; and rabbit marginal ear vessels (Jarrel *et al.* 1986, Folkman *et al.* 1979, Panula *et al.* 1978, Wagner and Matthews 1975, Davison *et al.* 1980a&b). These cultures are derived primarily from capillary ECs, and presumably reflect the specialised behaviour of capillary microvessels in-vivo.

PCV ECs are distinct from other microvascular ECs, in that they bind and allow extravasation of emigrating leukocytes, and are also responsive to agents causing vascular leakage in the earliest stages of the inflammatory response (Majno *et al.* 1961). Since the formation of both the fluid and cellular infiltrates are key events in the inflammatory response, PCV ECs should be used in the study these phenomena in-vitro to provide a physiologically relevant culture model. A difficulty with this, is that the number of PCV ECs relative to other microvascular cells is quite small. Also, methods for the separation of PCV ECs from their more plentiful fellows are not easily established. An exception to this, however, is found in tissues containing large numbers of HECs. As was seen in Chapter 5, the lumens of HEVs are lined by more ECs than are other PCVs lacking the specialised phenotype. In

addition, HECs have functional, antigenic and biochemical markers, creating the potential to identify and thus separate these cells from other microvascular ECs.

The HEC phenotype is known to be unstable in-vivo, in that when lymph nodes are deprived of their afferent lymph supply, HEVs undergo phenotypic change so that the ECs become flattened. This change is rapid, and is reversed by antigenic stimulation of the node (Hendricks and Estermans 1983). From this, it is likely that HECs, once isolated from their normal micro-environment, would quickly lose their activated appearance by undergoing a transition to cells with little functional resemblance to HECs. For this reason, any culture system for HECs should ideally use primary cultures of the cells at confluence, since it may be difficult to relate the behaviour of second and later generations of cultured HECs to their in-vivo function.

An additional advantage of using primary cultures of HECs, seeded at high density, is that the growth of contaminating cells may be limited by contact inhibition. This would help to maintain relatively high purity of the cultures, without recourse to manual weeding, which is both time consuming and tedious. Such manual weeding is often performed in microvascular endothelial cultures prior to preparing sub-cultures (Marks and Penny 1986, Zetter 1984), but would be impractical where primary cultures are required.

If it is assumed that 1×10^5 cells are required to seed a single $0.28cm^2$ culture well at confluence in a 96 well culture plate, then 1×10^7 purified HECs would be required to fill such a culture plate. The advantage of a multi-well system over the use of conventional culture flasks is that up to 96 separate units of data can be obtained from a single multi-well plate, with relatively few cells.

A survey of the literature revealed that a method for isolation and culture of HECs at high density in primary cultures has not been established. This chapter describes work done towards establishing such HEC cultures. A flow chart in Figure 7.1 provides an over-view of the strategy applied to isolating and culturing HECs.

Figure 7.1

Flow chart of the broad strategy applied in isolation and culture of HECs from rat lymph nodes.

	OBJECTIVE	METHOD USED
1	MAXIMISE THE YIELD OF HECS FROM RAT LYMPH NODES	Antigenic Stimulation
2	DEVELOP A TISSUE DISAGGREGATION PROCEDURE FOR LYMPH NODES WHICH RETAINS HEC VIABILITY	Proteinase Digestion with Mechanical Disaggregation
3	IDENTIFICATION OF HECS IN SINGLE CELL SUSPENSIONS	$^{35}SO_4$ Labelling Factor VIII Ag Alkaline Phosphatase
4	SEPARATION OF HECs FROM MAJOR CONTAMINANTS: MACROPHAGES & CAPILLARY ENDOTHELIAL CELLS	Percoll Gradients Differential Adherence
5	IDENTIFICATION OF SUITABLE ADHESION SUBSTRATE	Fibronectin
6	CHARACTERISATION OF ADHERENT CELLS	$^{35}SO_4$ Labelling Factor VIII Ag Alkaline Phosphatase
7	ATTEMPTS TO REMOVE LYMPHOCYTES	Complement Mediated Lysis Prevention of Lymphocyte Adhesion
8	THE NEED TO DEVELOP BETTER ISOLATION PROCEDURES	Discontinuous Gradients Elutriation

MATERIALS, METHODS AND RESULTS

Materials and Animals

Inbred Wistar Furth rats were obtained from the Blackburn Animal House at the University of Sydney. Collagenase type III was obtained from Worthington while collagenase type Ia was supplied by Sigma. Pronase and monensin were purchased from Calbiochem. DPB and DPB calcium-magnesium supplement was supplied by Oxoid. Percoll and density marker beads were obtained from Pharmacia. $Na^{35}SO_4$ was purchased from New England Nuclear, and anti-FVIII serum was supplied by Dakopats. Monoclonal antibodies were obtained from Serotec. Medium 199, FCS, trypan blue, antibiotics, 7.5% Na bicarbonate and guinea pig complement were supplied by CSL. Falkon tissue culture flasks and Miles 8 well plastic tissue culture slides were used. Extracellular matrix was purchased from Woods Scientific. Sterile pyrogen free plastic containers were supplied by Sterilin, while pyrogen free tissue culture water was purchased from Travenol. All other reagents were supplied by Sigma.

Rat Lymph Nodes as a Source of HECs

As discussed in earlier chapters, HECs in the post-capillary venules of lymph nodes have been extensively studied and characterised. Although a Mab (MECA-79) for HECs in mouse peripheral lymph nodes has been available for two years (Streeter *et al.* 1988), there are no reports of the use of this marker to isolate and culture HECs by antibody dependent separation techniques. It seems reasonable to assume that this reflects difficulties in obtaining sufficient numbers of cells from mouse lymph nodes. Mabs against mouse Peyer's patch HECs have been available for a similar amount of time (Streeter *et al.* 1988), but also have not been reported for the isolation of HECs. To overcome these problems, it was decided that rat lymph nodes would be used as a rich and accessible source of HECs for isolation and culture. Inbred Wistar Furth rats of both sexes and between 10 and 25 weeks old were used throughout this study.

Culture of High Endothelial Cells

Removal of Lymph Nodes from Rats

Lymph nodes from several sites were pooled to obtain a sufficient number of cells. To obtain sterile pooled lymph nodes, rats were shaved and all dissection was carried out in a laminar flow hood after soaking the animal for two to three minutes in absolute alcohol. The skin was reflected and held in position with skewers. Lymph nodes were dissected from their connective tissue attachments and pooled in DPB on ice. Animals were sacrificed by chloroform inhalation. The lymph nodes collected from rats were: submandibular, cervical, deep cervical, axillary, accessory axillary, popliteal, inner iliaci mediales, and nodes from the mesenteric lymph node chain. The pooled lymph nodes were weighed wet, in a sterile disposable petri-dish before further processing.

Stimulation of Rat Lymph Nodes

It is reported that antigenic stimulation of tissues drained by lymph nodes results in an increase in the number and extent of lymph node HECs proportional to the size of the lymph node (Anderson *et al.* 1975). On this basis, rat lymph nodes were stimulated with Freund's Complete Adjuvant in order to increase the number of HECs potentially available for isolation and culture.

Procedure

A 50 % emulsion of Freund's Complete Adjuvant in DPB was injected into multiple sites with 0.2 ml of emulsion deposited into each site. The emulsion had a final concentration of mycobacteria of 2 mg/ml. The sites injected were: the hind foot pads, the loose soft tissues of the upper forelimb, and the soft tissues immediately beneath the mandible. These sites were selected in order to stimulate the, popliteal, axillary, accessory axillary, submandibular, cervical and deep cervical lymph nodes, draining the injection sites. Injections were performed under chloroform anaesthesia. The animals tolerated the treatment well, and were able to feed and drink normally following treatment. Lymph nodes were harvested from the animals 7 days after stimulation.

Improvement in the Yield of Lymph Nodes with Stimulation

The total wet weight of lymph nodes pooled from a single unstimulated rat varied between animals, and ranged from 0.3 to 0.5 g per rat.

Injection of rats in multiple sites gave a significant increase in the weight of lymph nodes harvested from individual animals. The treatment resulted in a range of weights for rat lymph nodes of from 0.7 to 1.45 g per rat, with a mean lymph

node weight of 0.98 ± 0.22 g. Surprisingly, a strong correlation was not found between the sex and size of the rat, and the total weight of the lymph node mass. For example, a small female rat of 158 g weight yielded 0.78 g of lymph nodes tissue, while a large male rat weighing 350 g produced only 0.93 g of lymph node tissue, and a smaller 256 g male rat was the source of 1.38 g of lymph node tissue.

Disaggregation of Lymph Nodes to a Single Cell Suspension

A necessary prelude to the isolation of HECs is the establishment of a method for the preparation of a single cell suspension from lymph nodes. This was done using a combination of proteolytic and mechanical tissue disaggregation methods. The objective of the tissue digestion procedure was to provide the highest possible yield of viable lymph node cells in a single cell suspension, where viability was determined by exclusion of Trypan blue at 0.2%.

As an initial step, pooled wet lymph nodes were diced with sterile razor blades to form a fine slurry. Several proteinase digestion and mechanical disaggregation procedures were tried before a reliable method was found. In these experiments, the slurry was divided into quantities of equal weight, and subjected to varying treatments. The proteinases used were: pronase (7700 u/mg), trypsin type III (10200 BAEE U/mg), collagenase type III (102u/mg), and collagenase type 1A. The tissue slurry was suspended in 5 ml of DPB and incubated using a variety of digestion procedures. Where collagenase was used, Ca and Mg were included with the DPB solution, however, where trypsin or pronase were used in the absence of collagenase, these bivalent cations were not provided.

Early Experiments with Tissue Disaggregation

The tissue slurry was suspended in various enzyme preparations, and incubated for up to 2 hours at 37°C in a shaker incubator. Trypsin, pronase and collagenase types Ia and III varied from 0.02% to 0.4% (Marks *et al.* 1985, Folkman *et al.* 1979). Complete disaggregation of the tissues was not attained during a two hour incubation with any of these enzymes. Prolonged exposure to enzyme preparations gave low yields of viable cells. Overnight incubation at 4°C did not improve the viability or yield of cells, which did not exceed 1 X 10^8 cells from 1 gram of wet lymph node tissue.

Serum contains a number of protease inhibitors including α1-anti-trypsin, and α2-macroglobulin. These inhibitors have a wide range of specificity, and are effective against collagenase, as well as trypsin (Bennington *et al.* 1984). It was found that

cell viability was improved by collecting enzyme digest supernatants at frequent intervals and storing the cell rich enzyme preparations on ice with foetal calf serum (FCS). When tissues were treated with 0.4% collagenase type III for 30 minutes at 37°C, and the cell rich supernatant stored with 10 % FCS, complete tissue disaggregation did not occur. However, repeated rounds of digestion with this preparation in combination with mechanical disaggregation, by forcing tissues through a wire grid, gave cell yields of up to 7 X 10^8 cells/gram of wet lymph node tissue.

Mechanical disaggregation by forcing partially digested tissues through a wire mesh resulted in loss of tissue on the screen. It was thought that this would limit the potential yield of HECs, since stromal components would tend to be retained by the wire screen. The inclusion of trypsin at concentrations ranging from 0.01 to 0.2% with the collagenase type III solution gave complete tissue disaggregation, avoiding the necessity for disaggregation of tissues on the wire grid. Cell viability increased from 5.5 x 10^8 cells per gram of wet lymph node tissue at 0.2% trypsin, to 9.6 x 10^8 cells per gram of wet lymph node tissue at 0.01% trypsin.

The viability of capillary ECs could be determined in these preparations by observing the ability of capillary segments to exclude Trypan Blue. Although this did not give a direct indication of the viability of HECs, it was assumed that capillary ECs and HECs would have a similar capacity to survive the digestion procedure. Capillary segments were usually viable, however, the number of segments seen, and the viability of those found, varied between preparations. This suggested that although this approach gave higher cell yields than earlier methods, that EC viability was sub-optimal.

Andrews *et al.* (1980), reported that glucose was required for optimal uptake of the $^{35}SO_4$ label by HECs in-vitro. In light of this information, as well as variability in capillary segment viability, it was decided to include glucose in digestion and wash solutions in an attempt to aid HEC viability. To further aid HEC viability, cells freed by the enzyme preparation, were separated from undigested tissue fragments by allowing the tissue fragments to settle out of suspension. The cell rich enzyme solution was then centrifuged, and the resulting cell free enzyme solution was returned to the incompletely digested tissue fragments for a further round of proteolysis. The pelleted cells were stored with FCS. Bovine serum albumin (BSA) was included with the solution used to re-suspend and collect the pelleted cells, to avoid cell clumping. Viability of capillary segments was further aided by reduction in the concentration of collagenase type III to 0.1 %.

Culture of High Endothelial Cells

As described in Chapter 5, HECs incorporate $^{35}SO_4$ as a specific label for these cells in lymph nodes (Freemont 1988, Andrews *et al.* 1980). It is further reported that this label diminishes within thirty minutes of application (Andrews *et al.* 1980). This poses a logistical problem for the use of this label in the identification of HECs in suspension, as much of the label would be lost before manipulation of the cells is complete and the cells fixed. Inclusion of monensin at 10^{-6}M prevented loss of this label from HECs in whole lymph node slices (Chapter 5) as well as in suspension. Inclusion of monensin did not significantly alter the yield of viable cells in lymph node digests.

Final Digestion Procedure

A collagenase/trypsin enzyme preparation (CTEP) gave complete digestion of lymph node tissues, with good cell viability. This preparation consisted of 0.1% of collagenase type III, and 0.01% of trypsin in DPB supplemented with 0.11% glucose, 10^{-6} M monensin and phenol red.

The diced lymph node slurry from a single rat was incubated in 10 ml of CTEP in a 25ml sterile pyrogen free plastic tube. This container was incubated for 45 minutes at 37° C in a shaker incubator, shaking with a frequency of 125 cycles per minute. After 45 minutes, the solution was usually acid as determined by the colour of the phenol red indicator, and 1 to 2 drops of 7.5% sodium bicarbonate solution were added to restore pH to physiological levels. The tissues were then "pummelled" by repeatedly drawing the solution containing digested tissue fragments into a 10 ml syringe through a mixing cannula, and expelling the solution back into the sterile plastic container. This had the effect of mechanically disaggregating the digested tissue fragments, and washing loose otherwise free cells. The solution was then allowed to stand on ice for several minutes, while the remaining tissue fragments settled to the bottom of the container. The supernatant was then removed from the 25ml plastic container, leaving 1 to 2 ml of CTEP with tissue fragments behind. Cells were removed from the supernatant by centrifugation at 1000 rpm at 0° C. The cell free CTEP was then transfered to the container with the remaining tissue fragments and the digestion cycle repeated. In order to achieve complete digestion of lymph node tissue, this cycle was repeated from 4 to 5 times.

Cells pelleted from the CTEP were vortexed and transfered to a separate 25ml sterile pyrogen free plastic container with 3 ml of foetal calf serum with 10^{-6} M monensin. A solution of DPB supplemented with 0.11% glucose, 0.1% BSA and 1 X 10^{-6}M monensin was used to wash the remaining cells from the pellet. These cells

were stored on ice, and added to after each cycle of digestion, so that at the conclusion of the final digestion cycle, a preparation of from 10 to 15 ml of lymph node cells were available in a solution of DPB with glucose, BSA, and monensin with from 20 to 30 % FCS. Using this procedure, it was possible to obtain 6.6 ± 2.9 X 10^8 cells from 1g of wet lymph node tissue.

Identification of HECs

Earlier work described in Chapter 5 indicated that amongst ECs. HECs could be distinguished on the basis of the absence of APA and the uptake of $^{35}SO_4$. It was further found that rat lymph node ECs could be distinguished from other rat lymph node cells using the widely accepted EC marker FVIII. These criteria were used to distinguish HECs from other cells in films of single cell suspensions and subsequently, in cell cultures.

Factor VIII Associated Antigen

The FVIII anti-serum supplied by Calbiochem, used to probe PHyM in Chapter 4 did not label ECs in rat lymph nodes. However, antisera against FVIII supplied by Dakopats gave strong labelling of ECs in lymph node sections which had been processed by freeze-substitution. In this study, 8 μm sections were trypsinised for 10 minutes (Mepham *et al.* 1979), and incubated overnight at 37°C with a 1/10 dilution of the antisera in DPB with 10% FCS. The sections were then washed three times with DPB at 37°C for 20 minutes each, and then incubated for 1 hr with a 1/50 dilution of alkaline phosphatase conjugated goat anti-rabbit serum in DPB with 10% FCS. Slides were then washed three times in DPB for 20 minutes each at 37° C, and stained for alkaline phosphatase activity as described for immuno-histochemistry in Chapter 4 (Figure 7.2).

Films and cultures of cells were fixed with 4% paraformaldehyde in DPB for 3 minutes and stained in a similar fashion to sections of lymph node cells, except that the primary antibody was applied at a concentration of 1/100 for a period of 1 hr at 37° C, and washing with DPB was carried out for 15 minute periods instead of for 20 minutes.

Control sections and films consisted of identically treated specimens which had not been exposed to the primary antiserum (Figure 7.2).

Alkaline Phosphatase

As discussed in the preceding chapters, APA is absent in lymph node HECs, but is present in capillary ECs in these tissues. APA was used as a negative marker

for HECs, and was detected in sections, films and cultures of cells using the method of Higuchi *et al.* 1979, described in previous chapters. Films and cultures of cells were fixed with ice cold methanol for 3 minutes before staining (Figure 7.2).

Radioactive Sulphate

The unique ability of HECs to incorporate large amounts of $^{35}SO_4$ has been reported by others (Freemont 1988, Andrews *et al.* 1980), and was confirmed by autoradiography in Chapter 5. For this reason, it was decided that $^{35}SO_4$ uptake could be used to identify HECs in cell suspensions. This had the advantage of allowing localisation of HECs in films and cultures by autoradiography, as well as in Percoll gradients using a scintillation counter.

Attempts to label HECs with $^{35}SO_4$ following the digestion procedure failed, and may reflect either tryptic digestion of the necessary cell surface apparatus for incorporation of $^{35}SO_4$, or some other toxic effect of the proteinase treatment. Also, it was found that without the ionophore monensin, $^{35}SO_4$ was quickly lost from HECs, leaving little or no label for detection. However, it was possible to label HECs prior to digestion with CTEP, by incubating the diced tissue slurry with 40 μCi of $^{35}SO_4$ in a solution of DPB supplemented with 0.11% glucose, and 10^{-6} M monensin and phenol red at 37° C for 1 hour. This was done, by incubating the tissue slurry in 3 ml of this DPB solution for 1 hr, and then adding 7 ml of DPB with the remaining constituents of the CTEP to achieve the final enzyme concentrations described for tissue digestion. In this way, HECs were exposed to the radioactive label during the entire digestion procedure, as well as to the monensin required for retention of the label. Labelled cells were then washed three times at 4° C in a solution of DPB supplemented with 0.11% glucose, 0.1% BSA and 10^{-6} M monensin. These cells were then stored on ice.

Films of cells and cell cultures were prepared on alcohol cleaned microscope slides and air dried. These were then fixed with 10% acrolein in DPB for 5 minutes and then washed 4 times in DPB prior to autoradiography as described in Chapter 5.

Cultures of cells in tissue culture slides (Miles), were exposed to 40 μCi of $^{35}SO_4$ for 1 hr, and then fixed with 10% acrolein in the same way as described for films of cells.

Control cells were prepared by incubating half of the tissue slurry in identical solutions to those used for test cells, but without the $^{35}SO_4$ label. Radioactivity was detected for individual cells in films by autoradiography in the same way as described

in Chapter 5 (Figure 7.3). Large numbers of HECs could be identified using a ß counter.

The Number of HECs Potentially Available From Cell Suspensions

Cell counts of films of $^{35}SO_4$-labelled lymph node cells indicated that up to 4% of the cells in the whole cell preparation were sulphate bearing. This proportion was consistent with the FVIII and alkaline phosphatase stains, which revealed many large cells which were often FVIII positive, but were always AP negative. With a mean yield of 6.1 X 10^8 lymph node cells using the established digestion protocol, up to 2.4 X 10^7 HECs should be available for isolation and culture from a single animal. With a plating efficiency of 50%, this cell number should be sufficient to seed a 96 well culture plate to confluence. From this, it was decided to proceed with the attempt to isolate and culture HECs from rat lymph nodes.

Initial Separation Procedures

A number of procedures were tried before achieving some degree of cell separation. The approach used at an early stage of the work was based on the assumption that at increasing concentrations of Percoll in gradients, a concentration of Percoll would be reached at which HECs would separate from other contaminating cells. Macrophages, capillary ECs and fibroblasts were considered to be the most important potential contaminants. The approach was to exploit the association between HECs and lymphocytes, so that HECs could be separated in a dense fraction from buoyant macrophages and other large cells. It was also considered that the association between lymphocytes and HECs could be easily broken, and that contaminating lymphocytes could be removed from the HEC cultures.

Use of Percoll Gradients

Standard isotonic Percoll solution (SIP) was prepared as a 90% mixture of Percoll with 10% of a 10 times concentrate of DPB. This was then further diluted into working concentrations of Percoll using DPB supplemented with a number of additives.

Glucose at 0.11% was always included in the diluting solution. Also, since leukocyte adhesion is calcium and magnesium dependent, these divalent cations were included with the diluting solution. In early separations, cell clumping prevented cells from entering the Percoll gradient, however, inclusion of 0.1% BSA in the diluting solution prevented this problem.

Culture of High Endothelial Cells

The composition of the diluting solution that was used to give separation of cells (DS1) was: DPB supplemented with 0.11% glucose, 0.1% BSA, 10^{-6} M monensin with calcium and magnesium.

Application of Cells to the Diluted Percoll Solutions

50 ml of diluted Percoll solutions of a range of SIP concentrations were prepared in 50 ml sterile disposable centrifuge tubes. Tubes containing Percoll solutions were chilled on ice, and then 1 ml of the labelled lymph node cells were layered onto the top of the Percoll solution in each tube. Tubes were then spun at 800g for 20 minutes, and immediately placed on ice.

Sampling Percoll Gradients for Sulphate Bearing Cells

Ten 5 ml aliquots of Percoll were taken from each tube, so that the first 5 ml aliquot represented the top 5 ml of Percoll, and the tenth aliquot the bottom 5 ml of Percoll. From each aliquot of Percoll, three 100 µl volumes of Percoll were sampled and counted in a ß counter. The average number of counts per minute (cpm) for each 5 ml aliquot was recorded as a percentage of 1/50th of the total cpm applied to the centrifuge tube. This provided a means of accurately determining the distribution of $^{35}SO_4$ and presumably HECs throughout the Percoll gradient.

45% SIP in DS1 gave Separation of HECs with Small Lymphocytes

The distribution of $^{35}SO_4$ bearing cells through a series of centrifuge tubes with varying dilutions of SIP is shown in Table 7.1. Density marker beads were often distributed over several fractions. Where this occurred, most of the beads were concentrated in the central fraction. HECs varied greatly in density, with a range of from 1.016 to 1.076 g/ml. However, at least 60% of the radioactivity was associated with cells with a density between 1.062 and 1.076. It is possible that some of the radioactivity associated with cells of very low apparent density was artifactual, reflecting the failure of clumps of cells to enter the gradient. A difficulty with this interpretation is that no such clumps were visible in fractions of low density.

In the percoll preparation consisting of 45% SIP, a dense cell pellet was formed which contained 72% ± 8.5% of the whole cell preparation. Most cells in the films of cells from of this fraction were small lymphocytes. Amongst these small cells, larger cells were found as well as occasional short capillary segments. There were clearly far fewer capillary segments in this dense cell preparation as compared with the starting cell suspension.

When tested for APA, the contaminating capillary segments were found to bear the enzyme, however, the large cells did not have this activity. Also, stains for

FVIII revealed that most of the large cells were FVIII positive as were the short capillary segments (Figure 7.2). Autoradiography indicated that most of the large cells had incorporated the $^{35}SO_4$ label (Figure 7.3). Approximately half of the available HECs appeared to have pelleted with the dense small lymphocyte pellet. It was assumed that this reflected association between HECs and lymphocytes, which were thought to drag HECs into the dense pellet. The presence of occasional sulphate bearing and FVIII positive cells with apparently adherent lymphocytes supported this interpretation. However, true rosettes of HECs were rarely seen, suggesting that HECs separated purely on the basis of cell density and size. This possibility was supported in part by the tendency for there to be more small FVIII positive and sulphate bearing cells in the dense cell preparation than in the unfractionated cell preparation.

Cell Adhesion Substrates

Miles tissue culture wells were pre-treated with a number of different substrates to aid cell adhesion, including fibronectin isolated from rat plasma by the method of Rhuoslati *et al.* (1982), commercially available extracellular matrix and fresh rat serum. These were tested for their ability to aid the binding of capillary segments during primary adhesion.

Fibronectin was applied in a solution of DPB at 20µg/ml for 10 minutes and then washed with DPB three times before plating cells. Similarly, fresh rat serum was applied to wells for 10 minutes before washing three times with DPB and applying cells. Extracellular matrix was applied as a 1/5 dilution with medium 199 overnight at 37°C, and then washed three times with medium 199. Also, a combination of ECM and fibronectin was tried in which wells pre-treated with ECM were subsequently treated with fibronectin.

Fibronectin treatment alone resulted in complete removal of capillary segments, whereas fewer cells were adherent to surfaces exposed to other treatments. Pretreatment of plastic cell culture wells and flasks is widely used in the culture of ECs (Marks *et al.* 1985, Davison *et al.* 1980). Because of this, and the good adhesion of capillary segments to fibronectin coated surfaces, it was decided to use fibronectin treatment routinely for plastic ware for both primary and secondary adhesion cultures.

Primary Adhesion to Remove Contaminating Macrophages and Capillary Segments

As discussed above, the major contaminants of HEC cultures are likely to be macrophages and capillary ECs. Macrophages are reported to be buoyant, and it is likely that most of these cells did not pellet with the small lymphocytes and HECs. However, as an additional measure to remove macrophages which are noted for their adherence properties, cells in the dense cell preparation were allowed to adhere to a cell culture flask that were, pre-treated with fibronectin. Also, contaminating capillary segments were lost during this stage, and formed adherent capillary loops in these primary adhesion cultures.

Dense cells obtained using the 45% SIP preparation were diluted with DS1 without monensin, and were washed three times with DS1 lacking monensin. These cells were then re-suspended in 5 ml of Medium 199 with 10% FCS. This cell suspension was then incubated overnight under 5% CO_2 at 37° C in a 25 cm^2 culture flask. Non-adherent cells were washed from the overnight culture, and the remaining cells resuspended into Medium 199 with 20% FCS to a final concentration of $1X10^8$ cells per ml.

HEC Cultures Contaminated with Adherent Lymphocytes

Cells washed from the primary adhesion cultures were incubated at 37°C under 5%CO_2 in fibronectin-coated Miles tissue culture wells. $2x10^6$ cells were suspended in 200 µl of Medium 199 with 20% FCS supplemented with penicillin/streptomycin were applied per cm^2, and allowed to attach for 24 hrs. The following day, non-adherent cells were removed by gentle washing with DPB supplemented with 0.1%BSA, and then further cultured with medium 199, 20% FCS and penicillin/streptomycin.

Adherent cells in these cultures were composed of FVIII positive, alkaline phosphatase negative cells with large numbers of adherent lymphocytes bound to their surfaces (Figure 7.4). These cells also incorporated $^{35}SO_4$ (Figure 7.5). By these criteria, it was concluded that the adherent cells in the secondary adhesion cultures were HECs.

The morphology of adherent cells in secondary adhesion cultures was fibroblastic, rather than the typical cobble-stone morphology reported for most EC cultures. However, it has been established that ECs in cell culture will adopt a fibroblast like morphology when exposed to some inflammatory cytokines, including g-Inf and IL1 (Montesano *et al.* 1985). The presence of large numbers of contaminating lymphocytes in these cultures could explain the fibroblast like

appearance of these cells.

Importantly, occasional cells in these secondary adhesion cultures expressed slight APA as fine dots of activity. This was interpreted as the first signs of phenotypic reversion from the specialised HEC phenotype.

Attempts to Remove Contaminating Lymphocytes From Secondary Adhesion Cultures

It was clear that the major contaminant of secondary adhesion cultures were lymphocytes, bound to the surface of adherent cells. Apart from being obvious contaminants of the culture system, it was possible that the lymphocytes interfered with HEC adhesion to the culture well surface. To try and remove unwanted lymphocytes, several approaches were tried.

On the assumption that the majority of lymphocytes in preparations are T cells, attempts were made to kill lymphocytes using the rat T cell markers Mabs OX52 and OX44 and complement. The method used was identical to that used by Hedberg and Hunter (1988) to remove thymocytes from whole thymus preparations. Antibody was used at a concentration of 2.5µg/ml of cell preparation, while complement was used at 2% of the final volume. Briefly, cells were incubated with antibody at a concentration of 1 X 10^8 cells/ml on ice for 1hr in DPB with Ca and Mg, and then exposed to guinea pig complement for 30 minutes at 37°C, before counting cells with a haemocytometer. Controls consisted of cells treated with complement only, cells treated with antibody only, and cells treated with DPB with Ca and Mg only. No specific cell death, however, occurred using this technique. Increasing antibody and complement concentrations up to 10ug/ml and 25% respectively, as well as raising the temperature at which cells were incubated with antibody up to 20°C and 37°C did not improve specific cell killing. Control tests of complement fixation using an anti-sheep red blood cell serum and a sheep red blood cells, indicated that the complement fixation procedure was effective for anucleate cells. Immuno-histochemistry revealed negligible staining of T cells from rat lymph nodes for Mab OX52, and poor staining with Mab OX44. PAGE gel electrophoresis revealed that the Mab OX52 preparation did not contain intact antibody, whereas Mab OX44 appeared to have both degraded and intact antibody molecules.

It is reported that nucleated cells require considerably more membranous damage as compared with red blood cells for lysis to occur (Koski *et al.* 1983). In light of this, attempts to compromise the metabolic activity of lymphocytes following

complement treatment were made. Treatment of cells with complement at 37oC for 4 minutes, followed by overnight incubation at 4°C failed, however, to effect specific cell death. Amplification of the specific Mab signal by using a second stage rabbit anti mouse antibody at a dilution of 1/10 also did not improve cell killing.

The reasons for the failure of complement to lyse these cells are difficult to determine. It is possible that a low concentration of the relevant antigens on the surfaces of cells, with insufficient antibody levels and the cells intrinsic ability to remove complement attack complexes combined to foil specific cell killing.

Another approach tried was based on the idea that by breaking HEC-lymphocyte bonds, HECs in suspension after primary culture could be separated from lymphocytes, and then further cultured as near pure HEC secondary adhesion cultures. To try and achieve this a number of treatments aimed at disturbing cell adhesion were tried. These included treatment of cells washed from primary adhesion plates with EDTA 0.02% at 4°C, trypsin 0.01% at 37°C and trypsin with EDTA at 37°C, and the CTEP solution for 1 hr at 37°C. It is reported that fucoidan strongly inhibits HEC-lymphocyte binding both in vivo and in vitro (Brenan and Parish 1986, Stoolman and Rosen 1983), and so this compound was included at 1mg/ml in the Percoll separation solutions. On the assumption that HECs entered the dense cell pellet as a result of association with bound lymphocytes, attempts to isolate HECs from the resulting cell suspensions using two stage Percoll gradients consisting of 40,45,50,55 % SIP with fucoidan, overlayed with a 20% SIP solution were made. HECs however did not separate from small lymphocytes at the density interface, and were found in the dense cell pellet. Also, fucoidan was included with cells during the primary and secondary adhesion steps in an attempt to prevent lymphocyte-HEC binding and aid HEC binding to the culture well surface. This, however, did not increase the number of cells binding to culture wells.