CHAPTER 8

GENERAL DISCUSSION: THE DIRECTION OF FUTURE WORK

INTRODUCTION

Chapter 3 details a quantitative study of the anatomical changes occurring in human gingival blood vessels, with progression of periodontal disease from health to the advanced lesion. Development of the lesion was accompanied by dilation of blood vessels in all fields studied. Also, the number of vessel profiles in that part of the gingival biopsy adjacent to the bacterial plaque irritant increased with progression of the disease. It was concluded that expansion of the gingival vasculature occurs in periodontitis, and that this may contribute to some of the tissue degradation seen in this disease.

In the course of performing this survey, a perivascular hyaline material was observed in close relation to the pocket or sulcular epithelium. This unexpected material was the subject of a further study (described in Chapter 4), in which the incidence, extent, ultrastructure and immuno-histochemistry of PHyM was determined. The bulk of PHyM was found to consist of MBL material, and it was suggested that the formation of PHyM may be due to the combined effects of angiogenic and injurious agents upon vessels close to the bacterial plaque. A hypothesis was developed, that PHyM contributes to development of periodontitis, by limiting access of PMNs to the bacterial plaque. In this model of the disease, PHyM is proposed as a key factor in development of CAP in patients with normal PMN function. Also, this hypothesis attempts to explain the sporadic nature of pocket deepening, which occurs independently in different sites within the mouth.

Phenotypic specialisation of ECs was also noted in the quantitative survey of vascular changes. PHELVs were found to increase in number with development of periodontitis. These vessels were most prevalent in Fields 1 and 2, so that it was decided to focus on these fields to further characterise these vessels in the study described in Chapter 5. In this study, the morphology, ultrastructure and histochemistry of PHELVs was determined, and compared with that of the well

characterised HEVs of rat lymph nodes. The ability of these vessels to incorporate $^{35}SO_4$, as well as their leukocyte exchange properties, were also studied as functional markers of HEVS. It was found that PHELVs had similar properties to the HEVs and HELVs reported by others. However, PHELVs clearly exchanged PMNs rather than lymphocytes as is seen in other vessels with HECs. It was concluded that HECs have functions independent of leukocyte exchange, and that the morphology of these cells may reflect a role in the synthesis of factors relevant to the development of chronic inflammatory lesions.

In the study of the histochemistry of PHELVs, the absence of AP in these leukocyte exchange vessels suggested that AP could play a role in controlling the adhesion of leukocytes to EC surfaces. The idea that the control of leukocyte adhesion molecules may be a specific example of a general regulatory role for AP is developed in Chapter 6. Endothelial AP was identified as the liver/bone/kidney isoenzyme as an initial step in addressing this question.

It was concluded that in order to study both the synthetic capacity of HECs, as well as the role of AP in controlling leukocyte adhesion molecules, that a method for the isolation and culture of HECs had to be established. The work described in Chapter 7 produced useful methodology for the isolation and culture of HECs from rat lymph nodes. Techniques for digestion of lymph nodes that preserved the viability of HECs were established, as were methods for the identification of these cells in suspensions and cultures. This is critical information required before a definitive method for the preparation of high density primary cultures of HECs can be established. Also, an indication of the variability of HEC density, as well as of the ability of HECs to bind to various substrata was obtained. Rat fibronectin was found to be a good substrate for adhesion of HECs to culture wells, and fibronectin pretreatment of culture wells would seem to provide an effective and convenient means of maximising HEC adhesion in future attempts to isolate and culture HECs. Concurrently with work on HEC culture, the availability of suitable probes made it possible to address the question of the synthetic activity of HECs, using the technique of in-situ hybridization. This work is described in the appendix.

In carrying out these investigations, as part of the attempt to focus on the most interesting course of study, decisions were made not to pursue potentially productive lines of work. In this chapter, some of these unfavoured investigations will be outlined. Also, the path of work which it is hoped will proceed from this thesis, will be discussed.

ENDOTHELIAL CELLS AS SCAVENGERS OF BACTERIAL PRODUCTS

As discussed in Chapter 3, it is possible that an important defence function of small venular ECs is to scavenge bacterial products, decreasing the systemic load of potentially harmful toxins.

In Vivo Experiments to Determine the Scavenging Role of ECs

A direct way of investigating this possibility, would be to induce localised lesions in rats using Freund's complete adjuvant or some other persistent irritant, and to then inject radiolabelled bacterial products into the body of the lesion. The path of radiolabelled toxins could be followed by: collecting thoracic duct lymph, collecting blood by cardiac puncture, and surgical removal of the lesion, and testing for the presence of radiolabel at regular times following the injection. If it is found that significant amounts of radioactivity remain in the lesion, the precise location of the material could be determined using auto-radiography. ECs in culture have receptors for FMLP (Rotrosen *et al.* 1987), and as a typical bacterial product, ^{3}H labelled FMLP available from Du-Pont would be used in these experiments. Radiolabelled LPS would also be used as a clearly important bacterial product.

If the hypothesis that microvascular venular ECs scavenge bacterial products is correct, the radiolabelled toxin would be found to be present not only in local macrophages and lymph vessels, but also on the surface and within venular ECs.

In Vitro Studies of the Effects of Flow Rate Upon EC Trapping of Bacterial Products

Methods have been established to test the effects of flow rate on EC behaviour (Frangos *et al.* 1985). In-vivo observations, could be further examined in-vitro, using these established techniques. Briefly, human umbilical vein ECs would be cultured in parallel plate chambers and exposed to known flow rates of medium. Cells would then be exposed to radiolabelled bacterial products for a standard time period, and uptake of labelled material assessed by measuring released radioactivity after cell lysis, as well as by auto-radiography. It would be expected that labelling would increase in proportion to flow rate.

ANGIOGENESIS AND PERIODONTAL PATHOGENS

As discussed in Chapter 3, the origin of angiogenic factors in periodontitis is difficult to define. There are many studies identifying a range of endogenous factors which induce angiogenesis. Taichman *et al.* 1984 have reported the effects of some potential periodontal pathogens on EC proliferation, however, little is known about the possible role of such factors in inducing other aspects of the angiogenic response. This is particularly in regard to the migration and the proteolytic activity of ECs. It is possible that some aspects of the tissue degradation seen in periodontitis are due to angiogenic events.

In Vitro Studies of EC Proliferation

EC cultures could be used to study the interactions between ECs and bacterial products, and activated leukocyte supernatants, to determine if angiogenic factors are produced in the subgingival bacterial plaque. Angiogenesis could be assessed by the combined assays for proliferation and migration. In periodontitis, the vessels most affected by angiogenic events are capillaries, in that these vessels have an increased diameter and vessel profile number. For this reason, it is desirable to do these experiments using capillary EC cultures. Accepted methods could be used to establish human foreskin capillary EC cultures (Davison *et al.* 1980a) for use in these experiments. Also, capillary ECs could be obtained from rat adipose tissue, as well as cerebral microvessels (Wagner 1975, Folkman *et al.* 1979, Panula *et al.* 1978) to compare the results of the in-vivo studies with in-vitro observations.

Potential periodontal pathogens could be cultured, and their cell wall extracts and supernatants tested for the ability to initiate EC proliferation, migration and proteinase synthesis. Bacterial products potentially responsible for EC proliferation could be further characterised by column chromatography.

In-Vivo Tests for Angiogenic Factors

Factors which are potentially angiogenic could be tested for their actual angiogenic capacity using one of a range of widely accepted in-vivo tests for angiogenesis, such as the chick chorio-allantoic membrane assay, or the corneal pocket assay (Ishikawa *et al.* 1989, Polverini *et al.* 1977).

PERIVASCULAR HYALINE MATERIAL

In Chapter 4, a perivascular hyaline material was described. It was postulated that this could contribute to the development of the advancing periodontitis lesion by inhibiting PMN emigration. At the time that this work was done, it was decided that other lines of research had higher priority, and so further study of PHyM was not performed. However, several questions relating to the pathogenesis, structure and significance of the material need to be addressed.

Structure of PHyM

In relation to the structure of PHyM, it is necessary to understand the nature of the fine fibrils and membrane blebs which are distributed throughout the material. Immuno-histochemistry at the light microscope level did not reveal any labelling of the material for serum amyloid P protein, keratin or Factor VIII-associated antigen. However, it is possible that the respective antigens were present in PHyM in amounts that were insufficient for detection with the light microscope. It could be further argued that any successful labelling would be difficult to interpret, as the relation of labelling to ultrastructural features is not determined with light microscope sections. An approach to solve these problems would be the use of immuno-gold labelling with electron microscopy. This sensitive technique would not only allow detection of smaller quantities of antigen, but would also relate labelling to ultrastructural features.

Other antigens which should be searched for using this technique include, several plasma proteins, such as fibrinogen, albumin and immunoglobulin, as well as Type IV collagen. This would establish the presence or otherwise of degraded plasma proteins within the PHyM, as well as confirm or refute the contention made in Chapter 4, that the apparently structureless PHyM is in fact degraded MBL material. The possibility that the fine fibrils observed in PHyM with the electron microscope are type IX collagen basement membrane anchorage fibrils could also be tested by immuno-gold labelling.

Endothelial Cell Injury and Basement Membrane Production

EC cultures similar to those described in discussion of the study of angiogenic factors, could be used to study the ability of ECs to produce basement membrane material in response to plaque products. Potential periodontal pathogens could be cultured, and their supernatants and cell wall extracts tested for the ability to initiate

extracellular matrix synthesis by ECs. Antibodies against many basement membrane components are now available, including collagen types VII and IV as well as for fibronectin and laminin. ECs would be cultured in 96 well plates, and fixed cultures tested for basement membrane products by ELISA assays performed directly in culture plates. The production of glycosaminoglycans in response to potentially injurious agents, by ECs grown in micro-well cultures would also be studied. Glycosaminoglycan would be monitored using a modification of the detection system described by Forndale *et al.* (1986). By monitoring the appearance of intracellular enzymes such as lactate dehydrogenase, in culture supernatants, as well as by using electron-microscopy, ECs could be assessed for the toxic effects of defined bacterial products. This would allow correlation to be made between EC toxicity and the ability of toxins to stimulate production of basement membrane material. Using this approach, it may be possible to explain the appearance of PHyM.

Proteinase Secretion in Response to Bacterial Products

ECs produce several proteinases in response to angiogenic factors (Gross *et al.* 1983). These include collagenase (Moscatelli *et al.* 1980), plasminogen activator (Levin and Loskutoff 1982), and stromeolysin (Herron *et al.* 1986). The production of these enzymes would be monitored for EC cultures exposed to angiogenic factors identified in the above experiments. The production of angiogenic factors capable of inducing proteinase secretion by ECs could partially explain the degraded appearance of PHyM in inflamed gingival tissues.

PHyM and Suppression of Local Defence Mechanisms

As discussed in Chapter 4, it is possible that by inhibiting PMN emigration into the bacterial plaque, that PHyM might compromise the local defence of the host against periodontitis. It is difficult to test this hypothesis directly, since there is no way of assessing the presence or absence of the heavy deposits of PHyM in patients without removing the tissues involved.

In Chapter 5, it was shown that PMNs emigrated from PHELVs draining the superficial network of vessels beneath the sulcular and pocket epithelium. It is these superficial vessels which have the potential to form PHyM. A linear regression analysis, in which the percentage of superficial vessels affected with PHyM was compared with the number of invtravascular leukocytes in each biopsy failed to reveal any relationship between these parameters. Although this would seem to refute the

hypothesis that PHyM inhibits PMN emigration, it is clear on further consideration, that any effect of the material on leukocyte emigration would occur only when deposits become sufficiently extensive to significantly interrupt the diffusion of chemotactic agents into the tissues and vasculature, as well as to interfere with trans-epithelial migration of leukocytes. Within the framework of the hypothesis under discussion, this would occur infrequently, and would not be evident in the long intervals that separate bursts of pocket deepening. The biopsies used in this study were obtained from patients having teeth extracted for a variety of clinical conditions. Because of this, the lesions were at all stages of development, and it would be exceptional if any of the periodontal pockets studied was in the process of undergoing a burst of pocket deepening.

To test the hypothesis, it would be necessary to perform a long term, longitudinal study of many hundreds of patients. Periodontal pocket depth would be monitored in an accurate and non-invasive way at regular and frequent intervals, using radiological methods to avoid disruption of the microbial plaque (Tanner *et al.* 1984). Once a burst of pocket deepening is seen to have occurred, a gingival biopsy could be obtained and the lesion studied. Although this would only reveal the lesion after pocket deepening had occurred, the distribution of PHyM along the length of the pocket wall could be easily assessed. If the hypothesis is correct, there would be a strong bias for very extensive deposits of PHyM to occur up to 2 mm distant from the most apical portion of the pocket wall, with no material being present at the leading edge of the pocket. On the other hand, if the hypothesis is incorrect, PHyM in recently deepened periodontal pockets would vary in its extent and distribution to the same degree as in gingival biopsies obtained randomly from sites which are not monitored.

Should such a study support the hypothesis, it would be interesting to perform a further study of the microbial changes associated with the pattern of PHyM deposits, both on randomly selected sites and from monitored sites which have recently progressed. This could reveal the microbial changes that are associated with pocket deepening. There would then be a reasonable chance of identifying relevant microbial virulence factors, that are responsible for pocket deepening.

HIGH ENDOTHELIAL CELL CULTURE

Isolation and Culture of High Endothelial Cells

It is probable that the use of discontinuous density gradients will provide a means for the isolation of HECs from other lymph node cells. Alternatively, elutriation of cells, a method which separates cells according to size, may allow purification of HECs either from whole cell preparations, or from partially purified lymph node cell preparations. In Chapter 7, HECs were separated from some of the contaminating cells by differential adhesion to fibronectin-coated culture wells. Although this was partially effective, in that adherent cells remaining after an overnight culture of the dense cell preparation were primarily HECs, HECs were also lost in the primary adhesion step. This suggests that the method used selected for a poorly adherent subpopulation of HECs, which may not be representative of the majority of cells. For this reason, it would be desirable not to include such a differential adhesion step in future purification protocols, unless it is found that few HECs are lost with such a procedure.

Once a method has been established in which primary cultures of HECs at confluence are available in a multi-well format, it will be necessary to characterise the cultures. Apart from the need to define such factors as the optimal seeding density, the identity and degree of contamination with other cells, as well as optimal substrata, culture medium and serum requirements, of critical interest will be the stability of the HEC phenotype.

The Stability of HEC Phenotype In-Vivo

De-afferentization of lymph nodes causes a reversible loss of the specialised HEV phenotype (Hendricks and Estermans 1983). As HEC phenotype is reversible in-vivo, it is expected that it will be lost from HECs in culture, since cells will be isolated from the specific signals which normally maintain their specialised status.

The criteria currently available for the identification of HECs as against other ECs are: the ability to bind lymphocytes, the absence of alkaline phosphatase and the uptake of large amounts of radioactive sulphate. These are the variables that could be monitored in HEC cultures to determine the time course for the loss of the specialised HEC phenotype.

To confirm that the acquisition of APA, as well as the loss of $^{35}SO_4$ uptake by HECs occurs in HECs during this phenotypic reversion, an experiment could be performed, in which Wistar-Furth rats are subjected to the necessary surgery to de-

afferentize their lymph nodes, and the APA, $^{35}SO_4$ uptake and lymphocyte binding capacity of HECs monitored over a period of time. APA and $^{35}SO_4$ uptake would be monitored using freeze-substituted tissues and auto-radiography as described in Chapter 5. Lymphocyte binding would be monitored using the frozen section lymphocyte binding assay (Stamper and Woodruff 1976).

Time Course of Loss of Phenotypic Specialisation of HECs in Culture

On the premise that HEC phenotype is reversible in-vitro, and that the in-vivo experiment indicates that APA is acquired while $^{35}SO_4$ uptake and lymphocyte binding are decreased with loss of HEC phenotype, an experiment could be performed in which this loss of phenotype is monitored in HEC cultures.

Total $^{35}S0_4$ uptake by HEC cultures would be monitored in the microwell culture system by measuring released radioactivity from detergent lysed cells. Total APA would be measured in an ELISA reader, by observing changes in the optical density of methanol-fixed HEC cultures incubated with a p-nitrophenol phosphate (Sigma) in Tris-HCl 0.01M pH 9.5. Autoradiography to detect ^{35}S, and histochemistry for AP would be used to compare the changes measured in microwell cultures with the appearance of cells grown on tissue culture slides (Miles). Lymphocytes would be labelled with ^{51}Cr and binding to HEC cultures assessed after washing the cultures, by lysing the adherent cells with detergent and measuring the released radioactivity.

Factors Required to Maintain HEC Phenotype

There are reports that HEVs are dependent for maintenance of their specialised phenotype on lymphocyte emigration (Nightingale and Hurley 1978), macrophage products (Hendricks and Estermans 1983), and T-cell products, with gamma interferon (g-Inf) being especially implicated as a potentially important factor (Duijvestijn 1986). Extensive long-term monocytic, PMN as well as lymphocytic emigration have been reported in the absence of HEVs (Nightingale and Hurley 1978, Jeurissen *et al.* 1987). HEVs are thus not a prerequisite for prolonged leukocyte emigration, and it seems unlikely that PHELVs are maintained purely in response to the prolonged emigration of PMNs. These observations indicate that the HEV phenotype is both inducible and reversible, and is likely to be the result of modification of existing PCVs by locally produced inflammatory factors. However, the specific identity and source of these factors is as yet unknown.

A recently identified and partially purified Mr 36,000 protein, released by LPS

stimulated macrophages, has been shown to bind to the class II major histocompatibility antigen (Ia) as its receptor (Hedberg and Hunter 1988). This macrophage factor (MF) may have the potential to control many aspects of the inflammatory and immune response.

Activated T-cells produce g-Inf and it is well established that g-Inf induces the expression of Ia by ECs (Pober *et al.* 1983). In relation to this, human HEVs are reportedly rich in class II antigens (Turner *et al.* 1987), suggesting a role for g-Inf in their control. It is possible, that in lymphoid organs and in sites of chronic inflammation, antigenic stimulation generates g-Inf, causing local PCVEs to express Ia, and thus allowing MF to bind to the EC surface, perhaps acting as the final signal for the expression of the HEC phenotype.

This model reconciles conflicting views regarding the importance of lymphocyte emigration and antigenic stimulation in HEV maintenance, as lymphocyte emigration is critical only in as much as the supply of T-cells are involved. This model also helps to explain the presence of HEVs exchanging PMNs in periodontal tissues, as it is not dependent upon emigrating lymphocytes. To test this hypothesis, attempts could be made to maintain HEC cultures undergoing phenotypic reversion. HEC cultures could be exposed to combinations of commercially available recombinant rat g-inf (Amgen Biologicals), and MF supplied by Dr Hunter. The use of OX6 (Serotec), a monoclonal antibody which recognises the binding site of MF to Ia, would be used to mimic the effect of MF in preventing the loss of HEC phenotype, and the Fab fragment would be used to block the effect of MF. The presence or otherwise of Ia in HEC cultures could be tested both by immuno-histochemistry, and using the more sensitive ELISA system. The re-expression of HEC phenotype in cultures of cells which have lost it could be tested to confirm the findings of these experiments.

Should the hypothesis that g-Inf and MF are required to maintain HEC phenotype prove incorrect, experiments would be performed using activated leukocyte supernatants and commercially available recombinant cytokines, to identify the cytokines required to maintain and induce HEC phenotype.

The Synthetic Capacity of HEC Cultures

If lymphocyte exchange is not a requirement for PHELV induction and maintenance, and if HEVs are clearly not necessary to allow prolonged leukocytic emigration, there must be doubt regarding the currently held dogma explaining the large size of HECs as a specialised adaptation to assist with the emigration of lymphocytes (Kraal *et al.* 1987).

It is hypothesised that a major function of HEVs is the synthesis of inflammatory mediators, playing a role in both the local and systemic control of chronic inflammation.

The Synthetic Potential of HECs Determined In-Vitro

The synthetic potential of HEC cultures would initially be tested by examining the supernatants of cell cultures for the presence or absence of the cytokines expected to be produced by the cells. Bio-assays for IL1, IL6, PDGF and the CSFs would be performed, as well as complement fixation tests using C3 depleted serum to test for this product. Also, Northern blot analysis for these products should be performed. Once the panel of cytokines which HECs are capable of producing has been determined, the next phase of the work could commence.

Comparison of Synthetic Activity of Phenotypically Active with Reverting HEC Cultures

Since the hypothesis to be tested is that HECs are significantly more competent in the synthesis of inflammatory cytokines than are their flat endothelial counterparts, it is important to compare the synthetic activity of HEC cultures which are phenotypically active, with cultures which have lost the HEC phenotype. To do this, it would be necessary to divide the multi-well cultures from any given batch of cultured HECs into two groups. One group would be treated with the factors required to maintain phenotype, as determined in the earlier experiments outlined above, while the other would be deprived of these factors, providing the necessary control cultures for synthetic capacity.

The synthesis of specific cytokines by phenotypically intact HEC cultures could then be compared with that of reverted cultures. It would be expected that both the constitutive and induced secretion of specific cytokines would be greater for active HEC cultures than for the attenuated cultures. Bio-assays of supernatants, and Northern blot analysis of cell cultures would be used to compare the synthetic potential of the two types of HEC cultures. Constitutive production of these factors would be compared with that of LPS and IL1 stimulated cultures.

An important control in this experiment is to determine the activity of attenuated cultures that are exposed briefly to HEC inductive factors, as it would be possible that any increased synthesis of specific cytokine is related to activation by the factors maintaining phenotype, rather than to the HEC phenotype itself.

Also, the total protein synthesis of active as against attenuated HEC cultures could be compared using ^{3}H leucine incorporated into acid insoluble material. The total secreted protein produced by the two types of cultures could be compared with the cell associated protein, as determined by lysing the cells. This would give an indication of the relative activity of the cells devoted to the house-keeping activities required in maintenance of the specialised phenotype.

Role of the Sulphated Glyco-Lipo-Protein

One product of HECs which has been extensively studied is the sulphated glyco-lipo-protein (Andrews *et al.* 1980, 1982 & 1983) that forms the basis of labelling of HECs with $^{35}SO_4$. It should be possible to isolate and purify this compound from HEC cultures, and to study its action on leukocyte binding to HEC cultures. It is possible that the effect on leukocytes is independent of the vascular endothelium, since although this compound was found to increase the number of lymphocytes found in tissues into which the sulphated HEC product was injected (Andrews *et al.* 1980), it is not clear whether this was due to an increase in the migration of lymphocytes into the tissues, or to an increased retention of recirculating lymphocytes. It has been suggested that sulphated carbohydrates may play a role in positioning lymphocytes within lymphoid tissues (Parish *et al.* 1984), and it is possible that the sulphated compound synthesised by HECs plays a role in this process. For this reason, the effects of the sulphated compound upon lymphocyte migration in chemotaxis assays, as well as the aggregation of lymphocytes with one another and with other cells in suspension, should be tested. Chemotaxis assays could be performed using the micro-chemotaxis assay system, while cellular aggregation could be tested with an aggregometer. The further possibility that lymphocytes treated with this factor have an increased affinity for extracellular matrix components could be tested using micro-wells coated with a range of extracellular matrix components, and by testing the ability of labelled lymphocytes to resist washing from these surfaces.

Characterisation of Proteins Synthesised by HEC Cultures

A problem with the approach described above, is that only cytokines which are expected of being produced by the cells would be studied. This raises the possibility that cytokines, which are not currently known to be produced by ECs may not be detected in cultures. One way of dealing with this problem, would be to label all of the proteins secreted by the cells, and to characterise these proteins using two dimensional gel electrophoresis. By comparing the gels of proteins produced from phenotypically active with attenuated cultures, it should be possible to identify proteins unique to each of the culture types.

Phenotypically active HECs would be cultured in tissue culture flasks and ^{35}S methionine-labelled proteins collected from the supernatant and concentrated by dialysis. Newly synthesised labelled proteins would be separated on the basis of their Mr and iso-electric points by two dimensional electrophoresis with isoelectric focusing in the first dimension, and PAGE gel electrophoresis in the second dimension. Gels produced in such a way could be then compared with reference gels, in which radioactively labelled reference cytokines have been separated. Any secreted protein which can not be accounted for in the reference gel, would be a potential candidate for further characterisation by N terminal sequencing. This should determine the identity of the protein, if it has been characterised by earlier workers. If it is an unknown product, then oligonucleotide probes could be prepared to identify cDNA clones prepared from HEC cultures which code for the protein concerned, allowing the eventual sequencing of the gene, and the production of the gene product in large quantities for the study of its biological properties.

In-Situ Hybridization to Confirm the Production In-Vivo of HEC Products Identified In-Vitro

To confirm that the in-vitro observations have biological relevance, in-situ hybridization studies would be performed using lymph node tissue from untreated and de-afferentized rats, as well as human and mouse lymph node tissue, for which probes for a large number of relevant genes are now widely available.

The Role of Alkaline Phosphatase in the Control of Leukocyte Binding

As discussed in Chapter 6, it is possible that AP could play a role in the control of cell surface receptors involved with leukocyte adhesion to ECs. It was established that the endothelial isoenzyme of AP is the LBK form, and that kidney tissues constitute a rich source of the relevant isoenzyme.

Using the fractogel affinity column system available from Merk, rat kidney AP would be purified, and used for experimentation. The effects of AP upon leukocyte binding to EC cultures could then be investigated with a purified preparation of the identical isoenzyme of AP to that found in ECs.

Use of HEC Cultures to Study the Effects of AP on Leukocyte Binding

For several reasons, the use of HEC cultures would be of special relevance to such a project.

Firstly, HECs are AP negative, so that the addition and removal of AP should be a simple matter, whilst capillary EC cultures are AP positive (Panula *et al.* 1978). Further, it is clear that leukocyte emigration takes place from post-capillary venules. As representatives of PCV ECs, HEC cultures provide a physiologically relevant culture system for the study of leukocyte binding. Finally, HEVs are sites of extensive leukocyte emigration in vivo, so that if it is assumed that the cultured HECs have similar properties to their in-vivo counterparts, it should be easy to measure changes in leukocyte binding from relatively high basal levels.

Effects of Purified AP on Leukocyte Binding to HECs

As described in Chapter 7, HEC cultures are AP negative. The effects of a preparation of kidney AP in the pre-treatment of purified leukocytes, or HEC cultures could be compared with the effects of AP in co-incubation experiments with leukocytes and HECs. If the hypothesis that AP inhibits leukocyte binding is correct, a decrease in the binding of leukocytes to HEC cultures would be seen. This would determine whether or not AP plays a role in the control of leukocyte binding to ECs, and further identify the cell type upon which AP has its effect. Further experiments comparing the behaviour of purified populations of different leukocytes would identify any differences in the role of AP in the emigration of different types of leukocytes.

Effects of Inhibitors of AP Upon Leukocyte Binding to HEC Cultures

LBK isoenzyme inhibitors such as levamisole, could be used to confirm that the effect of AP on leukocyte binding to ECs, is a result of the activity of the enzyme. In this way, marked inhibition of the effect of AP on leukocyte binding would be expected by levamisole as compared with L-Leu-Gly-Gly. Inhibitors such

as L-Phen-Gly-Gly, which are ineffective against the LBK isoenzyme, however, would not be expected to alter adhesion. Further experiments could be performed using the AP positive phenotypically attenuated HEC cultures, showing that inhibitors of AP are capable of increasing the binding of leukocytes to these cultures. Inhibitor experiments could also be performed using other AP positive microvascular EC cultures. However, since PCVs are the site of leukocyte emigration in-vivo, it would seem more appropriate to use HEC cultures for the examination of this question.

To determine if the mechanism of action of AP is cleavage of phosphate groups from cell surface-associated molecules, cells pulsed with ^{32}P would be exposed to AP, with and without inhibitors of the enzyme serving as controls. The amount of liberated ^{32}P could be measured with a ß counter. It is expected that AP treated cultures would release significantly greater amounts of radioactivity as compared with untreated cultures. Also, in experiments with inhibitors of AP, the concentration and type of inhibitor used would be expected to be reflected in the amount of radioactivity released from HEC cultures.

Effect of AP Upon Mel-14 Dependent Lymphocyte Binding

As mentioned in the literature review in Chapter 6, Mel-14 antigen appears to bind at a mannose-6 phosphate site (Yednock 1987a&b). The lectin-like region of the related ELAM-1 vascular addressin, also raises the question of a possible role for mannose-6 phosphate in leukocyte binding. It would be interesting to determine whether or not AP affects Mel-14 antigen dependent binding of lymphocytes to ECs, by including AP in the experiments (briefly reviewed in Chapter-1), in which the role of Mel-14 antigen in lymphocyte trafficking was established.

Identification of Differences in the Complement of Cell Surface Proteins in Phenotypically Active and Attenuated HEC Cultures

Using a method obtained from Dr D. Newgreen of the Dep. of Paediatrics Westmead Hospital in Sydney, surface proteins of phenotypically active and attenuated HEC cultures could be labelled with N-hydroxy succinimide-biotin under mild conditions. Cytosolic proteins are not labelled using this technique. Biotinylated proteins would then be further labelled with ^{35}S-avidin and released from the cell membranes using detergent lysis. The labelled complexes would be analyzed by two dimensional electrophoresis. Although characterisation of proteins is not be possible using this method, due to the association of proteins with complexes of avidin, it would be possible to identify differences in the complement of surface proteins of

phenotypically active and attenuated HEC.

Using this approach, it should be possible to identify the critical HEC surface proteins that are unique to the activated phenotype of HECs. These proteins could be involved with leukocyte docking, the maintenance of phenotype or have a role in the transport of selective molecules, such as SO_4 that are important for HEC function.

The Presence of Phosphate in Cell Surface Proteins Used to Help Identify Key Leukocyte Docking Molecules

If AP is found to play a role in the control of leukocyte binding, this fact could be exploited to help in the identification of key proteins for leukocyte binding. This would be done by comparing the two dimensional maps of ^{35}S-avidin labelled proteins with similar maps prepared from cells pulsed with $^{32}PO_4$, and surface labelled with cold avidin. This should provide an identical map to that of the surface proteins, but would instead be a map of phosphorylated cellular proteins. A potential difficulty with this is that the number of phosphorylated proteins may be so high, that clear identity of individual proteins is not possible. If this is the case, cell membranes may be separated from biotin treated cell cultures, and proteins prepared from these membrane preparations.

Production of Monoclonal Antibodies To Defined Cell Surface Determinants

Cell surface proteins identified as of potential interest in the above experiment, would then be further studied. Mabs are an invaluable tool in the study of proteins, and methods are available (Dr Newgreen Personal communication) for the production of antibodies from the two dimensional gels used to identify proteins of interest. Briefly, the relevant part of the gel is cut out and emulsified for intrasplenic injection of the emulsion into mice. When serum antibodies binding to cells in stored HEC microwell cultures are detected by ELISA techniques, the mice would be sacrificed and spleen cells hybridised with the NS1 hybridoma. Resulting clones of antibody secreting cells would be screened by the ELISA method, as for the mouse serum samples. Positive antibodies would be validated by testing for binding of antibodies to HECs, using an immuno-phosphatase method on frozen and freeze-substituted sections of both normal and antigen stimulated Wistar-Furth lymph nodes.

$1x10^8$ cells in culture is sufficient to apply this method for both protein identification, and the production of monoclonal antibodies. If it is assumed that 1 X 10^7 HECs in culture can be obtained from a single rat, then 10 cultures stored

and pooled for electrophoresis should provide enough cells for a single two dimensional gel. This method has been found to greatly accelerate the time consuming process of Mab production, as only the specific proteins of interest are used as antigens. Also, the use of ELISA systems as a screening tool allows for the rapid assessment of clones, whereas the use of immuno-histochemistry to screen clones is inevitably more time consuming.

Mabs have played a large role in the study of cell surface receptors involved in leukocyte binding to endothelial surfaces (Makgoba *et al.* 1988). Using these reagents as probes for the gene products, it has been possible to clone and sequence the genes responsible for these important proteins, as well as to study the nature of leukocyte binding in blocking experiments. However, the production of these antibodies has largely been determined by the antigenicity of the molecules involved, with Mabs being raised to whole tissue and cell preparations. It is possible that this approach may have left important cell surface proteins undetected.

It is hoped that by performing a systematic study of the cell surface proteins of HECs, that a more complete understanding of endothelial leukocyte docking molecules will be achieved.

APPENDIX

IN SITU HYBRIDIZATION

INTRODUCTION

As discussed in Chapters 1 and 5, ECs in cell culture systems are capable of synthesising a large number of inflammatory mediators. The physiological relevance of this potential is, however, difficult to determine. There is no clear evidence that ECs synthesise such factors in-vivo. HECs are always found in either inflammatory sites or lymphoid tissues. Also, they are noted for their voluminous cytoplasm and extensive synthetic organelles. It is possible that HECs are ECs specifically adapted for the synthesis of inflammatory mediators.

In Chapter 7, work was described towards establishing a HEC culture model with which this hypothesis could be tested. A further means of addressing the hypothesis is to use the recently developed technique of in-situ hybridization. This methodology allows the detection of mRNA for specified proteins in paraffin (Morley and Hodes 1988, Pringle *et al.* 1989), cryostat (McCabe *et al.* 1986) and plastic sections (Guitteny *et al.* 1988). The availability of suitable probes allowed application of this technique to test for the relative abundance of mRNA for some cytokines suspected of being produced by HECs. This aspect of the work is described in this appendix.

in-situ hybridization has the advantage over in-vitro cell culture studies, that the synthesis of specific proteins by cells in tissues can be directly related to their spatial and temporal position in tissues and lesions. This removes an element of speculation from the extrapolation of in-vitro data to explain in-vivo phenomena. However, a pre-requisite in the use of this technique, is that the identity and genetic sequence of the proteins suspected of being synthesised by the cells must be known, since in-situ hybridization will only reveal the presence of mRNA for proteins encoded for by the DNA or RNA probes used in hybridizations. From this, the role of in-situ hybridization lies perhaps in the confirmation of possibilities raised by in-vitro studies identifying cell products in culture.

Selection of Probes for Testing

There have been no reports of studies investigating the production of cytokines by HECs in-situ. There is a single paper, in which cryostat sections of mouse lymph nodes were tested for the presence or absence of mRNA for IL1α and IL1ß (Taka'cs *et al.* 1988). These authors noted the absence of mRNA for these cytokines in HECs. However, a more extensive survey of potential endothelial products was not carried out, leaving the synthetic properties of these cells unresolved. Also, considering the importance of IL1 in the literature, it was decided that IL1α and IL1ß probes should be tested for in this study of human tissues.

Haemopoietic factors, apart from stimulating proliferation and differentiation of haemopoietic stem cells, are also known to have a wide range of effects upon mature leukocytes (Nicola 1989). The production by cultured ECs of G-CSF, GM-CSF and multi-CSF (Zsebo *et al.* 1988, Broudy *et al.* 1986, Segal 1987) has been reported. It is generally assumed that these factors act over short distances in the haemopoietic environment, and that the principal site of synthesis for these factors is in the bone marrow and other haemopoietic sites. Although this may be the case, intravenous injection of CSFs significantly increased haemopoiesis in myelosuppressed animals (Welte *et al.* 1987). Also, serum levels of some CSFs are elevated in mice after injection of LPS and following bacterial infection (Nicola 1989). This suggests that the synthesis of such factors by cells distant from haemopoietic tissues could modulate the production of leukocytes in haemopoietic tissues.

Leukocyte numbers, particularly PMNs, in peripheral blood are highly variable in human populations, with PMN numbers varying from 51% to 67% of leukocytes in normal blood films (Ciba-Geigy 1970). This presumably reflects differences in both genetic and environmental factors between individuals. If environmental factors are at least partly responsible for this variation, then some mechanism must exist for modulating haemopoiesis according to environmental need. The lymphoid tissues within which HEVs reside, are strategically placed to collect and process antigen from the environment. It is clear that at any given time, different sites will be exposed to differing amounts of antigenic stimulation both within individuals and between individuals. From this, it could be argued that lymphoid tissues are a sensitive gauge for the total antigenic, and presumably total bacterial load of the animal. If this is the case, and if HECs synthesise CSFs, these cells might account for the variability in leukocyte numbers seen amongst individuals.

For these reasons, it was decided to include two of the CSFs which are

produced by ECs in this in-situ hybridization study. The two CSFs tested were GM-CSF and Multi-CSF.

MATERIALS AND METHODS

Enzymes and Chemicals

Restriction and ligation enzymes and buffers were purchased from IBI, as was Na ATP. Agarose was obtained from NE Pharmaceuticals. Hind III digest of lambda DNA was purchased from Pharmacia. The non-isotopic DNA labelling kit, as well as the DNA tailing kit were purchased from Boehringer Manheim. Also, RNAsin, glycine and proteinase K were purchased from Boehringer Manheim. Hybond nylon membrane and 3MM filter paper were obtained from Amersham. Kleenex tissue paper was used. Aminoalkyl silane was purchased from Sigma, while repel silane was supplied by Pharmacia. Foetal calf serum was obtained from CSL. DPB was obtained from Oxoid. Hybridization solution was purchased from Sigma, as were all of the remaining reagents used in this study.

DNA Probes

The plasmid, pcD-hGM-CSF was a gift from Dr F Lee of the DNAX research institute at Palo Alto USA (Lee *et al.* 1985). Oligonucleotide probes for IL1α, IL1ß and Multi-CSF were purchased from British Biotechnology. Each of these was a cocktail of three 30mer oligonucleotides, with each oligonucleotide coding for a separate exon of the gene concerned.

Restriction Enzymes

Where necessary, plasmids were cut using the restriction enzymes: Hind III, Xho-1 and Apa-1. The same digestion regime was applied for each of these enzymes, in which 10 ul of plasmid DNA was mixed with 40µl of a 1/5 dilution of buffer. The buffer varied with each restriction enzyme used. 1 µl of restriction enzyme was introduced to this preparation, and incubated at 37°C for 4 hours. The buffers used were: 25mM Tris-HCl pH 7.4, 50mM NaCl, 10mM $MgCl_2$, 100µg/ml BSA, 2mM ß-mercapto-ethanol for Hind III; 25mM Tris-HCl pH 7.8, 100mM NaCl, 10mM $MgCl_2$, 100µg/ml BSA, 2mM ß-mercapto-ethanol for Xho-1; and 25mM Tris-HCl pH 7.8, 10mM $MgCl_2$, 100µg/ml BSA, 2mM ß-mercapto-ethanol for Apa-1. The resulting cut plasmid was checked for complete digestion by agarose gel electrophoresis, and then precipitated with ethanol.

Agarose Gel Electrophoresis

The mini-gel system by Hoeffer was used for electrophoresis. Briefly, 0.15% agarose was dissolved in Tris Borate EDTA (TBE) by boiling, and allowed to cool to 50°C before adding 0.005% of stock ethidium bromide (10mg/ml) and pouring into a gel former with a comb. TBE was diluted from a 5 times concentrate prepared with 54g Tris Base, 27.5g of boric acid and 20 ml of 0.5M EDTA made up to 1000ml. Once solid, the gel was placed into the electrophoresis apparatus, submerged with TBE containing 0.005% stock ethidium bromide, and loaded with samples. Wells received 8µl of each sample with 2µl of tracking dye. Molecular weight standards consisting of a Hind III digest of lambda DNA were used. Gels were run at a constant voltage of 120V till the tracking dye was near the end of the gel. The gel was then visualized under a short wavelength UV transilluminator and photographed with a polaroid camera.

Ethanol and Isopropanol Precipitation of DNA

DNA was precipitated with ethanol where possible, and isopropanol when larger volumes of DNA solutions were used. Ethanol precipitation was performed by adding 1/10th volume of 2.5M sodium acetate pH 5.2, and then 2 volumes of 100% ethanol. Mixtures were then chilled at -70°C for 15 minutes and centrifuged at 13,000 RPM for 5 minutes. Pellets were then dried with a vacuum desiccator before re-suspension.

Isopropanol precipitation was carried out by adding 1 volume of isopropanol to the DNA solution and centrifuging at 12,000 RPM. The resulting pellet was washed briefly with ice cold 70% ethanol before desiccation and re-suspension.

Amplification of pcD-hGM-CSF

Transformation

The E. coli strain NM522 was transformed with plasmid pcD-hGM-CSF using the method described by Chung *et al.* (1989). Briefly, 10 ml of LB Broth (bacto-tryptone 10g, bacto-yeast extract 5g, NaCl 10g in 1l of H_2O) was inoculated with E. coli and grown overnight with agitation at 37°C. The following morning, a further 10 ml of LB broth was inoculated with 200 ul of the overnight culture, and was incubated with agitation to an optical density of 0.3. 100µl of this bacterial culture was then diluted in an Eppendorf tube with 100µl of 2 X TSS (Chung *et al.* 1989) on ice, and 0.25µg of DNA in 1 µl of 10mM Tris-HCL with 1mM EDTA at pH 8 (TE). The tube was stored at 4°C for 30 minutes and then 900µl of YT broth added.

This was then incubated for 1 hr at 37°C to allow expression of antibiotic resistance, and then 200 ul spread onto an ampicillin-L broth plate (L-Amp Plate). Overnight incubation at 37°C selected for transformants, which were further purified by streaking out on a second L-Amp plate for overnight culture.

Amplification and Purification of the Plasmid

Amplification was performed using the method described by Maniatis (1982). Briefly, a transformant colony was selected, and cultured overnight at 37°C with agitation in selective media consisting of 10 ml LB-broth with 50µg/ml ampicillin. The following morning, the 10 ml culture broth was divided into two 5 ml quantities, and centrifuged at 9000 RPM for 5 minutes. After decanting off the supernatant, the bacterial pellets were vortexed and suspended in 200µl of a lysozyme solution before being transferred to an Eppendorf tube for 5 minutes at room temperature. The lysozyme solution consisted of 50mM Glucose, 10mM EDTA, 25mM Tris pH 8 with 5mg/ml lysozyme. 400µl of 0.2M NaOH with 1% Sodium Dodecyl Sulphate was added to each Eppendorf tube and the tubes rocked gently till the solutions were translucent. 300µl of potassium acetate solution was added and thoroughly mixed by gentle rocking. The potassium acetate solution was prepared by mixing 60 ml of 5M potassium acetate, with 28.5 ml of glacial acetic acid, and 11.5 ml of water, with the final pH adjusted to 4.8. The Eppendorf tubes were then stored for 5 min at -70°C, and centrifuged for 15 min at 13,000 rpm. 0.75 ml of the resulting supernatant was carefully removed, and the plasmid DNA precipitated by isopropanol precipitation, and resuspended in 200 µl of TE (10mM Tris HCl, 1mM EDTA at pH8).

The plasmid was further purified using the Quiagen tip-20 mini-prep kit. The protocol normally recommended for the use of these columns includes a step in-which the preparation is treated with RNAase. Since RNAase contamination is a major problem with in-situ hybridization for mRNA, it was decided not to perform this step with the plasmids prepared for this experiment. This system deproteinises the plasmid preparation and removes RNA, resulting in a very pure plasmid preparation. The purified plasmid was precipitated with isopropanol and resuspended into 100µl of TE.

Production and Amplification of Empty Vector

It was intended to use the whole plasmid preparation to probe for mRNA for GM-CSF. A potential difficulty with this is the possibility that plasmid sequences could give false positive results. It was decided to use a clone of the pcD plasmid, which had the hGM-CSF insert removed, as an empty vector control against this possibility.

Removal of the Insert

The restriction map of pcD-hGM-CSF is shown in Figure A.1. From this it is clear that removal of the insert can be achieved by digestion of pcD-hGM-CSF with the restriction enzyme Xho-1, followed by ligation and amplification of the resulting plasmid. After treatment with Xho-1, the resulting cut plasmid was checked for complete digestion by agarose gel electrophoresis, and then precipitated in ethanol.

Ligation of pcD-Xho-1 Digest

Ligation of the cut plasmid was carried out by resuspending the precipitated DNA into 20µl of a ligation solution (prepared by mixing 10µl of ligation buffer with 87µl of H_2O and 3µl of NaATP)(Maniatis 1982), with the ethanol precipitate of the cut plasmid and 1µl of T4 ligase at 4°C overnight. The following morning the ligated DNA was precipitated in ethanol.

Transformation, Amplification and Purification of pcD-Xho-1

The ethanol precipitate was dissolved in 10µl of TE. 2µl of this was then mixed with E. coli NM522 and the cells pulsed in a Biorad electroporator with pulse controller fitted, with 2.4kV and 100Ω resistance. The cells were rapidly resuspended in 1000µl of 2 X YT broth and incubated at 37°C for 30 min before brief centrifugation to pellet cells and resuspension in 200µl of LB broth. The resulting cell suspension was then spread onto a L-Amp plate, which was incubated overnight at 37°C.

8 transformant colonies were selected from this plate, and purified by streaking out on a L-Amp plate before amplification and purification of their respective plasmids using the same protocol as described above for pcD-hGM-CSF. The only difference was that plasmid preparations were not purified with the Quiagen column, until successful removal of the insert was verified by digestion with Hind III and gel-electrophoresis.

Gel Electrophoresis and Restriction Enzyme Digests Identify pcD-Xho-1

Linearization of samples of plamsids by Hind III digestion allowed identification of transformants with plasmid lacking the insert. Agarose gel

electrophoresis indicated that pcD-hGM-CSF was larger than plasmid which had undergone digestion with Xho-1 followed by ligation and amplification. The empty vector clone which had the highest yield was then purified with a quiagen column and used as a control in hybridization reactions (Figure A.2). A further test for the successful removal of the insert from pcD-hGM-CSF, was digestion of a sample of both pcD-hGM-CSF and pcD-Xho-1 with the restriction enzyme Apa-1. There is only one site for this restriction enzyme in pcD-hGM-CSF, and this is located in the insert (Figure A.1). Apa-1 digestion resulted in linearization of pcD-hGM-CSF but not of pcd-Xho-1 as determined by agarose gel electrophoresis.

Labelling of Probes

Probes were labelled using the non-radioactive labelling kit available from Boehringer Manheim. This system is based upon the incorporation of an antigenic hapten into DNA or RNA probes, which can then be detected using a polyclonal AP-conjugated antibody specific for the hapten with which the probe has been labelled. The labelled nucleotide analogue used to label DNA was digoxigenin-11-deoxy-UTP.

The non-radioactive labelling method has several advantages over conventional isotopic labelling for in-situ hybridization studies. Autoradiography inevitably entails a degree of uncertainty as to the specific site labelled. As was seen in Chapter 5, grains of activity are often found scattered adjacent to the labelled cell, due to lateral tracking of high energy particles. In auto-radiography, the concentration of grains is typically highest over the site of origin of radiation, allowing identification of the labelled cell. However, in in-situ hybridization, where adjacent but quite different cell types may be capable of producing the same protein, lateral tracking would make interpretation of autoradiographs very difficult where cell density is high. In this study for example, the production of Multi-CSF by both lymphocytes and endothelial cells has been reported, so that heavy labelling of tissues for mRNA coding for this protein would be difficult to interpret if radioactive labelling is used. Non-radioactive labelling techniques on the other hand, give good localization of label to the cytoplasm of the cell concerned.

A further advantage of the non-radioactive system is that results of a particular hybridization experiment are available within 1 day of the experiment, where-as autoradiography may take a week or more to provide the same information. Also, there is the additional advantage that the health risks of dealing with radioactive compounds are avoided.

Labelling of Plasmid

Plasmid probes are labelled by nick translation and random priming. Briefly, half of the yield of a plasmid preparation was linearized by Hind III. After checking that linearization was complete by agarose gel electrophoresis, the linearized plasmid was precipitated by ethanol precipitation and re-dissolved in 5µl of TE before being denatured by heating at 95°C for 10 minutes and quenching on ice. Then 2µl of hexanucleotide mixture, with 2µl of dNTP labelling mixture were added to the DNA, and made up to 19µl with sterile H_2O, before adding 1µl of Klenow DNA polymerase. The resulting solution was then incubated at 37°C overnight. The reaction was stopped with 2µl of 0.2M EDTA pH 8, and the DNA precipitated with 2.5µl of 4M LiCl and 75µl of ethanol which had been pre-chilled to -20°C. After storing at -70°C for 30 min, the precipitated DNA was centrifuged at 13,000 RPM, and resuspended in 50µl of TE.

Labelling of Oligonucleotide Probes

Oligonucleotide probes were labelled at the 3' terminus using a DNA tailing kit (Boehringer Manheim). Briefly, 200 ng of oligonucleotide in 2µl of TE were suspended in a solution prepared with 4µl tailing buffer, 6µl $CoCl_2$, 2µl Dig-11-dUTP (Boehringer Manheim), 2µl dATP, and 4µl of H_2O. 1µl of terminal transferase was added to this mixture, and incubated at 37°C for 5 minutes, before stoping the reaction by chilling in ice. The labelled oligonucleotides were then precipitated with ethanol using glycogen as a carrier, before washing briefly with 95% ethanol, desiccating and then resuspending the pellet in 10µl of H_2O.

Detection of Label

Following In-situ Hybridization

Slides were washed briefly with Buffer 1 (100mM Tris-HCl, 150 mM NaCl pH 7.5) before being treated for 30 minutes at room temperature with a blocking solution. The blocking solution consisted of a 0.5% solution of blocking agent dissolved in buffer 1, with 0.5% Tween-20 and 10% FCS. Pilot studies had shown that without FCS and Tween-20, significant non-specific binding of the AP conjugated antibody occurred, resulting in high background levels. Slides were then briefly washed in buffer 1, and placed in a humidified slide chamber. 100µl of a 1/200 dilution of AP conjugated anti-body in blocking solution was then applied to each slide, and the slides incubated at 37°C for 2 hrs. Slides were then returned to a Copland jar, and washed three times with DPB for 15 min each at 37°C. Slides

were then developed, with a developing solution at room temperature and protected from light. The developing solution consisted of 40 ml 100mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5, with 180µl of 4-nitroblue-tetrazolium-chloride solution, 140µl of 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Manheim) and 50mg of levamisole (Sigma). The reaction was stopped with TE when intense staining of individual cells was apparent, and background staining began to appear. This usually took from two to four hours of incubation.

Confirmation of Labelling

To confirm that the probes had been successfully labelled, and that there was negligible unincorporated label present in the labelled DNA preparations, a blot procedure similar to a Southern blot was performed. An agarose gel was prepared, in which 1µl of each of the labelled probes was run from a single well with 7µl of TE and 2µl of tracking dye. To avoid the loss of small nucleotides, electrophoresis was stopped when the tracking dye was half way down the gel. Wells were trimmed away from the body of the gel and the trimmed gel placed on 6 sheets of 3MM paper soaked with 10 X SSC (1.5M NaCl, 0.15M Na-Citrate pH 7.0). Plastic wrap was placed around the gel, so that only the gel was exposed. A further 2 pieces of 3MM filter paper were trimmed to the dimensions of the gel, as was a single sheet of nylon Hybond, and carefully placed over the gel, with the Hybond in immediate contact with the gel. Wads of Kleenex tissue paper were trimmed to the same size as the gel, and stacked on top of the gel, so that most of the contents of a box of tissue paper was available to absorb the transfer solution. A 500 ml conical flask was filled with water, and balanced on the stack of tissue paper. This was left overnight. On the following morning, transferred DNA was fixed to the Hybond membrane by exposure on both sides to short wavelength UV light for 5 min each. The membrane was then treated with antibody and developed in a similar way to that described for slides above, except that no FCS or Tween 20 was used in the blocking solution, and a 1/500 dilution of antibody in buffer 1 was used to detect labelled probe. A photograph of the Hybond membrane after development is shown in Figure A.3. Labelling was successful for each of the probes used, but was week for Multi-CSF. Nick translation produces a wide range of labelled DNA fragments, and this is observed for both pcD-hGM-CSF and pcD-Xho-1 probes. 3' tail end labelling produces a more narrow range of nucleotide sizes, and this is reflected by the relatively narrow bands for oligonucleotide probes. There seem to be multiple bands

for the oligonucleotides, suggesting that different oligonucleotides in the cocktail label with differing efficiencies. The smallest band of oligonucleotides could be interpreted as representing unincorporated label. However, since each of the probes was labelled at the same time, and the same amount of labelled dig-11-dUTP was used in each reaction, if the smallest band were unincorporated label, it would be expected to be of equal density for each probe labelled. This was not the case. Also, the positions of the lowest molecular weight bands appear to be slightly different for different probes, which would not be the case if this represented unincorporated label. From this, it was concluded that these probes could be used in hybridizations, with the exception of that for Multi-CSF which was re-labelled due to the weak signal observed in the blot.

Treatment of Glassware, Plasticware and Solutions

Anti-RNAase Treatment

mRNA is very labile to degradation by RNases. All glassware, spatulas and tweezers used in the preparation of solutions and manipulation of slides, as well as Copland jars, microscope slide and coverslips, were baked at 300°C for 4 hours prior to use (Maniatis *et al.* 1982). In addition, microscope slides and coverslips were acid cleaned with 4M HCl prior to baking. Solutions used in pre-hybridization procedures were treated overnight with 0.1% diethyl-pyrocarbonate (DEPC) and autoclaved (Maniatis *et al.* 1982), as was the water used to float freshly cut paraffin sections. Microscope slide boxes used to store paraffin sections were also treated with DEPC. Solutions used in post-hybridization procedures were not treated with DEPC, but were autoclaved prior to use. Eppendorf tubes and pipette tips were also DEPC treated, and autoclaved prior to use.

Silane Treatment

To maximize section adhesion, microscope slides were treated with aminoalkylsilane (Rentrop *et al.* 1986). This was done by dipping slides into a 2% solution of silane in acetone for 5 seconds, and then washing slides twice with acetone and twice again with DEPC treated water (Rentrop *et al.* 1986, Pringle *et al.* 1989). Slides were then dried at 37°C in a warm oven. Cover-slips were treated with repel silane by pouring repel silane neat over the cover-slip and allowing it to dry, before washing briefly with DEPC treated water.

Tissues

Six gingival biopsies were obtained from 4 separate patients with gingivitis and 2 additional patients with periodontitis. These were fixed with 4% paraformaldehyde in DPB on ice for 2 days, and after trimming, were dehydrated with graded alcohols before embedding with paraffin to form a composite block, containing all six specimens.

Two paraffin blocks of lymph nodes obtained at surgery and fixed with paraformaldehyde were obtained from the Department of Anatomical Pathology at the Royal Prince Alfred Hospital in Sydney. These nodes had been recently removed from patients undergoing surgery for malignant tumours, but were free of neoplastic cells.

8μm Sections were prepared from these tissues, and collected on aminoalkylsilane treated microscope slides. Slides were dried at 42°C for 2 days to ensure adhesion of sections, and stored at room temperature until use.

Removal of Proteins Associated with Nucleic Acids

Many different protocols are reported for in-situ hybridization. Since contamination with RNase is considered to be a major problem with the technique, every attempt was made to minimize the number of manipulative steps in the procedure, and so decrease the opportunity for contamination.

Slides were placed in a Copland jar, and de-paraffinized with two changes of xylene at 10 min each, followed by two changes of absolute alcohol of 10 minutes each. Residues of alcohol were removed from the Copland jar, by placing it into a vacuum desiccator.

Once dried, 50 ml of 0.2N HCL was poured into the Copland jar, and the slides incubated at room temperature for 20 minutes. The objective of this step was to remove basic proteins associated with nucleic acids. The slides were then washed with 3 changes of DPB at room temperature, before digestion with proteinase K at 50μg/ml in DPB at 37°C. The objective of proteinase K treatment is to remove proteins associated with nucleic acids which would otherwise prevent hybridization. For this reason, proteinase K should be present in the solution at maximal levels. It was found that 50μg/ml was optimal, in that 100μg/ml of proteinase K resulted in poor section adhesion. Proteinase K digestion was performed by removing slides from the Copland jar, and incubating 100μl of the enzyme in DPB on sections held in a humidified slide chamber for 30 minutes. Slides were then returned to the Copland

jar and the enzymatic reaction stopped with 0.2% glycine in DPB for 30 seconds. A brief wash with DPB was then followed by post-fixation in 4% paraformaldehyde in DPB for 10 minutes at room temperature. After three 15 min washes with DPB, slides were desiccated by washing twice with absolute alcohol and placing the Copland jar into a vacuum desiccator.

Pre-hybridization

Slides were then removed from the Copland jar, and placed into a humidified slide chamber, for pre-hybridization. This was necessary to minimize non-specific binding of labelled probes during hybridization. Where this was not done, labelled probe bound to both nuclear and cytoplasmic structures, giving high background levels. Each slide received 11µl of pre-hybridization solution. This pre-hybridization solution was prepared by mixing 10µl of hybridization solution (containing: Denharts solution, salmon sperm DNA and 20 X SSC) with 10µl of formamide and 2µl of RNAsin equivalent to 60 units of RNAse inhibitory activity. Sections were covered with pre-hybridization solution under a siliconized coverslip, and incubated at 42°C for 1 hr prior to hybridization.

Hybridization

The humidified slide chamber was removed from the incubator, and floated on water heated to 51°C. Where this was not done, background labelling, similar to that observed in the absence of a pre-hybridization step, occurred. It is possible that this was due to low affinity interactions occurring at room temperature during application of the labelled probes.

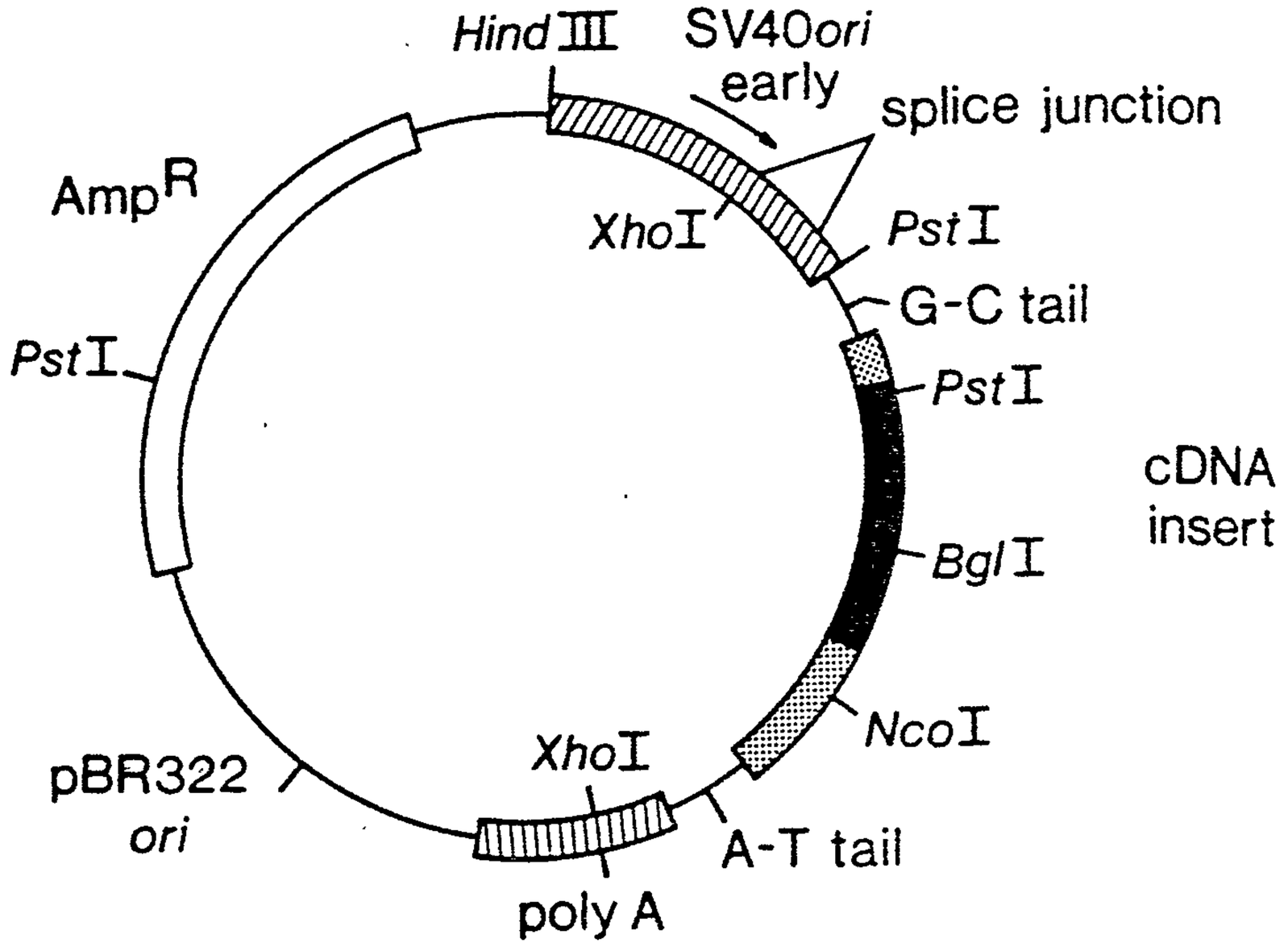
DNA probes were then applied to each slide by sliding the coverslip, covering the pre-hybridization solution, off the slide, and replacing this with a new coverslip bearing an 11µl drop of hybridization solution. Bubbles were expelled with gentle pressure on the surface of the cover-slip. The hybridization solution was composed of 10µl of the pre-hybridization solution plus 1µl of DNA. This gave a final DNA concentration of at least 500ng/ml, with 10ng being applied to each slide. Probes prepared from plasmids were first denatured by heating to 95°C for 10 minutes and cooling in ice, before use. Hybridization was carried out overnight at 42°C in a humidified slide chamber.

Washing Procedure Prior to Detection of the Hybridization Signal

To remove coverslips, slides were dipped in 2 X SSC, and then immediately transferred to a Copland jar with 2 X SSC. In order to remove non-specifically bound probe, slides were washed for 30 min at room temperature in 2 X SSC, and then with decreasing concentrations of SSC at 68°C. Each of these high temperature wash steps was for 30 minutes, starting with one wash with 2 X SSC, two washes with 1 X SSC and finally two washes with 0.5 X SSC. Label was then detected as described above.

Controls

The labelled empty vector was used as a control against labelling of cells with plasmid sequences, in hybridizations probing for RNA coding for GM-CSF. To control against false positive signals resulting from non-specific binding of probe DNA to individual cells, consecutive sections were treated with RNAase type 1-A (Sigma). This treatment was performed immediately after treatment with glycine, for 1 hr at 37°C at a concentration of 1µg/ml in 20mM Tris-HCL, 1mM EDTA pH 7.6 (McCabe *et al.* 1986). Consecutive sections were treated with hybridization solution in the absence of labelled probe, and processed in the same manner as test sections, to control against non-specific labelling of cells with antibody.


Hind III
SV40ori
early
splice junction
Amp^R
XhoI
PstI
G-C tail
PstI
PstI
cDNA
insert
BglI
NcoI
XhoI
pBR322
ori
A-T tail
poly A

B

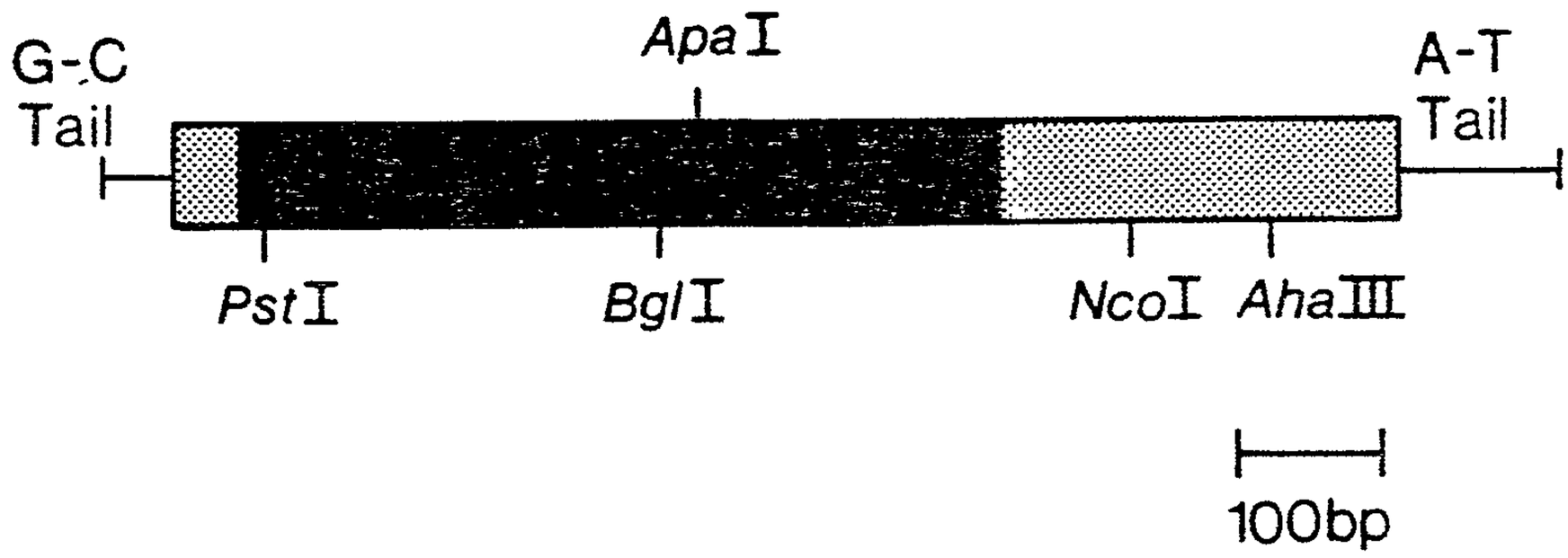
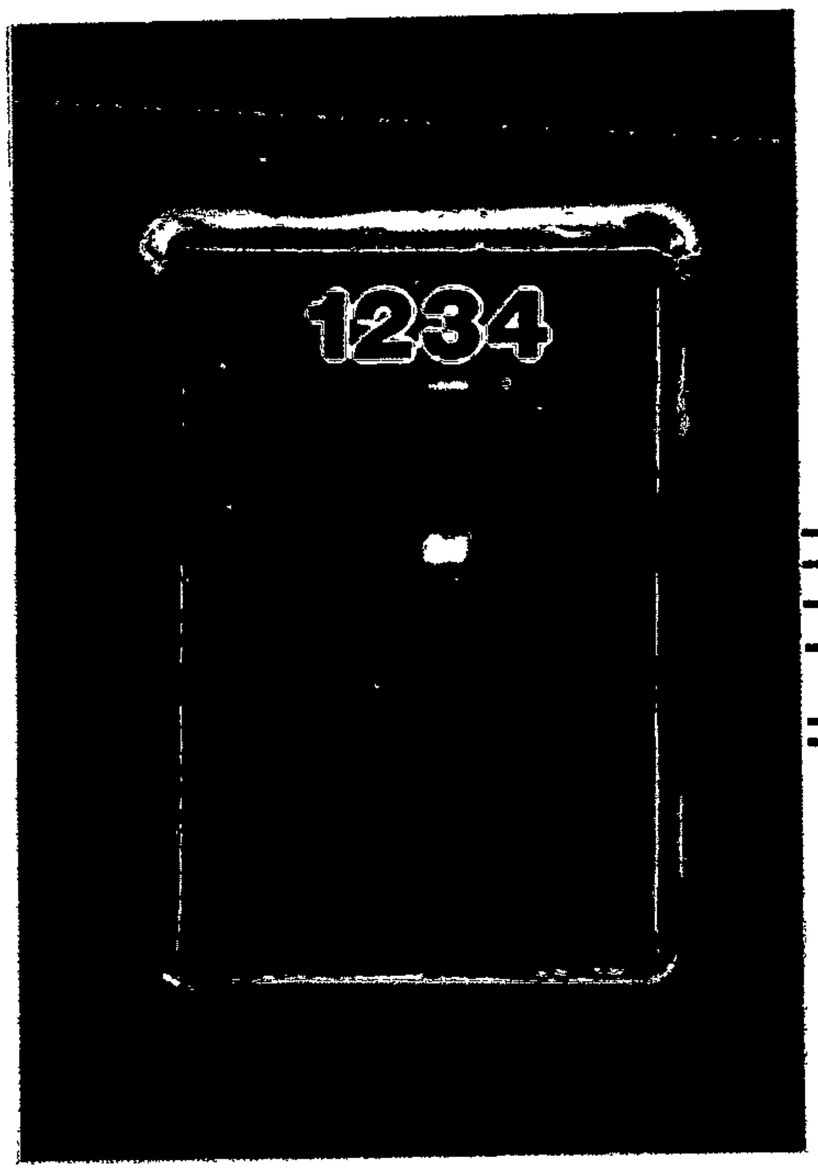

G-C
Tail
ApaI
A-T
Tail
PstI
BglI
NcoI
AhaIII
100bp

Figure A.1

Restriction map of the plasmid pcD-hGM-CSF (Lee *et al.* 1985).

The insert coding for GM-CSF is flanked by Xho-1 sites. This was exploited to clone out the insert, so as to prepare the plasmid pcD-Xho-1. pcD-Xho-1 was used as a control against binding of plasmid sequences in hybridization experiments. Also, the insert has a unique Apa-1 site, so that an inability to demonstrate an Apa-1 site in pcD-Xho-1, using pcD-hGM-CSF also helped to confirm that pcD-Xho-1 was free of the insert.



kbp

23.1
9.4
6.6
4.4
2.3
2.0

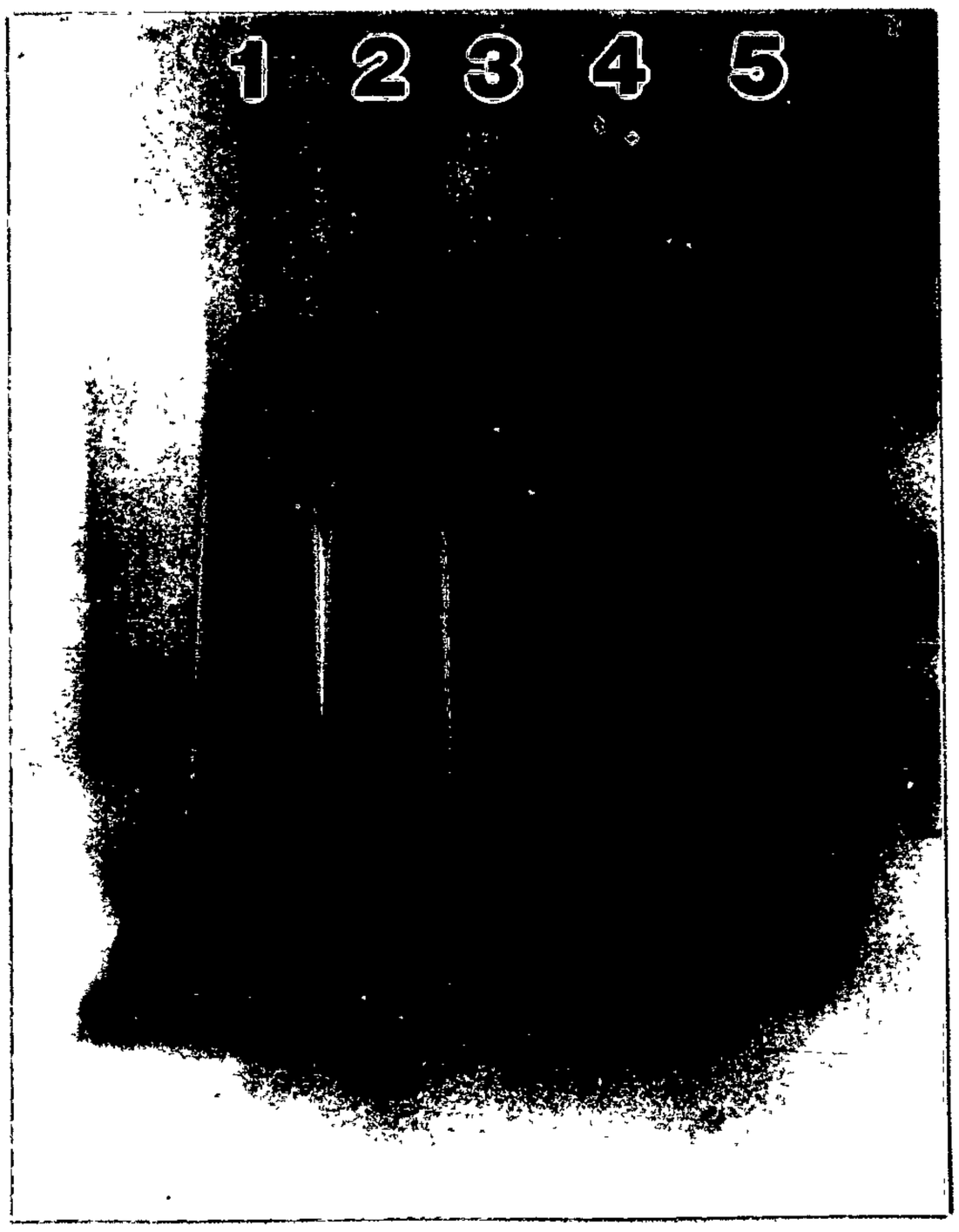


Figure A.2

Photograph of an agarose gel confirming removal of the GM-CSF insert from pcD-hGM-CSF. Samples have been treated with Hind III to linearize the plasmid prior to agarose gel electrophoresis. Lane 1 has pcD-Xho-1, lane 2 has pcD-hGM-CSF, lane 3 has MW marker and lane 4 has pcD-hGM-CSF.

pcD-Xho-1 is clearly smaller than pcD-hGM-CSF, indicating that the insert cloning for GM-CSF has been cloned out of the plasmid.

Figure A.3

Photograph of the Hybond Membrane blot used to confirm successful labelling of probes. Lane 1 has pcD-Xho-1, lane 2 has pcD-hGM-CSF, lane 3 has Multi-CSF, lane 4 has IL1ß, and lane 5 has IL1α.

Each of the probes has been successfully labelled, except for that for Multi-CSF, which is relatively weakly labelled. Nick translation with random priming has resulted in a wide range of sizes for the plasmid probes. Oligonucleotide probes have multiple bands, suggesting that different oligonucleotides in the probe cocktails label with differing efficiencies. The differing intensities of the smallest MW band in each lane, as well as the small differences in the positions of the smallest molecular weight band suggest that little or no unincorporated label remains in the labelled probe preparations.

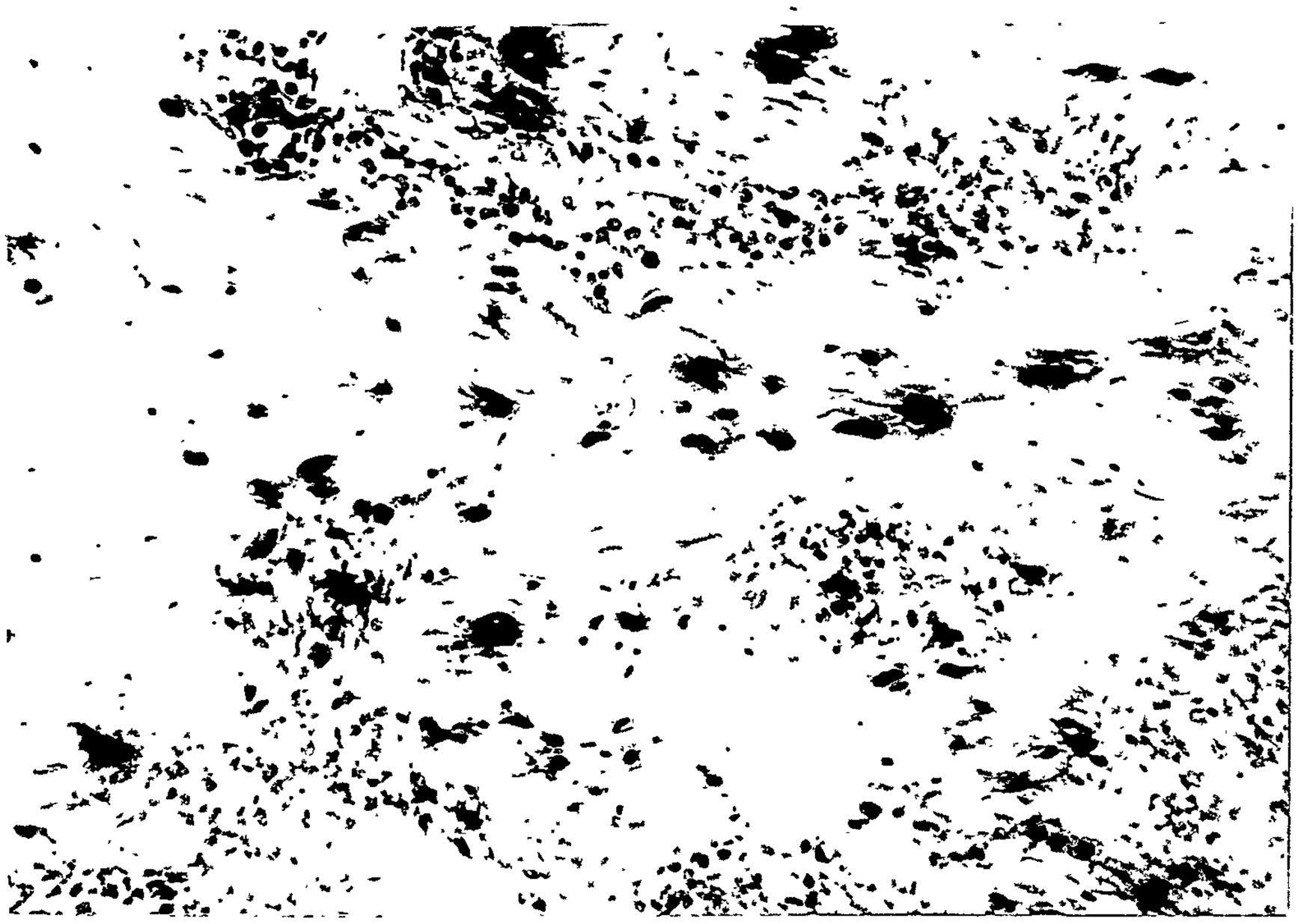
RESULTS

Periodontal Tissue

As seen in Figure A.4, isolated cells in the deeper connective tissues were strongly positive for each of the probes tested. The empty vector also labelled these cells, however, so that specificity of labelling for GM-CSF can not be claimed. PHELVs did not label, nor did any of the cells close to these vessels. Antibody alone did not give any labelling, so it can be concluded that something in the cells concerned, bound labelled DNA. However, when RNAse treated sections were studied, there was no detectable reduction in label. Raising the concentration of RNAase to 20 mg/ml also did not reduce labelling, suggesting that labelled DNA was bound to some cellular component other than mRNA.

Lymph Node Tissue

In lymph node tissues, as in periodontal tissues, isolated cells were positive for each of the biopsies studied. Some of these isolated cells were found in the walls of HEVs (Figure A.5). However, as in periodontal tissues, controls indicated that labelling was not specific for mRNA.



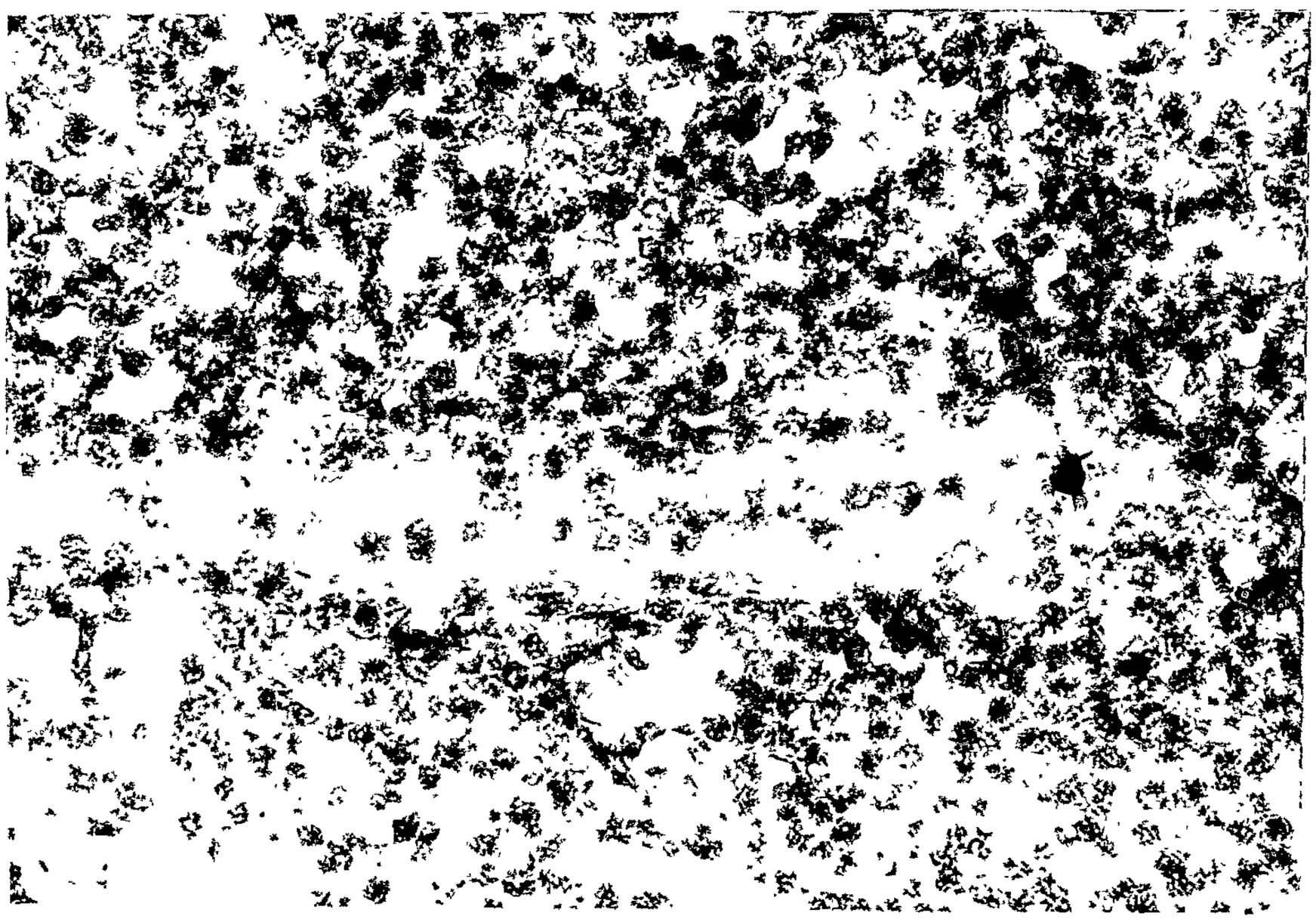


Figure A.4

Photomicrograph of a gingival biopsy from a patient with periodontitis, treated by in-situ hybridization for mRNA coding for IL1α.

Many cells within the body of the deeper connective tissues were positive. However, this labelling did not differ for any of the other probes, including the control pcD-Xho-1. This suggested that labelling was non-specific. (IL1α probe + Haematoxylin X 312)

Figure A.5

Photomicrograph of a lymph node specimen tested by in-situ hybridization for mRNA coding for IL1α.

Occasional cells were positive for the label, including cells in the walls of HEVs. However, control sections indicated that this labelling was non-specific. (IL1α + Haematoxylin X 312)

DISCUSSION

The specific labelling of cell cytoplasm, suggests that the labelling system used provides sufficient resolution to positively identify the exact cells producing specific proteins. This is a considerable advantage over radioactive techniques, which would not give the resolution required to be certain of the location of label amongst closely packed cells.

The hypothesis that PHELVs and HEVs in lymph node tissues produce IL1α, IL1ß, Multi-CSF and GM-CSF has not been conclusively tested in the experiments described in this appendix, as control sections indicated that non-specific labelling of cellular components other than mRNA could not be excluded.

The inability to prevent labelling with RNAase digestion, indicates that either: cells which were positive had labelled DNA bound to some component of their cytoplasm other than mRNA, or that positive cells had such large amounts of specific mRNA, that RNAase digestion could not eliminate enough signal to effect a detectable reduction in labelling. In regard to the latter possibility, some authors report a reduction rather than a total loss of signal with RNAase digestion (McCabe *et al. 1986*). With the non-radioactive labelling system used in this study, signal was amplified by the AP enzymatic reaction, so that if mRNA is not completely digested by RNAase, then perhaps enough signal could survive for the reduction in labelling to escape detection.

In the paper by Taka'cs *et al.* 1988, IL1α and IL1ß were found in many cells scattered throughout the pulp of mouse lymph nodes. In most cells this labelling was not strong. It is possible that the pattern of labelling seen in the study described in this appendix could be the result of both a loss of weak mRNA signal due to sub-optimal fixation procedures, and perhaps RNAase contamination of tissues or solutions during processing. On the other hand, signal in cells with very large amounts of mRNA could have survived the reduction of signal, leaving only a few strongly positive cells in sections.

However, two lines of evidence suggest that this optimistic view is incorrect. Firstly, each of the cytokines tested resulted in a similar pattern of labelling. This has particular relevance to the presence of Multi-CSF in periodontal cells which appear to be macrophages. This product is not reported to be produced by macrophages in-vitro, and casts doubt on the validity of this and other positive results. Further, labelling of similar cells with inappropriate DNA from the empty vector suggests an absence of specificity.

Regardless of the disappointing result of this experiment, it would seem reasonable to continue with this project. Although paraffin blocks from hospital archives have been used in such studies (Morley and Hodes 1988, Walker *et al.* 1989) suggesting that standard fixation and embedding procedures are sufficient to preserve mRNA for in-situ hybridization, other workers report considerable loss of signal during routine fixation and paraffin embedding (Penschow *et al.* 1987). It is likely that some if not all of the specific mRNA was lost during tissue processing of both periodontal and lymph node tissues. Perhaps alternative processing techniques such as frozen tissue cryostat sectioning, freeze-substitution, or even more optimal fixation and processing regimes with paraffin, would give more encouraging results.

Ideally, positive controls should be included in such a study. Initially, it was hoped that these would be internal, in that it seemed inevitable that some cells would produce these cytokines in the section. However, as was found in this study, isolated cells can have quite discrete labelling, and may still not bear the relevant mRNA. In retrospect, it would have been wise to establish cultures of cells, which could have been stimulated to produce specific cytokines. The production of the relevant cytokines could have been confirmed using bio-assays and southern-blots, and the cells then suspended in agarose or gelatine for processing into paraformaldehyde blocks. This should be done, if this project is continued.

In-situ hybridization is a very exacting technique, in which contamination of solutions or slides with RNAse can eliminate specific signalling. Once optimised, it is likely that this technique would provide convincing data regarding the synthetic activity of HECs. The identity of proteins potentially synthesised by HECs can be inferred from the literature describing non HEC EC products in-vitro, but would best be determined by the study of HEC cell cultures.

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EMENDATIONS

1) In Chapters 7 and 8 as well as in the Reference section, "Arger" should read "Ager".

2) The following passage should be inserted between the second and third paragraphs on page 7.17.

Cells obtained by the method of Ager (1987) display differential adhesion for lymphocyte subsets and have been used to show that endothelial cells must be viable in order for leukocytes to penetrate the vascular barrier (Ager and Mistry 1989). Also, a cell line has been established from similar cultures by Ise *et al.* (1988), who report that cell lines are easily prepared from passaged HECs. Ise *et al.* (1988) have also demonstrated differential adhesion of lymphocytes to their HEC cell line. Difficulties in the culture of passaged HECs were noted, however, in that the surface marker OX2 was lost with prolonged culture of the cells (Ise *et al.* 1989). Also, up to 10% of cells in earlier passages were fibroblasts, so that the need to select populations of cultured cells with a low incidence of fibroblastic cells was emphasised. Primary cultures of HECs were sensitive to batch variability in FCS, while passaged cells and cell lines could be cultured with many preparations of FCS (Ise *et al.* 1988). Although the incorporation of $^{35}SO_4$ was used as a marker of HEC phenotype and is significantly higher in a HEC cell line as compared with fibroblast cell lines (Ise *et al.* 1988), no data is provided comparing the relative labelling of primary cultures of HECs with passaged cells. In these studies HECs retaining some functional markers are successfully cultured as passaged cells (Ager 1987, Ager and Mistry 1988, Ise *et al.* 1988), however, the changes described with prolonged culture of HECs suggest loss of the specialised HEC phenotype.

Ager A, Mistry S. 1988. Interaction between lymphocytes and cultured high endothelial cells: an in vitro model of lymphocyte migration across high endothelial venule endothelium. Eur J Immunol 18:1265

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3) In Chapter 5 as well as in the References section, "Thome'" should read "Thomē".

4) On pages 5.1, 5.2 and 5.5, incorrect reference is made to a paper by Thomē (1898). This paper was miss-interpreted in that the phagocytic cells with cytoplasmic fibrillar networks described by Thomē (1898) were lymphatic endothelial cells and not HECs as stated in the text of the thesis. From this, the suggestion made on page 5.5 that the phagocytosed red blood cells reported by Thomē (1898) may have actually been the nuclei of lymphocytes in HEVs is clearly incorrect.

5) In the legends to Figures 7.2 and 7.4, "Anti-FVII" should read "Anti-FVIII".