ULTRASTRUCTURAL LOCALIZATION AND QUANTITATION OF BASAL LAMINA LAMININ AND TYPE IV COLLAGEN IN NORMAL RAT TONGUE MUCOSA AND INDUCED ORAL CARCINOMAS

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ERRATA

Page 5:	The following hypothesis has been added at the end of Chapter one (page 5) Hypothesis: There is no difference in the quantitative expression of basal lamina laminin and type IV collagen when normal oral mucosal epithelium and induced oral mucosal carcinomas are compared
Page 59, line 20:	"makers" should read "markers"
Page 130, line 21:	PH should read pH
Page 142, line 1:	delete "the"
Page 171, line 9:	"neuclei" should read "nuclei"
Page 173, line 6:	Insert "out" after "ruled"
Page 174, line 4:	Comma after carcinoma, <u>in that</u> ...delete comma after "that"
Page 179, line 1:	"representing" should read "representative"
Page 186, line 11:	Replace semicolon with colon after "planes"
Page 191, line 16:	"4 cm" should read "4 centimetre"
Page 218, line 2:	"Ultrastructural" should be ultrastructural
Page 219, line 1:	delete a after "is"
line 3:	Insert comma after "levels"
Page 220, line2:	Replace semicolon with colon after "aspects"
line 4:	Insert comma after "Accordingly"
line 7:	Insert comma after "satisfactory"
line 15:	Insert comma after "sections"
Page 221, line 6:	Insert comma after "(1988)"
line 10:	Delete to after "were"
line 16:	Insert comma after "methodology"
Page 224, line 5:	Insert comma after "image"
line 15:	"can not" should read cannot
Page 226, line 11:	Replace semicolon with colon after "exist"
line 13:	Insert inverted commas after "component"
line 23:	Delete "and" after "subsequently"
Page 232, line 3:	Add "the" after "out"
Page 258, line 11:	"neoplartic" should read "neoplastic"
Page 259, line 8:	"Enzyme-labeled" should read "Enzyme-labelled"

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ABSTRACT

In this study, special methods for the ultrastructural localization of basal lamina laminin and type IV collagen in animal oral mucosa were developed in a series of experiments aimed at determining optimum methods for tissue fixation, dehydration, embedding and immunoincubation. Furthermore, the distribution of laminin and type IV collagen in normal rat tongue mucosa and induced carcinomas was characterized. Quantitative descriptions of basal lamina laminin and type IV collagen in normal rat tongue mucosa and experimentally induced oral carcinomas were also established. The results of these studies provide a tool enabling further understanding of the molecular organization of normal oral mucosal basal lamina and basal lamina in squamous cell carcinomas.

To establish optimum tissue preparation and immunostaining protocols for the ultrastructural demonstration of basal lamina laminin and type IV collagen in rat tongue tissues, post-embedding (L.R.White resin) immunogold techniques were employed as basic methods to investigate the effect of a number of variables in tissue preparation and in immunostaining relative to morphological preservation and antigen retention. The variables investigated included:

1). Different fixatives (glutaraldehyde, paraformaldehyde and glutaraldehyde-paraformaldehyde mixture).

2). Variable fixative concentrations.
3). Different fixation additives (picric acid, polyvinylpyrrolidone and sucrose).
4). Different buffer systems (phosphate buffer and phosphate buffered saline).
5). Fixation osmolarity.
6). Dehydration methods.
7). Temperature of resin polymerization.
8). Primary antibody and gold-complex variables.
9). Blocking agents.

The results of this study indicate that the antigen expression of basal lamina laminin and type IV collagen is related to fixative used, fixative concentration, additive types and the temperature of resin polymerization. The choice of primary antibody and gold-complex, also, in some cases, affects immunostaining. The morphological preservation of tissue is associated with fixative used, fixation concentration, additive types, buffer system, fixation osmolarity, dehydration and the temperature of resin polymerization.

Observations on the distribution of laminin and type IV collagen in normal rat tongue mucosa and experimentally induced oral carcinoma were carried out by correlating gold particle distribution with morphological detail. It was shown that laminin and type IV collagen were essentially confined to the lamina densa of epithelial basal lamina in normal tissues and induced oral carcinomas, and that some fibroblasts in normal tissues and

induced oral carcinomas and carcinoma cells also expressed laminin. Laminin appeared also to be distributed in the stroma of neoplasms.

Quantitative analyses of basal lamina laminin and type IV collagen in normal rat tongue mucosa and experimentally induced oral carcinomas were undertaken using morphometric methods combined with immunogold techniques. Prior to the formal establishment of quantitative data, a pilot study was performed to establish a specimen sampling pattern, the determination of optimum magnification, the selection of a measurement grid, the establishment of structural criteria and the determination of sample size. Statistical analysis of quantitative data obtained in this study indicates that laminin is significantly increased in tumour basal lamina; whereas type IV collagen is significantly decreased in tumour basal lamina.

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PUBLICATIONS FROM THESIS

During preparation of this thesis manuscript the following papers have been prepared

1. De-Jun Jiang, David F. Wilson, Peter S. Smith, Angela M. Pierce and Ole W. Wiebkin. Distribution of basal lamina type IV collagen and laminin in normal rat tongue mucosa and experimental oral carcinoma: Ultrastructural immunolocalization and immunogold quantitation. Oral Oncology (European Journal of Cancer Part B) (In press)

2. De-Jun Jiang, David F. Wilson, Angela M. Pierce and Peter S. Smith. Tissue preparation and basal lamina antigen. Journal of Histochemistry and Cytochemistry (Submitted)

CHAPTER ONE: INTRODUCTION

Cancer is one of major diseases leading to the death of patients. Although the incidence of oral squamous cell carcinoma is relatively low compared with the other forms of cancer, the mortality of oral carcinoma is still a significant problem world-wide. It is known that the lethality of oral cancer resides chiefly in its ability to invade and metastasize. The invasion and metastasis of neoplastic epithelial cells from epithelial tissues such as epidermis, cervical mucosa, breast, oral mucosa and other sites are believed to result from pathological interaction between the neoplastic cells and the extracellular matrix including the basal lamina.

Basal lamina is a "sheet" of extracellular matrix separating parenchymal cells from the adjacent connective tissue and is found in every organ of the body. To date, a number of components namely laminin, type IV collagen, heparan sulphate, fibronectin, entactin/nidogen and tenascin have been identified in the basal laminae of various tissues. Laminin and type IV collagen constitute the major components of basal lamina.

Although the role of basal lamina in relation to the behaviour of malignant neoplasms of epithelial origin and the significance of basal lamina changes is not well understood, it is considered (Rich and Reade, 1992) that investigation of basal lamina changes in epithelial neoplasms is of relevance to a better understanding of the biology of cancers and that elucidation of these changes will possibly provide reliable predictors of malignancy in its early stages (i.e. in its preinvasive stages as dysplasia and carcinoma in situ). Previous studies have described changes in

basal lamina morphology and its components including laminin and type IV collagen in a variety of malignant neoplasms. However, many descriptions of the changes of basal lamina components are based on the staining patterns seen using immunohistochemical techniques at the optical microscopic level. Moreover, most observations are qualitative. The ultrastructural identification of basal lamina components including laminin and type IV collagen have been reported using immunogold techniques in a variety of tissues. However, there is no information available regarding the ultrastructural localization of basal lamina laminin and type IV collagen in oral mucosa, and then there is a paucity of data describing the presence, distribution and quantitative aspects of these basal lamina components in normal human mucosa, human neoplasms and in experimentally induced carcinomas.

By establishing the characteristics of these basal lamina components in normal homoeostatic conditions and in neoplasms, it is felt that description of these basal lamina components in normal oral mucosa and in experimentally induced oral squamous cell carcinomas will be of value in providing a better understanding of the molecular organization of basal lamina in normal oral mucosa and in the understanding of the pathobiology of neoplastic epithelial cell invasion. This information may be of value in improving diagnostic and prognostic techniques in relation to malignant neoplastic epithelial lesions in mucosal sites such as the oropharynx and cervix and in the epidermis.

The ultrastructural identification and quantitation of most tissue antigens has been carried out using immunogold techniques. In

immunogold techniques, it is recognized that the immunolabelling of tissue antigens relies on two major factors namely tissue preparation techniques and immunostaining techniques. In pilot studies for the present investigations, it was found that no optimum tissue preparation and immunostaining methods described in the literature were directly applicable for the ultrastructural demonstration of basal lamina laminin and type IV collagen in rat tongue mucosa. Consequently in this study on the ultrastructural localization and quantitation of basal lamina laminin and type IV collagen in normal rat tongue mucosa and 4 Nitroquinoline1-oxide(4NQO)-induced oral carcinomas it was necessary to develop new techniques for tissue fixation and processing. To obtain optimum tissue preparation and immunostaining methods for the ultrastructural identification and quantitation of these basal lamina constituents, a number of investigations in relation to issues of morphological preservation and antigen retention were undertaken.

This thesis comprises five parts:

In part one (Chapter two), the biological properties and pathological changes of the extracellular matrix components including laminin and type IV collagen and associated enzymes are reviewed.

In part two (Chapter three), a series of experiments establishing special tissue preparation and immunostaining techniques are described. These experiments were carried out in order to

establish the optimum methods for the ultrastructural localization of basal lamina laminin and type IV collagen.

In part three (Chapter four), the methods developed in the previous section are used to localize and characterize the distribution of basal lamina laminin and type IV collagen in normal rat tongue mucosa and 4NQO-induced experimental oral carcinoma at the ultrastructural level.

In part four (Chapter five), quantitative analysis of the expression of basal lamina laminin and type IV collagen in normal rat tongue mucosa and 4NQO-induced experimental oral carcinoma is described using established morphometric methods and methods developed for this study.

In part five (Chapter seven), further studies are suggested related to issues such as tissue preparation, immunostaining and ultrastructural identification of other basal lamina antigens.

Hypothesis:

There is no difference in the quantitative expression of basal lamina laminin and type IV collagen when normal oral mucosal epithelium and induced oral mucosal carcinomas are compared

CHAPTER TWO: LITERATURE REVIEW

2.1.Introduction

Basal lamina is a "sheet" of extracellular matrix separating parenchymal cells from the adjacent connective tissue. Basal lamina is distributed in every organ of the body (Martinez-Hernandez and Amenta, 1983). To date, a number of components have been identified from basal laminae of various tissues including type IV collagen, laminin, heparan sulfate, fibronectin, entactin/nidogen, bullous pemphigoid antigen and tenascin (Stanley et al, 1982; Martinez-Hernandez and Amenta, 1983; Woodley, 1987; Fine, 1991). It is found that the composition of basal lamina and the quantity of the individual basal lamina components varies among tissues and among species. This phenomenon has been related to the architectural and functional heterogeneity of basal laminae in various tissues (Martinez-Hernandez and Amenta, 1983; Yurchenco and Schittny, 1990). Despite this, it is accepted that three major components namely type IV collagen, laminin and heparan sulfate proteoglycan are common to the basal laminae of various tissues (Hodge and Freeman, 1978; Thorgeirsson, Turpeenniemi-Hujanen and Liotta,1985; Lee, 1988; Yurchenco, 1990).

The visualization of basal lamina (basement membrane) has been effected by a variety of methods such as light microscopy {i.e. using haematoxylin and eosin (H & E) staining; periodic acid-schiff (PAS) staining; sudan black/acetone staining (Melcher, 1965)}, electron microscopy (Briggaman and Wheeler, 1975; Hodge and Freeman, 1978) and immunohistochemistry (i.e. optical

immunohistochemistry and immunoelectron microscopy) (Grimaud et al, 1980; Laurie et al, 1980; 1982).

Over the years, two terms have been used to describe the specialized extracellular matrix structure "separating" epithelia from connective tissue namely " basement membrane " and " basal lamina ". It is now recognized that the " basement membrane " as seen by light microscopy includes the " basal lamina " described by electron microscopy, reticular fibres and "ground substance" (Hodge and Freeman, 1978). It is now generally held that the term " basement membrane " is appropriately used in light microscopy; whereas the term " basal lamina " is preferably employed in electron microscopy (Squier, Johnson and Hopps, 1976).

The ultrastructural features of basal lamina depend on the tissue site being examined. In general, the basal lamina (except in the lung, kidney, lens capsule, Descemet's membrane, Reichert's membrane and murine parietal yolk sac) consists of two regions the lamina lucida (lamina rara in kidney) and the lamina densa and associated structures (i.e. anchoring fibrils, hemidesmosomes containing a basal attachment plaque, tonofilaments and sub-basal densa plate) (Briggaman and Wheeler, 1975; Thorgeirsson, Turpeenniemi-Hujanen and Liotta, 1985; Fine, 1991) (Fig.1). In normal situations, basal lamina as shown by electron microscopy is usually a continous layer (Chen and Squier, 1984)

The width of basal lamina varies from species to species and from tissue to tissue. Skin basal lamina is approximately 100 nm thick (Fine, 1991); aorta basal lamina (rodent and porcine) is 17-

55 nm thick (Heickendorff, 1989) and oral mucosa basal lamina is 100 nm thick (lamina densa 55 nm, lamina lucida 45 nm) (Chen and Squier, 1984). The lamina lucida and lamina densa in most tissues are considered to be approximately equal in width (approximately 40-60 nm)(Martinez-Hernandez and Amenta, 1983).

A variety of immunohistochemical techniques have been used for the demonstration of basal lamina (basement membrane) at the optical microscopic level and at the electron microscopic level namely immunofluorescence, immunoperoxidase, immunoalkaline phosphatase and immunogold methods.

The precise macromolecular organization of basal lamina is not well established. Possible models have been proposed by a few authors (Woodley, 1987; Yurchenco and Schittny, 1990). In Woodley's model, type IV collagen molecules form a lattice network in the lamina densa by means of their 7S domains; laminin transverses the lamina densa and lamina lucida and binds cells and type IV collagen by the central intersection of arms; heparan sulfate proteoglycan straddles the lamina densa. However, Yurchenco and Schittny (1990) considered that type IV collagen and laminin formed respective polymer networks and that the two polymer networks were linked by a sulfated glycoprotein (i.e. entactin). A heparan sulfate proteoglycan anchored to laminin and type IV collagen through their glycosaminoglycan chains.

The functions of basal lamina (basement membrane) may depend on its structure and composition. At present, it is recognized that

basal lamina plays many important roles including physical support, ultrafiltration (kidney), cell attachment, migration and morphogenesis (Martinez-Hernandez and Amenta, 1983; Szarfman et al, 1982; Timpl and Dziadek, 1986; Lee, 1988; Heickendorff, 1989; Yurchenco and Schittny, 1990).

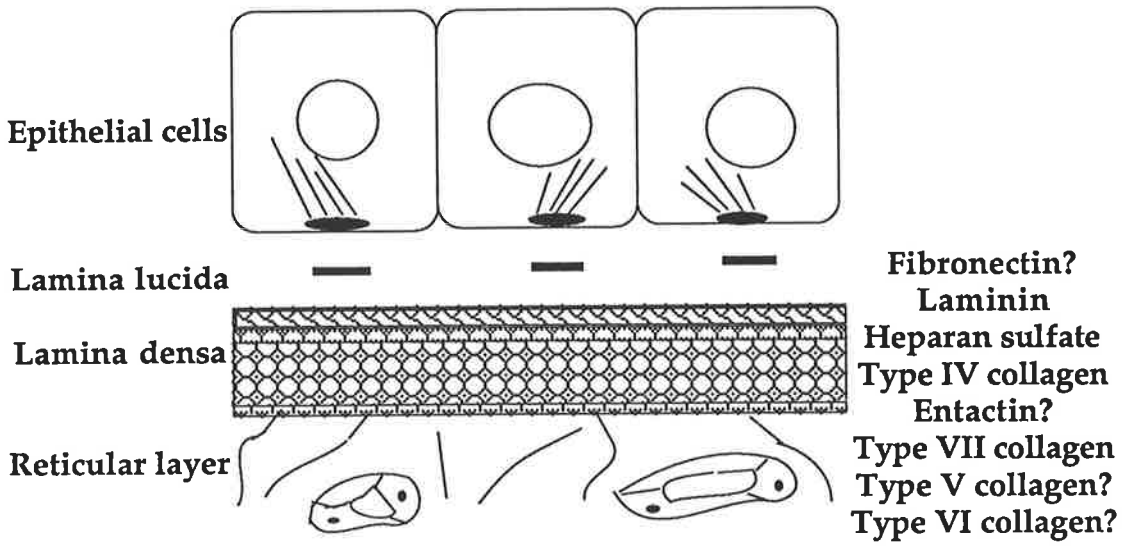


Figure 1. Schematic diagram of basal lamina (modified from Szarfman et al, 1982)

2.2. Basal lamina components

2.2.1 Type IV collagen

Type IV collagen is ubiquitously distributed in basal laminae. It has been found that the type IV collagen molecule contains two genetically distinct chains (Pollner et al, 1990) and is characterized by triple-helical domains interrupted by non-helical domains (Timpl and Martin, 1982). It is thought that the

structural features of the type IV collagen molecule allow it to be flexible and permit the formation of a mesh-like network in the molecular organization of basal lamina (Burgeson, 1988; Uitto, Olsen and Fazio, 1989). Owing to the existence of non-collageneous domains in the molecule, type IV collagen is thought to be sensitive to proteolytic enzymes (Stanley et al, 1982).

The exact organization of the type IV collagen molecule in basal lamina architecture is not yet fully understood. Several models have been proposed namely 1). an alternating arrangement of collagenous and noncollagenous protein sheets (Kefalides, 1973), 2). a regular network (Timpl et al, 1982), 3). an irregular polygonal network (Yurchenco and Schittny, 1990). Despite this, it has been agreed that the four amino-terminals of the collagen molecule are linked together to form a stable complex, the so-called 7S domain, in which the four short triple-helical strands are overlapped in a parallel fashion into four arm tetramers (Timpl and Martin, 1982; Yurchenco and Schittny, 1990) (Fig. 2).

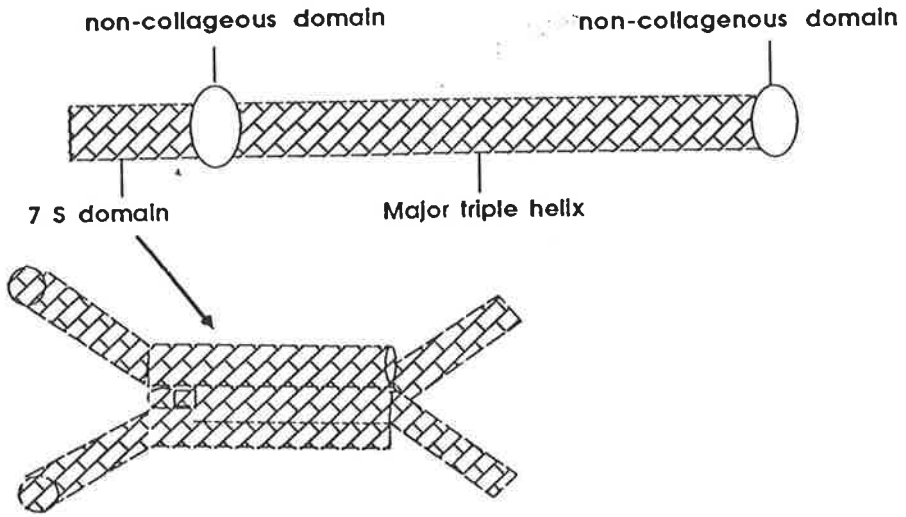


Figure 2. Schematic diagram of type IV collagen and 7S domain (modified from Timpl et al,1982)

The 7S domain is characterized by bacterial collagenases resistance and by stronger immunogenicity compared with intact type IV collagen (Risteli, Rohde and Timpl, 1981; Stanley et al, 1982). Several investigators have reported that type IV collagen in various tissues can be successfully localized using anti-7S collagen antibodies (Kallioinen, 1985; Soini, Autio-Harmainen and Miettinen, 1989).

The production of type IV collagen occurs in a variety of cells such as epithelial cells, endothelial cells, muscular cells, adipose cells and their malignant counterparts, carcinomas and sarcomas (Kleinman et al, 1982; Martinez-Hernandez and Amenta, 1983; Heickendorff, 1989). The main sources of type IV collagen for immunological and biochemical studies are usually Engelbreth-Holm-Swarm (EHS) tumour matrix and human placenta (Timpl et al, 1979; Bornstein and Sage, 1980). Specific fragments of type IV collagen molecule can be obtained by the digestion using various enzymes.

Type IV collagen has been demonstrated in the basal laminae of many tissues such as blood vessels, kidney, lung, intestine, muscle, epidermis, the enamel organ of developing teeth, nerves, lens capsule, human placenta, trachea, liver, hair follicles and sweat glands using immunofluorescence, immunoperoxidase light and electron microscopic techniques (Grimaud et al, 1980; Laurie et al, 1980; 1982). Different patterns of staining have been noted using specific monoclonal antibodies against type IV collagen in the different tissues. This phenomenon is thought to be due to the heterogeneity of molecular structure (Foellmer, Madri and

Furthmayr, 1983). The exact location of type IV collagen in basal laminae of various tissues has been identified by immunocytochemical techniques (i.e. immunogold electron microscopic methods). For instance, Bendayan (1985) and Grant and Leblond (1988) demonstrated that type IV collagen was concentrated in the lamina densa of the basal lamina in sites such as the kidney, colon and enamel organ. Jungell (1990) in an immunoelectron microscopic study on oral mucosa basal lamina using anti-type IV collagen antibody and a peroxidase techniques claimed to identify type IV collagen in the lamina densa and lamina lucida.

Type IV collagen in basal lamina may play important roles in the organization of basal lamina as a consequence of its structural features (eg flexibility) and specific binding sites for the attachment of other basal lamina components. In normal situations and pathological circumstances (i.e.tumour invasion and metastasis), type IV collagen has also been thought to have functions possibly related to cell differentiation (Kleinman et al, 1982; Szarfman et al, 1982).

2.2.2 Laminin

Laminin, a large glycoprotein, is also ubiquitously distributed in basal laminae. It is now recognized that there are at least three genetically distinct chains in the laminin molecule namely an A chain (GP1) and B1 and B2 chains (GP2). These chains are linked by disulfide bonds (Kleinman et al, 1985; Yurchenco, 1990; Yurchenco and Schittny, 1990).

Based on observation of the laminin molecule by rotary shadowing techniques, it has been suggested that laminin is a cross-shaped molecule comprising three short arms (MW 200,000) and one long arm (MW 400,000) (Lee, 1988). The four arms of the laminin molecule have the different binding sites for cells and other extracellular matrix components. The binding sites for type IV collagen are on the short arms; whereas the long arm serves as a binding site for heparin and heparan sulfate proteoglycan. The central intersection of the arms serves as the binding site for cells and entactin (Woodley, 1987; Campbell and Terranova 1988; Yurchenco, 1990).

The isolation of laminin was initially accomplished from an embryonic carcinoma cell line and EHS mouse sarcoma (Chung, Freeman and Braginski, 1977; Timpl et al, 1979). Laminin is susceptible to various tissue proteinases such as plasmin, cathepsin and stromelysin (Jones and Clerck, 1982; Pauli et al, 1983; Tryggvason, Höyhtyä and Salo,1987). Intact laminin is very difficult to isolate. Therefore, the fragments of laminin are usually obtained by the digestion of various proteinases such as pepsin, thrombin, serine protease, trysin and elastase (Risteli, Rohde and Timpl, 1981; Campbell and Terranova, 1988).

Laminin can be synthesized by epithelial cells, endothelial cells, muscle cells and fibroblasts (Jones and Clerck, 1982; Campbell and Terranova, 1988; Chung, 1991).

Using a variety of antibodies against the different fragments of the laminin molecule (i.e. P1 and P2), the distribution of laminin

in a number of tissues has been demonstrated (Risteli, Rohde and Timpl, 1981; Herken and Miosge, 1991). For instance, laminin is described as being localized in the lamina densa and the lamina lucida of glomeruli, muscle and intestinal basal lamina and in the lamina lucida of skin and oesophagus basal lamina using immunoperoxidase electron microscopic techniques (Foidart et al, 1980; Martinez-Hernandez and Chung, 1984). Laminin is reportely found in both the lamina densa and lamina lucida of basal laminae in glomeruli, colon and enamel organ using immunogold electron microscopic methods (Abrahamson, 1986; Grant and Leblond, 1988; Desjardins and Bendayan, 1989).

Based on the heterogeneous distribution of laminin in basal laminae in different sites, in different basal laminae of the same tissue and at different stages of tissue and organ development, it has been suggested that heterogeneity of the structure of laminin may be a feature of this molecule (Desjardins and Bendayan,1989; Yurchenco and Schittny, 1990).

A number of roles for laminin have been suggested. They include the maintenance of basal lamina components, cell attachment, cell morphology, cell growth, cell differentiation, cell migration, wound healing, autoimmune disease, bacterial adhesion, tumour cell invasion and metastasis and anti-bacterial and anti-tumour effects (Kleinman et al,1985; Campbell and Terranova,1988).

The mechanism for cell-laminin interaction has been attributed to specific membrane glycoproteins which are referred to as laminin receptors (Timpl and Dziadek, 1986; Campbell and Terranova,

1988). Laminin receptors have been isolated from a mammalian cell membrane (Malinoff and Wicha, 1983), normal tissue cells and neoplastic cells (Akiyama, Nagata and Yamada,1990). Interlinking between cytoskeletal elements and the extracellular matrix components in the process of cell growth and differentiation is thought to be main role of laminin receptors (Juliano, 1987; Akiyama, Nagata and Yamada,1990).

2.2.3 Heparan sulfate

Heparan sulfate is one of a number of macromolecular proteoglycans which are characterized by a core protein linking covalently to glycosaminoglycans. It has been established that heparan sulfate proteoglycan is distributed at the cell surface and within basal laminae (Comper and Laurent, 1978; Trelstad, 1985; Heickendorff, 1989; Horiguchi et al, 1989; Kogaya et al, 1990; Yurchenco and Schittny, 1990). Basal lamina heparan sulfate proteoglycan has been isolated from normal tissues (i.e. glomeruli, blood vessels) and EHS tumour (Kanwar and Farquhar, 1979; Hassell et al, 1980).

The molecule of heparan sulfate proteoglycan is characterized by polyanionic chains. This molecular feature confers its charge-dependence in basal laminae. As a consequence of this anionic charge characteristic of heparan sulfate proteoglycan, the demonstration of this molecule can be carried out using cationic probes (i.e. alcian blue, ruthenium red, poly-L-lysine-gold) (Scott, 1973; Lüllmann-Rauch, 1989; Goode, Shires and Davison, 1992).

Several methods have been used for localizing heparan sulfate proteoglycan in basal lamina. For instance, Kogaya and colleagues (1990) demonstrated by a high-iron diamine thiocarbohydrazide silver proteinate staining technique that heparan sulfate proteoglycan was present in the lamina densa of the basal lamina in nerves, muscle and capillaries, mainly in the lamina lucida of the basal lamina in gingival junctional epithelium and in the lamina densa and the lamina lucida of basal lamina in oral epithelium. Using antibodies against the core protein of heparan sulfate proteoglycan, heparan sulfate proteoglycan has been found in the lamina densa and the lamina lucida of the basal lamina in human epidermis using a immunoperoxidase electron microscopic method (Horiguchi et al, 1989) and in the lamina densa and the lamina lucida of the basal laminae in the kidney, colon, enamel organ and vas deferens using immunogold electron microscopic techniques (Desjardins and Bendayan, 1989; Grant and Leblond, 1988).

On the basis of the molecular structure and distribution characteristics of heparan sulfate proteoglycan, it is thought that heparan sulfate proteoglycan plays important roles in interlinking other extracellular matrix components (eg. collagen, laminin, fibronectin), acting as a charged-dependent selective sieve for filtration and serving as a repository for basic fibroblast growth factor in basal lamina (Trelstad, 1985; Yurchenco and Schittny, 1990).

2.2.4 Fibronectin

Fibronectin is also a glycoprotein and shares many biological properties with laminin. Two types of fibronectin which are referred to as plasma fibronectin (Vitronectin) and cellular fibronectin respectively are distributed in plasma and on cell surfaces (Pauli et al, 1983; Yamada, 1983). It has been shown in immunologic and biochemical studies that there are considerable similarities in molecular structure between the two forms of fibronectin within one species; However, there are considerable differences from species to species (Yamada et al, 1985). To date, there has been much controversy about its location in basal laminae among tissues (Madri et al, 1980; Jones and Clerk, 1982; Martinez-Hernandez and Amenta, 1983; Heickendorff, 1989). For instance, Madri and colleagues (1980) using immunoferritin electron microscopic staining techniques reported that fibronectin was in the lamina densa and the lamina lucida of glomerular basal lamina. Jungell (1990) using an immunoperoxidase electron microscopic staining technique described that fibronectin was distributed throughout the connective tissue of oral mucosa. Immunogold electron microscopic staining by Lin and Essner (1990) demonstrated that fibronectin was scarce in retinal capillary basal lamina.

Suggested roles for fibronectin may include cell-binding, fibroblast attachment to collagen and cell behaviour (McDonald, 1982; Pauli et al, 1983; Yamada, 1983; Yamada et al, 1985; Timpl and Dziadek, 1986; Heickendorff, 1989).

Specific fibronectin receptors have been identified on cell membranes (Yamada et al, 1985). These receptors have been isolated from avian and mammalian tissues (Akiyama, Nagata and Yamada,1990; Juliano, 1987).

2.2.5 Entactin/Nidogen

Entactin / nidogen is a noncollagenous protein. Both proteins are now considered to be the same macromolecule (Yurchenco and Schittny, 1990). Entactin/nidogen was initially isolated from a mouse teratocarcinoma cell line (Carlin et al. 1981). This protein is susceptible to proteolysis (Yurchenco and Schittny, 1990). Using immunoperoxidase electron microscopic techniques, entactin has been demonstrated in the basal laminae of some tissues such as kidney and intestine (Martinez-Hernandez and Chung, 1984). Yurchenco and Schittny (1990) suggested that entactin/nidogen might facilitate binding activity between matrix and cells.

2.2.6 Bullous pemphigoid antigen

Bullous pemphigoid antigen has been only identified in the basal lamina of stratified squamous epithelial insites such as the skin, gall bladder and trachea (Stanley et al, 1982; Woodley, 1987). Cell-cultured studies demonstrated that this antigen was synthesized by epidermal cells (Woodley et al, 1987; Stanley et al, 1982). Stanley et al (1982) reported that the bullous pemphigoid antigen was located in the lamina lucida of the basal lamina. Owing to the fact that the bullous pemphigoid antigen is

often identified in the serum of patients with the subepidermal blistering disease bullous pemphigoid, it is thought that bullous pemphigoid antigen may play an important role in epidermal cell-matrix component interaction (Stanley et al, 1982).

2.2.7 Tenascin

Tenascin once referred to as myotendinous antigen was first isolated from the conditioned media of chick embryo fibroblasts and muscle culture (Chiquet and Fambrough, 1984). It is now recognized that tenascin is an extracellular matrix glycoprotein and comprises several disulfide linked subunits (Chiquet and Fambrough, 1984).

Tenascin is not susceptible to bacterial collagenase and has a low affinity for fibronectin and collagen, but seemingly a high affinity for chondroitin sulfate proteoglycan (Chiquet and Fambrough, 1984). Using specific antibody and immunofluorescence techniques, tenascin has been demonstrated in muscle, periosteum, tendon, around budding epithelia in foetal hair follicles, teeth and mammary glands, in the papillary tips of connective tissue of the palate, gingiva and dorsal lingual mucosa and in the basal lamina of ventral tongue, buccal, labial and vestibular mucosa (Chiquet and Fambrough, 1984; Chiquet-Ehrismann et al, 1986; Sloan et al, 1990). Schalkwijk et al (1991) also described tenascin in the upper dermis adjacent to the basal lamina of skin using an immunoperoxidase light microscopic staining. Recently, it has been reported that the expression of tenascin in neoplastic tissues is different from that in normal

tissues using immunofluorescence and immunoperoxidase light microscopic staining techniques (Chiquet-Ehrismann et al, 1986; Howeedy et al, 1990; Schalkwijik et al, 1991). The roles of tenascin have been not well known, but it is believed that tenascin is an important extracellular matrix component in mesenchymal cell condensation, epithelial growth and mesenchymal-epithelial interactions (Chiquet-Ehrismann et al, 1986; Sloan et al, 1990).

2.3. Non-basal lamina extracellular matrix components

The extracellular matrix principally consists of a group of macromolecular fibrous and nonfibrous proteins which, in turn, can be divided into four categories: collagens, elastins, proteoglycan and glycoproteins. These macromolecules are characterized by their own specific distribution and conformation in various tissues (Jones and Clerck, 1982). The complicated and delicate interactions between the extracellular matrix components enable them to maintain their own integrity and establish a microenviroment for cell differentiation and growth in physiological situations (Uitto, Olsen and Fazio, 1989).

Aside from the specialized basal lamina extracellular matrix components already described, several other extracellular matrix components are also distributed in tissues and some of them have been related to basal lamina in distribution and biological properties. In this section several other extracellular matrix components (so-called non-basal lamina extracellular matrix

components) including other collagens and proteoglycans are reviewed.

2.3.1 Other collagens

Type I, II, III collagens

Type I collagen, type II and type III collagens are classified as interstitial collagens (Burgeson, 1982). Type I collagen is distributed in all connective tissues being more abundant in dermis, tendon, bone, dentin and periodontal ligament (Miller and Gay,1982; Jones and Clerck, 1982; Becker et al,1991). Several types of cell namely fibroblasts, osteoblasts and smooth muscle cells are thought to synthesize type I collagen (Kühn, 1982). Type III collagen is frequently distributed with type I collagen in tissues and is more abundant in foetal tissues. Some authors report that smooth muscle cells and myofibroblasts might be sources of type III collagen (Barsky et al, 1982).

The ratio of type I collagen / type III collagen in tissues has been related to the developmental stage of the tissue. A high ratio of type I collagen/type III collagen occurs in mature tissues (Burgeson, 1982). Ultrastructural immunoperoxidase staining has demonstrated that type I collagen and type III collagen are distributed in different regions of connective tissues (Grimaud et al, 1980). Becker et al (1991) using an immunogold electron microscopic method described that type I collagen was found on all cross-striated fibrils entering tooth cementum and in cementum proper; whereas type III collagen was demonstrated on

all cross-striated fibrils in the periodontal ligament remote from the cementum.

In the process of formation of collagen fibres, it is thought that type III collagen regulates fibre diameter by interference with lateral interaction of fibres by nonhelical structural domains (Burgeson, 1982; Uitto, Olsen and Fazio, 1989). Type III collagen together with type I collagen contribute to tissue extensibility (Burgeson and Morris,1987), .

Type II collagen is mainly distributed in hyaline cartilage and its fibres are of smaller diameter than type I collagen fibres (Jones and Clerk, 1982; Kühn, 1982). Burgeson (1982) reported that the distribution of type II collagen was separate from other types of collagen in cartilage, except in sites such as the intervertebral discs and in fibrocartilage and that type II collagen contributed to resistance to compression by being embedding in proteoglycan-rich matrix.

An additional form of type I collagen has been described. This is the so-called " type I trimer " which is different from the type I collagen in that its molecular structure comprises three identical chains. This collagen was isolated from cultured chondrocytes, gingival fibroblasts, rat odontoblasts, human skin and virus-induced neoplasm (Bornstein and Sage, 1980). The function of type I trimer is unknown. However, it is suggested that type I trimer may be associated with cell phenotype and cell differentiation (Bornstein and Sage, 1980).

Type V collagen

Type V collagen was initially isolated from human placental membrane (Chung, Rhodes and Miller,1976) and five molecular chains (A,B,C,E,F,) have been identified. This collagen has been demonstrated in the periodontal ligament and retinal capillaries by immunogold electron microscopic staining (Lin and Essner, 1990; Becker et al, 1991) and in the interstitial connective tissue of oral mucosa using immunofluorescence (Becker et al, 1986). Chondrocytes, smooth muscle cells and myofibroblasts may be involved in the synthesis of type V collagen (Barsky et al, 1982; Miller et al, 1982; Küha, 1982; Sage, 1982).

Type V collagen shares some features with the interstitial collagens and type IV collagen (Küha, 1982), and can form a fibre structure by the end-to-end interaction of molecular units with limited lateral interactions (Burgeson, 1982; Küha, 1982).

The function of type V collagen is poorly understood. Recently, there have been two suggestions. First, it is thought that type V collagen may play a role in the interaction between cells and matrix components (Burgeson, 1982; Heickendorff, 1989; Küha, 1982). Secondly, type V collagen fibres may serve as core for the formation of major fibrils in connective tissue (Becker et al, 1991).

Type VI collagen

Type VI collagen is also referred to as "intima collagen" (Chung,

Rhodes and Miller,1976). Three genetically distinct chains have been identified (Burgeson, 1988; Doliana, Bonaldo and Colombatti, 1990). Immunofluorescence staining by Becker and colleagues (1986) demonstrated that type VI collagen is distributed in the connective tissue of oral mucosa including the interstitial space between muscle and salivary glands, in dentine and in bone. The site distribution of type VI collagen on the unbanded microfibrils of periodontal ligament has been identified using immunogold electron microscopic techniques (Becker et al, 1991). It is thought that cultured fibroblasts and smooth muscle cells synthesize type VI collagen (Heickerdorff, 1989; Kielty et al,1990).

A possible role for type VI collagen has been suggested namely the interlinking of cells, basal lamina and larger banded fibre bundles (Burgeson, 1988; Heickendorff, 1989; Keene, Sakai and Burgeson, 1991).

Type VII collagen

Type VII collagen is thought to constitute the major component of anchoring fibrils (Regauer et al,1990). Keratinocytes and normal amniotic epithelial cells are believed to synthesise type VII collagen (Regauer et al,1990; Burgeson et al, 1990).

Using specific antibodies (i.e.LH 7.2), type VII collagen has been detected in the connective tissue adjacent to the epithelial basal laminae of many tissues such as skin, oral mucosa, oesophagus, bronchus, breast, vaginal mucosa and cornea (Burgeson, 1988;

Burgeson et al, 1990; Kirkham et al, 1989; Fine, 1991; Wetzels et al, 1991; Uitto, Olsen and Fazio, 1989). It has been suggested that type VII collagen extends into basal lamina amorphous anchoring plaques and there interacts with type IV collagen (Burgeson et al, 1990; Uitto, Olsen and Fazio, 1989). Thus the stabilization of basal lamina and the underlying connective tissue may be maintained by type VII collagen (Gammom, 1991; Wojnarowska et al,1991).

Type IX, and XII collagens

Type IX collagen was first isolated from cartilageous tissues and cornea (Gordon et al, 1989). The molecule of type IX collagen contains three triple-helical domains and four non-helical domains (Shimokomaki et al, 1990). The structural features of the molecule allow type IX collagen to be very flexible. The non-helical domains are susceptible to proteolytic enzymes.

Type IX collagen is found on the surface of type II collagen bundles and has interaction with type II collagen in cartilage (Gordon et al, 1989; Shimokomaki et al, 1990; Ye et al, 1991). This collagen may provide link sites for interacting with cells and other matrix components (Burgeson, 1982; Gordon et al, 1989).

Type XII collagen has many structural features in common with type IX collagen. For instance, both collagens contain triple-helical domains and non-helical domains (Gordon et al, 1990). Type XII collagen and type IX collagen do not form fibrils, but are associated with fibrils (Burgeson, 1988; Gordon et al, 1989;

1990). The distribution of type XII collagen is coincident with type I collagen in tissues and type XII collagen may provide potential interaction sites for cells or other matrix components (Gordon et al, 1989).

2.3.2 Other proteoglycans

Proteoglycans are a class of macromolecules in which one or more glycosaminoglycans are linked covalently to a core protein, with the possible exception of hyaluronic acid. They are very abundant in connective tissue. The glycosaminoglycans mainly comprise hyaluronic acid, chondroitin sulfate, dermatan sulfate, keratin sulfate, heparan sulfate and heparin (Comper and Laurent, 1978; Handley, Lowther and Mcquillan, 1985; Trelstad, 1985). These glycosaminoglycans, except heparan sulfate and heparin, consist of multiple repeating disaccharide units (Silbert, 1982).

Mammalian cartilage is the main source for the isolation of proteoglycans (Handley, Lowther and Mcquillan,1985). In addition to heparan sulfate proteoglycan, chondroitin sulfate has been found in basal laminae of some tissues such as EHS tumour and Reichert's membrane (Timpl and Dziadek, 1986; Yurchenco and Schittny, 1990).

2.4 Changes in basal lamina components in pathological situations

Basal lamina changes are thought to occur in a variety of diseases. These diseases include non-neoplastic conditions and

neoplastic lesions. Various pathological changes involving basal lamina have been related to the pathogenesis of some diseases.

2.4.1 Non-neoplastic conditions

In non-neoplastic diseases, pathological changes in basal lamina have been found in relation to a number of conditions including psoriasis, lupus erythematosus, lichen planus, blistering diseases such as pemphigoid, diabetes, Goodpasture syndrome and liver fibrosis (Hodge and Freeman, 1978; Timpl and Dziadek, 1986). These diseases involve a variety of organs including skin, kidney, liver and oral mucosa. Study of these diseases has shown a variety of pathological changes involving basal lamina including basal lamina duplication, focal gaps, discontinuities, thickening and increased deposition of basal lamina components. Based on observation on basal lamina changes and clinical correlation, it has been suggested that the pathological features of basal lamina are correlated with the activity of some diseases such as psoriasis and diabetes (Hodge and freeman, 1978; Timpl and Dziadek, 1986). Identification of changes to basal lamina have been served as diagnostic criteria in some diseases (eg. fibrinogen deposition along basal lamina in lichen planus)

2.4.2 Neoplasms

Morphological changes of basal lamina in neoplasms have been extensively studied using electron microscopy. A diversity of ultrastructural appearances including multiplication, discontinuities and thickening of basal lamina have been

described in relation to neoplasms of skin, cervix and oral cavity (Frithiof, 1969; Wood and Smith, 1970; Mckinney and Singh, 1977; Steven and Robert, 1978; Jiang, Wilson and Wiebkin, 1993; Tsujioka et al, 1991). The exact mechanisms for the alterations described in the basal laminae of neoplasms is still under investigation, though it has been suggested that the ultrastructural changes seen in basal lamina are correlated with lytic enzymes and the developmental stage of neoplasm (Hodge and Freeman, 1978; Liotta, 1984).

The pathological changes of basement membrane (basal lamina) in neoplasms have been further characterized by specific identification of basement membrane (basal lamina) constituents using immunohistochemistry (immunocytochemistry). In this context, laminin and type IV collagen, as two major components of basement membranes, have been extensively studied in a variety of neoplasms using optical immunohistochemistry. Using such techniques, it has been demonstrated that there are marked differences in the distribution of basement membrane laminin and type IV collagen when non-neoplastic tissues and neoplastic tissues are compared (Miettinen, Foidart and Ekblom, 1983; Barsky et al, 1983,; Carter, Burman and Barr, 1985; Kallioinen, 1985; Ormerod et al, 1985; Soini, Autio-Harmainen and Miettinen, 1989; Fisseler-Eckhoff et al, 1990; Hirota, Yoneda and Osaki, 1990; Verhoeven et al, 1990; Raymond and Leong, 1991; Cheng et al, 1992; Pitt, Hale and Buckley, 1992; Wilson et al, 1993).

A diversity of staining patterns for basement membrane laminin and type IV collagen in neoplasms have been described by a

number of investigations. For instance, Ormerod et al (1985) using an immunoalkaline phosphatase method reported that a continuously abnormal deposition of basement membrane laminin was demonstrated in dimethylbenzanthracene-induced rat mammary adenomas. Kallioinen (1985) employed a specific antibody against human laminin P1 fragment and immunoperoxidase light and electron microscopic methods for the demonstration of basement membrane (basal lamina) laminin in dermal cylindromas. Kallioinen showed that basement membrane (basal lamina) laminin around cylindroma islands was thick and continous. Fisseler-Eckhoff et al (1990) investigated preneoplastic lesions and early carcinomas of lung using immunofluorescence staining. They reported that laminin was seen as continuous staining along the basement membrane in preneoplastic tissues and that obvious laminin staining appeared not only around original basement membrane, but also around neoplastic islands and neoplastic cells within neoplastic islands. In contrast, an absence of immunoreactivity of basement membrane laminin in a variety of invasive carcinomas (i.e. breast, skin, pancreas, and prostate) was described by Barsky and co-workers (1983) using immunofluorescence and immunoperoxidase light microscopic techniques.

A number of studies have also shown different staining patterns for type IV collagen in relation to various neoplasms (Carter, Burman and Barr, 1985; Ormerod et al, 1985; Hirota, Yoneda and Osaki, 1990; Verhoeven et al, 1990; ten Velde et al, 1991; Pitt, Hale and Buckley, 1992). For instance, Carter and colleagues (1985) in an optical microscopic study reported that basement

membrane type IV collagen in squamous cell carcinomas of the head and neck exhibited evidence of focal thickening, aggregation and deficiency using a monoclonal antibody against human type IV collagen. Type IV collagen in squamous cell carcinoma of lung has been demonstrated by ten Velde et al (1991) using a polyclonal antibody against human type IV collagen and an immunoperoxidase light microscopic technique. In this study, it was described that continuous staining for type IV collagen appeared in the carcinoma periphery with expansive growth, while variable staining patterns namely limited deposition and complete absence for type IV collagen occurred in the central part of carcinoma . A variable staining intensity for type IV collagen but not of laminin has been reported in periductal elastosis of breast cancer by Verhoeven et al (1990) using a polyclonal antibody and an immunoperoxidase light microscopic method. In oral squamous cell carcinoma, a heterogeneous staining patterns of type IV collagen has also been reported by Hirota and co-workers (1990) using a monoclonal antibody and immunoperoxidase staining. These authors described irregular thickening, gaps and an abscence of staining in the basement membrane. Similar features were described by Wilson et al (1993) for induced rat tongue carcinomas.

The implications of different laminin and type IV collagen staining patterns in neoplasms is poorly understood. Nevertheless, some suggestions have been made by a number of investigators. For instance, it has been suggested that the variable expression of basement membrane laminin in neoplasms were correlated with neoplastic differentiation in that well-differentiated neoplasms

express strong staining for basement membrane laminin; while poorly-differentiated neoplasms express a less (or absent) staining of basement membrane laminin, but the presence of intracytoplasmic staining (Lee, 1988; Campbell and Terranova, 1988, Barsky et al, 1983, Soini, Autio-Harmainen and Miettinen, 1989). Ormerod and colleagues (1985) suggested that the deposition of basement membrane laminin in neoplasms was an indicator of abnormal cell proliferation and a loss of epithelial differentiation.

Numerous roles for laminin in neoplasms have been suggested. They comprise stimulating the release of type IV collagenase and altering metastatic cell phenotype and immune surveillance (Kleinman et al, 1985; Timpl and Dziadek, 1986; Campbell and Terranova, 1988). In the process of tumour cell metastasis, laminin has been thought to enhance tumour cells attachment to the basal lamina of vessels (Terranova, Rohrbach and Martin,1980; Liotta, Thorgeirsson and Garbisa, 1982). In *in vitro* studies on the effect of laminin on tumour cells, it has been shown that the attachment of metastatic tumour cell lines could be promoted by laminin and that laminin-attached tumour cells show more metastatic propensity. Moreover, these phenomena could be inhibited by anti-laminin antibodies (Liotta, Thorgeirsson and Garbisa, 1982).

Juliano (1987) and Akiyama, Nagata and Yamada (1990) have suggested that cell surface receptors might play important roles in laminin attachment to neoplastic cells. Using antibodies to block laminin receptors, it has been found that laminin

attachment to neoplastic cells is inhibited (Timpl and Dziadek, 1986; Juliano, 1987).

The significance of the presence and variable expression of type IV collagen in the basement membrane of neoplasms is not well understood. Verhoeven and co-workers (1990) suggested that the different amounts of type IV collagen observed were related to variation in type IV collagen production and destruction which in turn depended on epithelial proliferation and other systematic factors (e.g. hormones). Moreover, it was suggested that the staining pattern of type IV collagen was correlated with tumour invasion and the prognosis of patients. Carter and co-workers (1985) suggested that the expression of type IV collagen was related to disordered maintenance and different rates of destruction of type IV collagen in neoplasms. In a study on invasive carcinoma of uterine cervix, Pitt and colleagues (1992) suggested that there was a significant correlation between the presence of type IV collagen and prognosis and infiltrating growth pattern.

2.5. Changes to other extracellular matrix components in neoplasms

Tumour invasion and metastasis involve not only basal lamina but also other non-basal lamina extracellular matrix components. For instance, collagen loss, desmoplasia and changes in proteoglycan have been reported in relation to a number of neoplasms (Toole, Bisvas and Gross, 1979; Barsky et al, 1982; van den Hooff, 1983; Iozzo, 1985; Timple and Dziadek, 1986). Collagen loss has been

associated with high activity of collagenases and low synthesis of collagen in neoplasms (van Den Hoof,1983). In contrast to the phenomenon of collagen lysis, an increased production of collagen in tumour stroma (so-called desmoplasia) is also observed in many neoplasms (Fisseler-Eckhoff et al, 1990; Barsky, 1982; van den Hoof, 1983). This phenomenon is thought as a defence against neoplastic cell invasion (Barsky et al, 1982; van den Hoof, 1983). In neoplasms, proteoglycans also show alterations in structure and composition. For example, increased proteoglycans have been detected in cultured colon carcinoma cells (Iozzo, 1985). Degradation of heparan sulfate has also been identified in cultured tumour cells (Timple and Dziadek, 1986).

2.6. Malignant neoplastic invasion-associated enzymes

The pathological changes of basement membrane (basal lamina) laminin and type IV collagen and other extracellular matrix components in neoplasms have been related to a variety of enzymes (Liotta, Thorgeirsson and Garbisa, 1982; Pauli et al, 1983). Emonard and Grimaud (1990) described that a number of proteinases were involved in the degradation of extracellular matrix components. At present, the investigations on enzyme genes in neoplasms have also confirmed that various enzymes are involved in extracellular proteolysis during tumour invasion (Kusukawa et al, 1992; Poulson et al, 1992; Pyke et al, 1993) These proteinases have been classified on the basis of chemical and functional features into four groups: 1). Serine-proteinases. 2). Cysteine-proteinases. 3). Aspartic-proteinases(acid-

proteinases). 4). Metalloproteinases (Thorgeirsson, Turpeenniemi-Hujanen and Liotta, 1985; Emonard and Grimaud, 1990)(Table 1)

Table 1 Classification and function of proteinases

Category	Main composition	Function
Serine-proteinases	Plasminogen activators plasmin	Glycoprotein digestion Activatation of metalloproteinases
Cysteine-proteinases	Cathepsin B	Collagen digestion Activatation of collagenase
Aspartic-proteinases (acid proteinases)	Cathepsin D, Pepsins	Protein digestion
Metalloproteinases	Interstitial collagenase Type IV collagenase Type V collagenase Proteoglycanase	Collagen digestion Glycoprotein digestion Proteoglycan digestion

2.6.1 Collagenases

Collagenases are a group of proteinases which degrade collagen structure by cleaving the collagen molecule into fragments. Two distinct kinds of collagenases have been identified from normal tissues and neoplastic tissues (Liotta, Thorgeirsson and Garbisa, 1982; Pauli et al, 1983; Tryggvasion, Höyhtyä and Salo, 1987). The collagenases which degrade the interstitial collagens are referred to as vertebrate collagenases or interstitial collagenases (Emonard and Grimaud, 1990). These collagenases cleave the

interstitial collagen molecules at a single site into 1/4 and 3/4 size fragments which can be further degraded by collagenases themselves or other proteinases (Liotta, Thorgeirsson and Garbisa, 1982). Interstitial collagenases can be produced by various connective tissue cells, epithelial cells and inflammatory cells (Emonard and Grimaud, 1990). High collagenolytic activity has been found in human and experimental neoplasms (Liotta, Thorgeirsson and Garbisa, 1982; Tryggvason, Höyhtyä and Salo,1987). A number of substances are thought to stimulate the release of the interstitial collagenases. These substances include chemical agents, cytokines, several growth factors and the interstitial collagens themselves (Emonard and Grimaud, 1990).

Aside from the interstitial collagenases, another important group of collagenases are type IV collagenase and type V collagenase which degrade type IV collagen and type V collagen respectively (Liotta, Rao and Barsky, 1983). Type IV collagenase and type V collagenase have been identified in normal tissue cells, neoplastic cells and inflammatory cells (Liotta, Thorgeirsson and Garbisa, 1982; Krane, 1982; Kusukawa et al, 1992).

Type IV collagenase is a glycoprotein which cleaves the type IV collagen molecule at approximately 1/4 of the distance from the N-terminal end. Type IV collagenase is initially released in a latent form into the extracellular spaces and then is activated by other enzymes (Liotta, Thorgeirsson and Garbisa, 1982). Previous studies have shown that the type IV collagenase activity is correlated with neoplastic behaviour (Thorgeirsson, Turpeenniemi-Hujanen and Liotta, 1985). A high level of type IV

collagenolytic activity has been revealed in human and experimental neoplasms (Pauli et al, 1983). Kusukawa and colleagues (1992) using immunofluorescence staining, immunoblot analysis and gelatin-substrate gel enzymography reported that two cell lines of oral squamous cell carcinoma produced matrix metalloproteinases such as type IV collagenase and stromelysin. Using *in situ* hybridization techniques, gene expression of type IV collagenases has been found in stromal cells surrounding invasive carcinoma, but not in normal tissues (Pyke et al, 1993). It is believed that type IV collagenase plays important roles in the destruction of the basal lamina "barrier" in malignant neoplastic invasion, in the reconstruction of basal lamina and in the malignant behavior of carcinomas (Tryggvason, Höyhtyä and Salo, 1987; Kusukawa et al, 1992).

2.6.2 Other enzymes

Several other enzymes involved in the degradation of extracellular matrix have also been found in malignant tissues namely proteoglycanases, capthepsin B, plasmingen activators, plasmin, elastase and stromelysin. Enhanced activities of proteoglycanases have been demonstrated in neoplastic tissues (Jone and Clerk, 1982; Tryggvason, Höyhtyä and Salo, 1987). Liotta and colleagues (1982) described that plasmin was associated with the degradation of laminin. It has been also found that elastase and stromelysin are involved in the degradation of type IV collagen, type V collagen, laminin and proteoglycan (Pauli et al, 1983; Tryggvason, Höyhtyä and Salo, 1987).

2.7 Summary

As detailed in the preceding review it is evident that much of the knowledge describing the distribution and apparent alterations to basal lamina and its consituents laminin and type IV collagen derive from studies conducted at the optical microscopic level. Investigators such as Kallioinen (1985) and Havenith and colleagues (1990) have reported on laminin and type IV collagen in neoplasms at the ultrastructural level using immunoperoxidase and immunogold techniques respectively. Such studies have indicated the presence of these basal lamina consituents in tumours, but detailed analysis of distribution and quantitative data was not described.

A knowledge of the detailed distribution of these basal lamina constiuents in normal and neoplastic tissues would seem to be important given the existence of data emerging on these molecules from biochemical, cell culture and other sutdies. As described a knowledge of the distribution and relative quantities of these molecules in the basal lamina of normal tissues and malignant neoplasms may also provide the potential for improved human clinical-pathological diagnostic techniques as well as furthering our understanding of the biology and pathobiology of basal laminae.

2.8. Immunoelectron microscopic techniques

In order to satisfactorily identify tissue antigens using immunoelectron microscopy, it is necessary to retain antigens in tissues. Routine methods of tissue preparation for electronmicroscopy usually result in effective loss of many antigens. For this reason, immunoelectron microscopic techniques often require special tissue preparation techniques which preserve tissue antigenity and at same time an adequate degree of identifiable morphology allowing antigen sites to be recognized relative to cell and tissue morphology (Bendayan, Nanci and Kan, 1987).

In immunoelectron microscopy, two main techniques have been used, namely, pre-embedding techniques and post-embedding techniques. In pre-embedding techniques, the localization of antigens with antibodies occurs before tissue embedding. These techniques reduce the loss of tissue antigenity caused by the embedding process. However, in this technique there is the disadvantage of poor penetration of immunoreagents into tissue through cell membranes. Although several methods have been introduced to increase the permeability of cell membranes in pre-embedding techniques, it is thought that the tertiary structures of tissue are often destroyed and antigens mobilized (Stirling, 1990).

An alternative to the pre-embedding techniques are the post-embedding techniques. Post-embedding techniques detect the exposed antigens at the surface of sections of either frozen or

plastic embedded tissues after tissue processing and sectioning. It is generally considered that post-embedding techniques overcome many of the problems encountered in pre-embedding techniques. Postembedding ultracryomicrotomy is an excellent method for the preservation of tissue antigenity and has been used in previous studies (Madri et al, 1980; Jungell, 1990). However, resultant poor morphology and a high demand for technology preclude its wide application. The use of resin embedded sections in postembedding immunoelectron microscopic techniques often result in the loss of antigenity. However this technique does allow the preservation of "sufficient" tissue antigenity and the better preservation of morphology provided tissue preparation techniques are selected with care.

2.8.1. Tissue preparation

In immunoelectron microscopy, it is recognized that tissue fixation, dehydration and embedding conditions are associated with morphological preservation and antigen retention (Roth, 1986; Stirling, 1990).

2.8.1.1 Fixation

For ideal fixation, it is desirable that structural distortion and antigen destruction are minimized. In chemical fixation, several fixative variables are generally considered to be associated with preservation of tertiary structures and tissue antigenity. These include the nature of the fixative, the concentration and length of

fixation, additives, osmolarity, pH value and temperature (Bullock, 1984; Roth, 1986; Hayat, 1989).

Several types of fixative have been used in immunoelectron microscopy including glutaraldehyde, (para)formaldehyde and osmium tetroxide (Gosselin et al, 1986). Glutaraldehyde and (para)formaldehyde are more often used than osmium tetroxide.

The advantages and disadvantages of these fixatives have been reviewed by a number of authors (Bullock, 1984; Fox and Benton, 1987; Gosselin et al, 1986; Hayat, 1989). Hayat (1989) described that the most efficient preservation of protein structure and excellent stabilization could be obtained from tissues fixed in glutaraldehyde as a result of its cross-linking proteins. However, it is recognized that glutaraldehyde has a relatively slow penetration rate in tissue and greatly denatures proteins such that tissue antigenity is lost (Kuklmann and Krischan, 1981; Eldred et al, 1983; Bullock, 1984). In addition, it has been reported that residual glutaraldehyde on tissue sections can bind to immunohistochemical reagents, resulting in non-specific staining on sections (Gosselin et al, 1986).

The contrasting effects of glutaraldehyde in providing good morphological preservation but some antigen loss has caused many investigators to use it, albeit hesitatingly, in immunoelectron microscopy. Nevertheless, some studies have reported that satisfactory immunolabelling of antigens can be obtained using a mild concentration of glutaraldehyde (Bendayan et al, 1980; Timms, 1986). In addition, it has also been reported

that some antigens are still retained after the use of concentrated glutaraldehyde fixation (Hisano et al, 1986; Roth, 1986), and that the non-specific binding of immunoreagents with residual glutaraldehyde in immunostaining can be avoided by quenching methods using various agents such as cell culture medium, glycine, lysine, ammonium carbonate buffer and sodium borohydride after fixation or before immunostaining (Stirling, 1990; Gosselin et al, 1986; Roth, Taatjes and Warhol, 1989).

Analysis of the literature shows that (para)formaldehyde is more commonly used than glutaraldehyde in immunoelectron microscopy. Paraformaldehyde is a solid form of formaldehyde. Gosselin et al (1986) and Fox and Benton (1987) suggested that fresh formaldehyde solutions made from powdered paraformaldehyde should be used in immunohistochemistry owing to the fact that formaldehyde solutions during storage may form formic acid which decreases the pH. Hayat (1989) described that formaldehyde had a more rapid penetration rate than glutaraldehyde; but that a problem with formaldehyde was slow reactivity with tissue proteins. Gosselin et al (1986) reported that total cross-linking of formaldehyde with protein took about 2 weeks. In a study on formaldehyde, Fox and Benton (1987) considered that several factors might affect the tissue fixation properties of formaldehyde namely the concentration, pH, osmolarity and the length of fixation.

Karnovsky (1965) considered that a mixture of formaldehyde and glutaraldehyde was superior to formaldehyde or glutaraldehyde alone for morphological preservation. Karnovsky suggested that

proteins would be better preserved by the temporary fixation of the faster penetrating formaldehyde and the subsequently permanent cross-linking of glutaraldehyde. Tokuyasu (1984) also considered (para)formaldehyde and (para)formaldehyde-glutaraldehyde mixtures to be optimum fixation solutions for immunohistochemistry.

A number of studies have reported that a variety of antigens have been identified using formaldehyde or a formaldehyde-glutaraldehyde combination (Eldred et al, 1983; Grant and Leblond, 1988; Berryman and Rodewald, 1990; Stirling, 1990). Stirling (1990) suggested that fresh formaldehyde at a certain concentration was an alternative to glutaradehyde to preserve morphology and tissue antigenity. Some studies have reported that antigen inactivation caused by formaldehyde fixation can be reversed by some treatments such as extensive washing or the addition of sucrose in washing buffer (Deng and Beutner, 1974; Bullock, 1984). Despite this, the poor preservation of morphology caused by formaldehyde fixation has been revealed in some studies (Stephens, Bendayan and Gisiger, 1985; Smith, 1990).

Based on the nature of antibodies, Beesley (1989) suggested that the choice of fixative depended on the type of antibody to be used. Beesley considered that formaldehyde-glutaraldehyde mixture or formaldehyde alone was compatible with monoclonal antibodies; while glutaradehyde was compatible with polyclonal antibodies.

Osmium tetroxide is commonly used as a secondary fixative following glutaraldehyde in conventional electron microscopy. It

has been noted that osmium tetroxide can efficently preserve membrane protein and therefore provide good membrane contrast, but can result in tissue artifact and excessive antigen destruction (Berryman and Rodewald, 1990; Stirling, 1990). In general, the use of osmium tetroxide is avoided in immunohistochemistry. Despite this, the high degree of morphological preservation obtained using osmium tetroxide fixation attracts some investigators to use osmium-fixed materials for immunohistochemistry. Some satisfactory results have been shown in a number of studies (Bendayan and Zollinger, 1983; Bendayan, Nanci and Kan, 1987). However, it has been shown that pretreatment of sections is required for antigen exposure using oxidizing agents such as hydrogen peroxide, alcohol, sodium hydroxide and sodium metaperiodate when osmium tetroxide is used (Bullock, 1984; Bendayan, Nanci and Kan, 1987).

In immunohistochemistry, the concentration and the length of time of fixation also affect the preservation of tissue antigenity. A variety of fixation concentrations and periods have been used in immunohistochemistry and even then it would appear that the degree of antigen preservation varies from tissue to tissue and from antigen to antigen. From analysis of previous studies, it is found that a concentration of glutaraldehyde ranging from 0.5% to 5% has been used in immunohistochemistry, when glutaradehyde alone is employed as a fixative (Bendayan et al, 1980; Sisson and Vernier, 1980; Newman, Jasani and Williams,1983; Smart and Millard, 1985; Mahrle et al, 1989; Berryman and Rodewald, 1990). For instance, Stirling (1990) recommended that 2-4% glutaraldehyde for 1-2 hours was compatible with the labelling of

insensitive epitopes; while a mild concentration for a short period was compatible with the labelling of sensitive epitopes. In using a mild concentration of fixative, Kraehenbuhl and Jamieson (1973) recommended that 0.5% glutaraldehyde was optimum for tissue fixation in immunohistochemistry. However, some studies have shown that some antigens are still destroyed to become immunologically unrecognizable even at very low fixative concentration (White et al, 1988). Conversely in some instances, some antigens reveal a high resistance to a relatively high concentration of glutaraldehyde. For example, Mahrle et al (1989) reported that the antigenity of intermediate filaments in epidermis fixed in 5% glutaradehyde for over 2 hours still persisted.

Four percent formaldehyde has commonly been used in immunohistochemistry. Good retention of tissue antigenity has been reported in a number of studies (Stephens, Bendayan and Gisiger, 1985; Bendayan, 1985; Grant and Leblond, 1988). The period of formaldehyde fixation ranges from 1 to 22 hours (Stephens et al,1985; White et al, 1988).

The proportion of ingredients in glutaraldehyde-formaldehyde fixative mixtures used in immunoelectron microscopy ranges from 0.005% to 2.5% glutaraldehyde and from 1% to 4% formaldehyde (Sisson and Vernier, 1980; Eldred et al, 1983; Sternberger, 1986; Timms, 1986; White et al, 1988; Berryman and Rodewald, 1990; Lin and Essner, 1990; Herken and Miosge 1991). Tokuyasu (1984) and Bullock (1984) recommended a mixture of 0.1%-1% glutaraldehyde with 2%-4% formaldehyde as an optimum

concentration of fixation for immunohistochemistry. A length of time for glutaraldehyde-formaldehyde fixation ranging from 15 min to 24 hours has been described (Sisson and Vernier, 1980; White et al, 1988; Shida and Ohga, 1990).

In studies on the ultrastructural immunolocalization of basal lamina type IV collagen and laminin in various tissues reportedly satisfactory preservation of tissue antigenity has been obtained using different fixatives with variable concentrations and fixation times (Table 2).

2.8.1.2 Additives

It has been recognized that additives are a supplemental means of improving fine structure and preserving tissue antigenity during tissue preparation (Bullock, 1984). A number of substances have been used as additives including periodate-lysine, polyvinylpyrrolidone (PVP), picric acid, sucrose, tannic acid, uranyl acetate, imido-esters, parabenzoquinone, calcium ions, lithium ions, potassium and ferricyanide (Bullock, 1984; Hermanns et al, 1986; Berryman and Rodewald, 1990).

Periodate-lysine

Mclean and Nakane (1974) first reported the use of periodate-lysine as an additive for paraformaldehyde fixation. These authors indicated that a satisfactory preservation of antigenity was obtained by this method of fixation. The proposed mechanism was that periodate and lysine contributed to the stabilization of

Table 2: Summary of fixation, embedding and incubation parameters used in previous immunocytochemical studies of laminin and type IV collagen.

Tissue	Antigen	Fixative	Fixation period	Fixation Temp (°C)	Embedding materials	Embedding Temp (°C)	Embedding period	Antibody	Marker	Study
Rat Kidney	Type IV collagen	4%form-aldehyde	2h	4	Lowicryl	-20		Polyclonal	Protein A-gold	Bendayan, 1985
Mouse sketetal muscle	Type IV collagen Laminin	2% PLP	1-2h	4	Lowicryl	-20		Polyclonal	Protein A-gold	Stephens et al, 1985
Rat kidney	Type IV collagen	4%form-aldehyde	2h		Lowicryl	-20	>5-7 days		Protein A - gold	Bendayan et al, 1987
Rat tissues	Type IV collagen Laminin	4%form-aldehyde	16h	4	Lowicryl	-35	>3 days	Polyclonal	Protein A-gold	Grant & Leblond, 1988
Human oral mucosa	Type IV collagen Laminin	PLP or 4%paraform-aldehyde + 0.1% glutar-aldehyde	2h	4	OCT compound	-70		Polyclonal	ABC peroxidase	Jungell, 1989
Rat kidney	Type IV collagen Laminin	4% PLP	2h	4	Lowicryl	-20		Polyclonal	Protein A - gold	Desjardins & Bendayan, 1989
Human kidney	Type IV collagen Laminin	1% para-formaldehyde or 1% glutar-aldehyde	3h 30min	Room temperature	Lowicryl L.R.White	-25 55	>7 days 26h	Polyclonal	Protein A - gold	Smith, 1990
Human lung	Type IV collagen	2%paraformaldehyde			Lowicryl			Polyclonal	IgG-gold	Havenith et al, 1990
Rat retina	Type IV collagen Laminin	3-4%para-formaldehyde + 0.1-0.25% glutaraldehyde	2h	4	L.R.White	4 or-20	14h	Polyclonal	IgG-gold	Lin & Essner, 1990
Mouse Kidney	Laminin	4%paraformaldehyde + 0.5% glutaralde-hyde	15min or 30min	Room temperature	L.R.White or L.R.Gold	50	24-48h	Polyclonal or monoclonal	IgG-gold	Herken &Miose, 1991

protein carbohydate moietes by molecular oxidization and cross-linking. Since then, many studies have successfully used periodate-lysine-paraformaldehyde fixation for the immunolabelling of various antigens including basal lamina laminin and type IV collagen (Stephens, Bendayan and Gisiger, 1985; Desjardins and Bendayan, 1989; Jungell, 1990). In addition, other formulations (eg. periodate-lysine-paraformaldehyde-glutaraldehyde) have also been advocated (Hermanns et al, 1986).

Polyvinlpyrrolidone (PVP)

Polyvinylpyrrolidone (PVP) is a water-soluble non-ionic polymer with a molecular weight ranging from 2500 to 1 million and can be synthesized by radical polymerization of vinylpyrrolidone in aqueous and nonaqueous solution with an initiator of hydrogen peroxidase (Haaf, Sanner and Straub, 1985; Biswas, Philips and Williams, 1990). The molecular structure of PVP is characterized by repeating units of polymer which contain hydroxyl and carbonyl end groups (Fig.3).

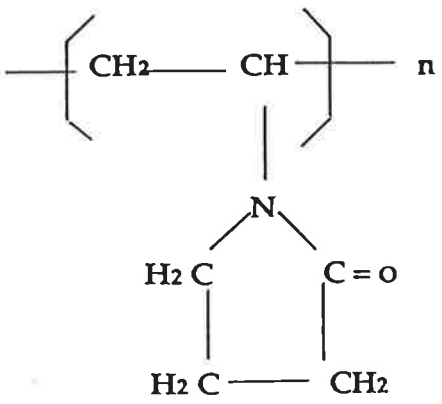


Figure 3. Molecular structure of polyvinylpyrrolidone (PVP)

These end groups containing charged nitrogen and oxygen are able to bind reversely to various compounds by hydrogen bonding (Haaf, Sanner and Straub,1985; Türker et al. 1990). Due to its broad range of molecular weights and biological properties PVP has been widely used for medical, industrial and biochemical purposes (Haff, Sanner and Straub, 1985; Barnard, 1987).

In electron microscopy, PVP as a non-electrolyte, has been used to adjust the osmolarity of solutions (Hayat, 1989). In immunohistochemistry, PVP has also been used for the cryoprotection of biological specimens and for the preservation of tissue antigenity (Kuhlman and Krischan, 1981; Hermanns et al, 1986; Barnard, 1987; White et al, 1988). There appears to have been inconsistent results describing the preservation of antigenity with PVP in previous studies (Table 3). The mechanism for antigen preservation exerted by PVP has been poorly understood. Kuhlman and Krischan (1981) explained that PVP preserved antigenity by the micellar formation of PVP with proteins. In their study, PVP was added into washing buffer and ethanols in tissue processings. Several investigators have used PVP as a additive of chemical fixations (Hermanns et al, 1986; White et al, 1988; Malecki, 1990).

Picric acid

Picric acid is 2,4,6-trinitrophenol (Pearse, 1980). It was first used as an additive for fixation to improve fine structure

Table 3: Characteristics of PVP shown in previous studies

Amount of PVP	MW of PVP	PVP added to:	Technique	Function	Study
5-10%	10,000	Fixative wash buffer Ethanol Embedding material	Immunohisto-chemistry	Preservation of antigenicity	Kuhlmann & Krischan, 1981
5%	10,000	Fixative Wash buffer Ethanol	Immunohisto-chemistry	No effect on immunoreactivity	Hermanns et al, 1986
4%	30,000	Buffer	Immunohisto-chemistry	Unreported	Roth, 1986
Unknown	Unknown	Cryoprotectant solution	Cryoultramicrotomy	Cryoprotection	Barnard, 1987
Unknown	Unknown	Fixation	Electron microscopy	Adjustment of osmolarity	Hayat, 1989
5%	10,000	Fixative	Immunoelectron microscopy	Unreported	White et al, 1988
2%	Unknown	Fixative Buffer	Immunohistochemistry	Used as a water-soluble embedding medium	Malecki, 1990

(Zamboni and Martino, 1967; Stefanini, Martino and Zamboni,1967). Since then, picric acid has been added to a number of fixatives resulting in several formulations such as picric acid-formaldehyde, picric acid-formaldehyde-glutaraldehyde and picric acid-glutaraldehyde. It has been reported that picric acid improves tissue morphology and the immunoreactivity of tissue antigens (Ito and Karnovsky, 1968; Mazurkiewicz and Nakane, 1972; Newman, Jasani and Williams, 1982, 1983; Eldred et al, 1983; Hermanns et al, 1986; Hisano et al, 1986; Gosselin et al, 1986; Newman and Hobot, 1987; Berryman and Rodewald, 1990)(Table 4). Concentrations of picric acid ranging from 0.0013% to 14% have been used. The formation of intermolecular salt links between picric acid and proteins or the coagulating properties of picric acid may contribute to the improvement of fine structure preservation and antigen retention (Pease, 1980; Bullock, 1984; Berryman and Rodewald, 1990). Despite this, Eldred et al (1983) pointed out that picric acid reduces tissue antigenity, especially when the concentration exceeded 0.5% for more than 30 minutes.

Sucrose

Sucrose, like PVP, has been used as a non-electrolyte for adjusting the osmolarity of solutions, or, as a cryprotectant agent applied to low temperature biological techniques (Hayat, 1989; Barnard, 1987). In immunohistochemistry, several studies have reported that sucrose can reverse the inactivation of tissue antigenity resulting from chemical fixatives and prevent the

Table 4: Characteristics of picric acid shown in previous studies

Concentration of Picric acid	Combined with fixative	Technique	Function	Study
0.02%	2% formaldehyde	Electron microscopy	Improvement of morphology	Stefanini et al, 1967
	Formaldehyde	Electron microscopy	Improvement of morphology	Zamboni & Martino, 1967
0.02%-0.1%	Formaldehyde-glutaraldehyde	Electron microscopy	Improvement of morphology	Ito & Karnovsky, 1968
0.15%	1% glutaraldehyde	Immunoelectron microscopy	Preservation of morphology & antigenicity	Newman et al, 1983
0.15-0.5%	4% paraformaldehyde	Immunoelectron microscopy	Improvement of morphology & Reduction of antigenicity	Eldred et al, 1983
0.2%	4% paraformaldehyde	Immunohistochemistry	Reduction of antigenicity	Hermanns et al, 1986
0.6%	2% paraformaldehyde-either 0.1% or1% glutaraldehyde	Immunoelectron microscopy	Antigenic retention	Hisano et al, 1986
14%	2% paraformaldehyde	Immunohistochemistry	Antigenic retention	Gosselin et al, 1986
0.2%	1% glutaraldehyde	Immunoelectron microscopy		Newman & Hobot, 1987
0.2%	1% glutaraldehyde	Immunoelectron microscopy	Preservation of morphology & antigenicity	Berryman & Rodewald, 1990

diffusion of enzymes by adding it to fixative and washing buffer solutions (Miller, 1972; Deng and Beutner, 1974).

2.8.1.3 Osmolarity

As defined by Hayat (1989), " osmolarity " or " osmolality " is referred to as the molarity (mole per litre of solution) or molality (mole per kilogram of solvent). In immunohistochemistry, it is generally considered that the osmotic pressure during tissue processing should be similar to the " *in vivo* " osmotic pressure of external fluids of the body in order to prevent tissue artifact and the diffusion of antigens.

Several factors are considered to be important in relation to fixation osmolarity including buffers, the fixative agents themselves and additives. The preservation of tissue morphology is associated with fixation osmolarity (Hayat, 1989). Effective osmolarity depends on impermeable molecules and ions such as buffers, salt, sucrose and PVP and buffers contribute to major effective osmolarity in fixation solution. (Bone and Ryan, 1972; Brunk and Ericsson, 1972; Cowan, 1986; Hayat, 1989).

Several buffer systems are used as fixative vehicles for electron microscopy namely cacodylate, phosphate buffer (PB), phosphate buffered saline (PBS), Caulfield's and Dalton's dichromate buffers (Hayat, 1989; Stirling, 1990). Among them, phosphate buffer(PB) and phosphate buffered saline (PBS) are more commonly used in immunoelectron microscopy. Although both buffer systems have been widely used by many workers, whether or not both buffer

systems have different effects on the preservation of morphology and antigenity remains obscure.

In relation to the issue of fixative osmolarity, it is thought that fixative agents contribute to a relatively small and less effective osmotic pressure in fixation solutions, and that the effect of fixative osmolarity on cellular morphology varies according to the types of fixative used, tissue density and cellular compartments (Bone and Denton, 1971; Bone and Ryan, 1972; Hayat, 1989).

In order to preserve fine structure and minimize the diffusion of antigens, Hayat (1989) suggested that fixation osmolarity should be at a " physiological level " and in some cases needed to be adjusted using a number of additives such as sodium chloride, calcium chloride, sucrose, glucose, dextan and PVP. In some instances, it is difficult to obtain an optimum osmolarity. For instance, some tissues contain several cell types (eg. kidney, skin, oral mucosa) requiring a different osmolarity for each cell type.

2.8.1.4. Dehydration

In immunoelectron microscopy, it is known that inappropriate dehydration will denature antigens. Stirling (1990) emphasized that the media used for dehydration should be compatible with resin systems and should be inert in relation to biological material. From analysis of the literature, it is evident that several media are used for tissue dehydration. These include ethanol, methanol, acetone, glycol methacrylate,

dimethylformamide and ethylene glycol (Hermanns et al, 1986; Hisano et al, 1986). Of these, ethanol is thought to be a satisfactory dehydrating medium for many resin systems (Stirling, 1990). A series of graded ethanols (up to 100% ethanols-full dehydration) is commonly employed in many studies (White et al, 1988; Mahrle et al, 1989; Shida and Ohga, 1990). However, partial dehydration (up to 70% ethanols) is considered to be compatible with L.R.White resin due to the hydrophilic characteristic of L.R.White (Newman and Hobot, 1987). Despite this, some investigators still use full dehydration with L. R. White resin and reportedly satisfactory immunolabelling has been obtained (Bendayan,Nanci and Kan, 1987; Lin and Essener, 1990; Herken and Miosge, 1991).

2.8.1.5 Embedding

Two categories of resin system have been used to date in immunohistochemistry namely epoxy resins and acrylic resins. Hydrophobicity is a characteristic common to epoxy resins including Araldites, Epons and Spurr. Epoxy resins have high chemical reactivity during polymerization and a high temperature is usually required for polymerization (Causton,1984). Although some investigators have successfully employed epoxy resins for the detection of various antigens (Kuhlmann and Krischan, 1981; Smart and Millard, 1985; Roth, 1986; Bendayan, Nanci and Kan, 1987), the pretreatment (" etching ") of sections with various agents is often a prerequisite for the exposure of masked antigens (Newman and Hobot, 1989; Hayat, 1989). This disadvantage of

epoxy resins precludes their wide application in immunohistochemistry.

The development of suitable acrylic resins provides compatible embedding materials for immunohistochemistry. The acrylic resins consisting mainly of the Lowicryl series, L.R.White and L.R.Gold have several advantages such as hydrophilia and the avoidance of "etching" treatments. The polymerization of acrylic resins can be carried out at a range of temperatures including low temperature curing, room temperature and high temperature curing (Hayat, 1989). Of these resins, methacryl-based Lowicryl resins and L.R. White resin have been widely used in previous studies (Timms, 1986; Bendayan, Nanci and Kan, 1987; White et al, 1988; Mahrle et al, 1989; Shida and Ohga, 1990). The satisfactory preservation of antigens with methacryl-based Lowicryl resins has been demonstrated in some studies (Kuhlmann and Krischan, 1981; Bendayan, Nanci and Kan 1987; Shide and Ohga, 1990). Despite this, Newman (1989) considered that it was inappropriate to polymerize methacryl-based Lowicryl resins under heat or catalytic conditions due to the absence of an activator in Lowicryl kits supplied by the company. Further it has been suggested that the distortion of fine structure caused by the polymerization process and the instability of ultra-thin sections in the electron beam are also problems associated with methacryl-based Lowicryl resins (Newman, Jasani and Williams,1982; 1983).

Newman and colleagues (1982) suggested that an alternative to methacryl-based Lowicryl resins was L.R.White and reported that

L.R.White had characteristics including hydrophilia and electron beam stabilization which were compatible with immunoelectron microscopy. In the application of L.R.White resin to this field, it has been demonstrated that satisfactory preservation of tissue antigenity can be obtained (Newman, Jasani and Williams, 1983; Timms, 1986; Bendayan, Nanci and Kan,1987; Newman and Hobot, 1987; White et al, 1988; Herrera, 1989; Smith, 1990; Fanning et al, 1990; Lin and Essner, 1990; Herken and Miosge, 1991). L.R.White resin can be polymerized at sub-zero temperature, at room temperature and be heat cured. However, it appears that heat polymerization of L.R.White resin is the more commonly technique. Newman (1987) suggested that the conditions of polymerization for L.R.White resin should be correlated with the immunostaining techniques to be used. Nevertheless Newman considered that the polymerization of L.R.White resin at different temperatures did not make a significant difference to immunolabelling provided immunogold techniques were employed. Smith (1990) compared the immunolabelling of N-acetyl-glalactosamine in rat kidney at -25^{0}C and 55^{0}C for the polymerization of L.R.White resin using protein-A gold techniques. It was reported that a greater degree of immnolabelling was achieved at -25^{0}C than at 55^{0}C.

Under normal conditions the polymerization of L.R.White resin requires a considerable time. It is indicated that under conditions of cold cure and heat cure the times approximatelly range from 12 hours (overnight) to 24 hours (Newman, 1989; Lin and Essner, 1990). However, rapid polymerization of L.R.White resin is able to be achieved by use of an accelerator at room temperature (catalytic polymerization) (Newman and Hobot, 1987). Newman

(1989) reported that catalytic polymerization could reduce tissue extraction and was useful for urgent diagnosis.

2.8.2. Immunostaining

As described in the previous sections reviewing tissue preparation variables, it is clear that the choice of an appropriate fixation, dehydration and resin system is critical for the retention of tissue antigenity in immunolabelling investigations.

A number of immunolabelling techniques have been used at the electron microscopic level namely immunoperoxidase, immunoferritin and immunogold techniques (Delellis, 1981). The success of immunostaining relies on the selection of an appropriate primary antibody, blocking agents and visualizing markers (Stirling, 1990).

In the selection of primary antibody, it is important that the availability, types and characteristics of the antibody to be used are taken into consideration. In many studies, polyclonal antibodies and monoclonal antibodies are usually available for the one antigen. Both types of antibody have their own characteristics. For instance, monoclonal antibodies are characterized by a high homogeneity and a specific reaction with only one molecule or a single antigen epitope. Moreover, it is generally held that the use of monoclonal antibody for immunolabelling produces more specific labelling and less nonspecific background staining. However, the high specificity of monoclonal antibody in some instances, can lead to a low level of

immunolabelling due to insufficient retention of antigen epitopes after tissue preparation. Owing to the property of polyclonal antibodies to bind to multiple antigen epitopes, it is considered that the use of polyclonal antibody may, in some cases, produce a better label density of tissue antigens compared to monoclonal antibody. However, polyclonal antibodies cause a high level of non-specific background staining compared with monoclonal antibody (Stirling, 1990). It has been suggested that the purification of antigen fragments used for raising polyclonal antibodies is critical for specific staining (Beesley, 1989; Stirling, 1990).

In immunostaining, a number of factors have been thought to result in non-specific background staining namely the use of "dirty" antibody, inappropriate concentration of antibody, the maintenance of reactive groups on ultrathin sections and the use of inappropriate markers (Roth, Taatjes and Warhol, 1989; Stirling, 1990). To reduce the level of non-specific background staining, it is necessary to select optimum antibody and antibody dilutions, to use blocking agents and to employ appropriate makers. In previous studies, a number of blocking agents have been used including bovine serum albumin (BSA), ovalbumin, defatted milk, Tween 20, Triton X-100, gelatin and glycine (Roth and Heitz, 1989; Roth, Taatjes and Warhol, 1989; Stirling, 1990). Of these, BSA and ovalbumin are commonly used. In some investigations, defatted milk has been combined with BSA or used as a substitute for BSA or ovalbumin to block non-specific binding (Roth and Heitz, 1989; Lin and Essner, 1990). Defatted milk is considered to have the advantages of being inexpensive and having

a high efficiency for acrylic resin-embedded sections (Roth and Heitz, 1989; Stirling, 1990). Similarly, gelatin alone, or used in combination with other blocking agents, in some studies, show more effeciency than BSA (Stirling, 1990). Recently, a number of investigators have used Tween 20, Triton-100 or glycine combined with other blocking agents in the incubation of antibody with sections and demonstrated effective reduction of non-specific background staining (White et al, 1988; Roth and Heitz, 1989; Berryman and Rodewald, 1990; Smith, 1990; Fanning et al, 1991).

Several probes namely enzyme, ferritin and colloidal gold are available as visualizing markers in immunoelectron microscopic investigations (Signer, 1959; Nakane and Pierce, 1966; Faulk and Taylor, 1971). Of these, it is generally considered that colloidal gold is a versatile and reliable marker. As described by Beesley (1986), gold probes have a number of advantages such as the availability of a wide range of sizes for multiple immunolabelling, diverse complexes which can be selected and high electron density allowing easy identification.

The gold probe used in immunostaining is usually colloidal gold coated with some substance such as immunoglobulin G (IgG), straphylococcal protein A, streptococcal protein G, avidin, enzyme, lectins and streptavidin (Stirling, 1990). Protein A derived from *staphylococcus aureus* cell wall is commonly used to coat colloidal gold. Protein A has a high affinity for immunoglobulin G (IgG) from several mammalian species such as rabbit, guinea pig and human, but a relatively low affinity for IgG

from rat, mouse, sheep and goat (Bendayan and Garzon, 1988; Roth and Heitz, 1989; Stirling, 1990). Due to this limitation, the use of protein A-gold complex in immunocytochemical studies is sometimes precluded, especially in those using monoclonal antibodies which are mainly raised from mouse and rat (Stirling, 1990).

Alternatives to protein A are protein G and IgG (Bendayan and Garzon, 1988; Grant and Leblond, 1988; Lin and Essner, 1989). Protein G can bind to immunoglobulin G (IgG) from a wide range of mammalian species and is compatible with antibodies raised from goat and mouse (Bendayan and Garzon, 1988). Colloidal gold can interact with immunoglobulin G to form IgG-gold complexes which may be used in some cases (Grant and Leblond, 1988; Havenith et al, 1990), especially in situations in which primary antibody is raised from goat and mouse (Lin and Essner, 1989; Stirling, 1990). For instance, Grant and Leblond (1988) in a study of basal lamina antigens found that the IgG-gold complex as an visualizing marker obviously exhibited a lower level of non-specific background staining than protein A-gold which often bound to nuclei and interstitial collagen. A phenomenon noted with the use of IgG-gold complexes is that of particle clustering. Stirling (1990) considered that particle clustering might enhance label density. As explained by Lin and Essner (1989), this phenomenon possibly results from the difficult adsorption of antibodies to colloidal gold during IgG-gold complex preparation.

Further attempts to improve tissue morphology after immunostaining have been made by some investigators. For

instance, Berrymann and Rodewald (1990) reported the use of osmium as a substitute for uranyl acetate for staining ultrathin sections and considered that this enhanced membrane contrast. Dingemans and van den Bergh Weerman (1990) reported that the use of tannic acid to stain ultrathin sections remarkably improved the contrast of extracellular matrix components.

2.8.3 Quantitation and immunogold techniques

Griffiths and Hoppeler (1986) divided quantitation into two categories namely relative quantitation and absolute quantitation. It is considered that absolute quantitation is never obtained from direct analysis of label density on sections, because label density estimated from given cellular or tissue compartments does not always correlate with the absolute density of antigen in the tissue and can be affected by many factors such as tissue preparation methods and immunostaining techniques used (Beesley, 1989; Stirling, 1990). Thus relative quantitation is the method used in quantitative immunoelectron microscopic studies.

In studies involving the quantitation of tissue antigens from ultrathin tissue sections, immunogold techniques are considered to be the most reliable (Bendayan, 1984). A number of quantitative studies have employed immunogold techniques to quantitatively analyse the expression of various tissue antigens (Beesley, 1984; Bendayan, 1984; Grant and Leblond, 1988; Haftek, Chignol and Thivolet, 1989; Okami et al, 1989; Roth and Heitz, 1989; Hammel and Kalina, 1991).

Bendayan (1984) and Griffiths and Hoppeler (1986) considered that the immunogold post-embedding method was more reliable compared with immunoperoxidase pre-embedding methods due to the restriction of gold particles to the surface of thin sections. In analysing the expression of tissue antigens using gold probes, Bendayan (1984) described that two common techniques the point-counting method (Weibel, 1969) and a modular system for quantitative digital image analysis can be employed, and that the label density of antigen might be represented as the quantity of gold particles per area or per length. These methods have been used in relation to the quantitative estimation of various tissue antigens (i.e. Na+, K+ -ATPase in rat ciliary epithelial cells; basal lamina laminin and type IV collagen in kidney, colon and enamel organ (Grant and Leblond, 1988; Okami et al, 1989).

2.8.4 Summary

Review of the literature on immunoelectron microscopy clearly reveals that the fixation protocols used for rountine electron microscopy are not appropriate for the majority of immunocytochemical investigations. However, it is also evident that no single easily reproducible protocol incorporating standardized fixation, embedding and immunostaining-labelling procedures yet exists for the identification of antigens.

The sucessful examination of immunoelectron microscopic investigation depend on careful analysis of a number of factors which include

1) Consideration of the tissue being studied.

2) The nature of the antigens to be detected.

3) Consideration of the most appropriate fixative and concentration.

4) Consideration of appropriate additives which might influence morphology and osmolarity.

5) Careful consideration of the type (i.e. whether monoclonal or polyclonal) and source of antibody to be used.

6) Consideration of the most appropriate "staining" (visualization) system.

CHAPTER THREE: ESTABLISHMENT OF OPTIMUM METHODS FOR THE ULTRASTRUCTURAL LOCALIZATION OF BASAL LAMINA LAMININ AND TYPE IV COLLAGEN IN RAT ORAL MUCOSA

3.1. Introduction

Although many studies have reported that a variety of tissues antigens are successfully localized using a variety of tissue preparation and immunostaining techniques (Roth, 1986; Sternberger, 1986; Stirling, 1990), immunolocalization of tissue antigens still remains a tedious and often unpredictable process, particularly at the electron microscopic level. In immunoelectron microscopy (immunocytochemistry) post-embedding immunogold techniques have become more popular compared with pre-embedding peroxidase techniques for the ultrastructural localization of tissue antigens. A review of the literature reveals that a number of investigators have successfully immunolocalized laminin and type IV collagen in animal and human tissues using a range of fixation protocols and embedding agents and immunogold techniques.

Most of these studies have employed low-temperature embedding in Lowicryl. A relatively new resin that has been recommended for use in the ultrastructural identification of various antigens including laminin and type IV collagen is L.R.White resin (Newman, Jasani and Williams, 1982; 1983).

In the ultrastructural immunolocalization of tissue antigens, it is well recognized that there are tissue processing variables from tissue to tissue and from antigen to antigen (Bullock, 1984; Bendayan et al, 1987). During pilot studies of the immunocytochemical distribution of laminin and type IV collagen in basal lamina of rat tongue tissues, it became evident that the

development of appropriate, specific and standardized tissue processing protocols was a prerequisite for the ultrastructural localization of laminin and type IV collagen in normal mucosa and experimentally induced oral carcinoma.

In this section of the study, several experiments designed to test the effect of variable tissue preparations and immunostaining techniques on the morphology and immunolabelling of laminin and type IV collagen in rat lingual mucosa were performed. In the first experiment, several different tissue preparation methods were employed to determine the quality of identification of basal lamina laminin. It was found that varying fixation conditions and embedding procedures affected the demonstration of laminin. Further, epithelial cell morphology was unsatisfactory using the protocols described and poor polymerization of L.R.White resin at room temperature occurred in some tissue blocks. In view of these findings, a second series of investigations were carried out with a wide range of fixatives, different dehydration protocols and different polymerization conditions of resin embedding. In order to improve epithelial cell morphology, the fixation osmolarity which was considered to be related to the preservation of morphology was adjusted by additives. From these investigations, it was concluded that satisfactory immunolabelling could be achieved by several fixation protocols combined with certain embedding techniques. Additionally, it was noted that the fixatives containing PVP resulted in a higher level of labelling than those without PVP. Therefore a question arising from this second series of experiments was whether PVP itself preserved tissue antigen or whether the fixation osmolarity

contributed by PVP indirectly affected the antigen preservation. To answer this question, a third series of experiments was designed in which the osmolarity of fixation without PVP was adjusted by sucrose to a level similar to those containing PVP.

Two different dehydration procedures for L.R.White resin have been used in previous studies (Newman and Hobot, 1987; Lin and Essener, 1990). To clarify which protocol to use, the label density of tissue antigens resulting from two different dehydration procedures was compared.

It is known that the temperature during tissue processing for electron microscopy and immunocytochemistry is critical for preservation of tissue antigen (Bullock, 1984; Roth, 1986; Hayat, 1989). With L.R.White resin, two different temperatures for polymerization have usually been used for immunocytochemistry namely room temperature and 50^{0}C (Newman and Hobot, 1987). To investigate the effect of L.R.White polymerization temperature on immunolabelling of laminin and type IV collagen in the present study, a comparative study was performed using polymerization temperatures of room temperature and 50^{0}C.

During the various experiments described more problems (for example poor labelling; high non-specific background staining) were encountered with type IV collagen than laminin. Several experiments aimed at rectifying this problem were designed and performed and the results are described in this section.

This section describes 9 experiments as follows:

1). (Section 3.3) Effect of different fixatives on the morphology of lingual mucosa and immunolabelling of laminin and type IV collagen.

2) (Section 3.4) Effect of fixative concentration on the morphology of lingual mucosa and immunolabelling of laminin and type IV collagen.

3) (Section 3.5) Effect of fixation additive on the morphology of lingual mucosa and immunolabelling of laminin and type IV collagen.

4) (Section 3.6) Effect of buffer system on the morphology of lingual mucosa and immunolabelling of laminin and type IV collagen.

5) (Section 3.7) Effect of fixation osmolarity on the morphology of lingual mucosa and immunolabelling of laminin and type IV collagen.

6) (Section 3.8) Effect of dehydration condition on the morphology of lingual mucosa and immunolabelling of laminin and type IV collagen.

7) (Section 3.9) Effect of polymerization temperature for L.R.White resin on the morphology of lingual mucosa and immunolabelling of laminin and type IV collagen.

8) (Section 3.10) Investigation of causes of poor labelling and high non-specific background staining in immunolabelling for type IV collagen.

9) (Section 3.11) Establishment of controls for immunolabelling of laminin and type IV collagen.

3.2. Materials and methods

Materials and methods in this section were used to investigate the effect of the different tissue preparation and immunostaining techniques on morphology of rat lingual mucosa and immunolabelling of laminin and type IV collagen. Materials including animals, chemicals and immunoreagents and basic methods used in all experiments are described here.

3.2.1. Materials

All animals used in this phase of the project comprised normal female Albino Wistar rats (average weight 180gm, 13 weeks old). Following sacrifice by an overdose of nitrous oxide-halothane tongues were dissected free from the animals. Strips of tongue tissue consisting of epithelium, connective tissue and muscle were cut into approximately $1mm^3$ size blocks in a few drops of phosphate buffered saline (PBS). For all studies described in this thesis the approval of the University of Adelaide Committee on Animal Esthics was obtained using approved procedures. All handling and experimentation was carried out in the Medical School Animal House, University of Adelaide, Director Dr. B. Burke.

3.2.2. Chemicals

Twenty five per cent glutaraldehyde was purchased from Merck, Germany. Paraformaldehyde, aqueous picric acid, sodium dihydrogen orthophosphate, di-sodium hydrogen orthophosphate,

sodium chloride and ammonium chloride were obtained from BDH Chemicals, Australia. Polyvinlpyrrolidone (PVP) (MW 30,000-40,000) was purchased from May & Baker LTD, Dagenham, England. Sucrose was bought from Jomar Diagnostics, Australia. Hard-grade L.R.White resin and accelerator were purchased from London Resin Company, London. Phosphate buffer and phosphate buffered saline were made as described by Glauert (1974).

3.2.3. Immunoreagents

1). Rabbit anti-human laminin polyclonal antibody was purchased from EY Labs, CA, USA (Catalog No.AT-2404).

2). Purified human laminin was obtained from Sigma, USA (Catalog No.2404).

3). Rabbit anti-human type IV collagen polyclonal antibody was obtained from Eurodiagnostics, Netherlands (Catalog No. PCO).

4) Rabbit anti-mouse type IV collagen polyclonal antibody was purchased from Chemicon, CA, USA (Catalog No. AB756).

5). Rabbit anti-human type IV collagen polyclonal antibody and purified rabbit anti-human type IV collagen polyclonal antibody were provided by Mrs. B. Reinboth (Department of Pathology, University of Adelaide, South Australia).

6). Purified goat anti-human and bovine type IV collagen polyclonal antibody (Catalog No. 1340-01), purified human type IV collagen (Catalog No. 1250-01) and purified bovine type IV collagen (Catalog No. 1260-02) were purchased from Southen Biotechnology, Birmingham.

7). Bovine serum albumin was purchased from Sigma (Catalog No. A-1662). Tween 20 was bought from BDH, England (Catalog No. 66368).

8). Protein A-gold (20nm) were provided by Smith (IMVS, South Australia).

9). Rabbit anti-goat IgG-gold (20nm) was purchased from ICN Biochemicals, USA (Catalog No. 678961)

10). Goat anti-rabbit IgG-gold (20nm) was bought from Biolin, UK (Catalog No. 029203).

11). Ovalbumin, fish gelatin and glycine were offered by Fanning (CMSSA, University of Adelaide, South Australia).

12). Trypsin was purchased from Hopkin & Williams, England (Catalog No.883600).

3.2.4. Basic methods common to all experiments:

3.2.4.1. Tissue preparation

1). Tongue tissues were immediately fixed by immersion in a variety of fixatives as described in subsequent sections for 1 hour.

2). Tissues were washed in 0.01M phosphate buffered saline (PB) with 50 mM NH_4CL and 3% sucrose for 1 hour, after fixation.

3). Tissues were dehydrated in 70% ethanol for 5 minutes, 90% ethanol for 5 minutes and 100% ethanol twice for 50 minutes (except Section 3.8).

4). Tissues were infiltrated in pure, hard-grade L.R.White resin thrice for 3 hours at room temperature.

5). Tissues were embedded in gelatin capsules with pure, hard-grade L.R.White resin at 50^0C for 24 hours (except Section 3.9)

3.2.4.2. Sectioning

1). Silver or gold ultrathin sections were cut using a diamond knife with a Reichert-Jung Ultramicrotome.

2). Ultra-thin sections were mounted on formvar-coated 150 mesh parallel lines nickel grids (Probing & Structure, Qld, Australia).

3.2.4.3. Immunostaining

Grids mounted with ultrathin sections were each inverted on drops of solutions as described below.

1). 1% BSA in PBS for 5 minutes (except Section 3.10.4).

2). Primary antibodies diluted (1:10 for Laminin) or (1:5 for type IV collagen) with PBS containing 1% Tween 20 and 1% BSA for 3 hours (except Sections 3.10.3; 3.10.4).

3). 3 X PBS wash for 15 minutes.

4). 1% BSA in PBS for 5 minutes (except Section 3.10.4).

5). Protein A-gold (1:200, OD_{530}=0.1) or IgG-gold (1:20) diluted with PBS containing 1% Tween 20 and 1% BSA for 30 minutes (except Section 3.10.4).

6). 3 X PBS wash for 15 minutes.

7). Wash with distilled water for 5 minutes.

8). 2% aqueous uranyl acetate for 2 minutes.

9). Reynold's lead citrate for 30 seconds.

3.3. Effect of different fixatives on the morphology of lingual mucosa and immunolabelling of laminin and type IV collagen.

Methods: Approximately 1 mm^3 size tissues harvested from normal rat tongues were immediately immersed in following fixatives:

1). 0.5% glutaraldehyde in 0.05M PB, PH 7.2
2). 1% glutaraldehyde in 0.05M PB, PH 7.2
3). 4% paraformaldehyde in 0.05M PB, PH 7.2
4). 4% paraformaldehyde-0.1% glutaraldehyde in 0.05M PB, PH7.2

for fixation and processed following the protocol described in Sections 3.2.4.1 and 3.2.4.2.

Ultrathin sections were immunostained using the protocol described in Section 3.2.4.3. Semi-quantitative evaluations of epithelial cells and basal lamina morphology and label density of laminin and type IV collagen were undertaken by randomly selecting 5 fields of epithelial cells and 5 fields of overlapped basal lamina areas at an original magnification of 20,000 times. A scoring system for morphology and label density describing four categories of results was established (Table 5)

Table 5 Scoring system used to assess label density and morphology

Label density		Morphology	
Very dense	++++	Excellent	++++
dense	+++	Good	+++
Moderate	++	Average	++
Weak	+	Poor	+

++++ More than 15 gold particles in a viewed field; good cell morphology and distinct basal lamina structure

+++ Approximately 10-15 gold particles in a viewed field; reasonable cell morphology and distinct basal lamina structure

++ Approximately 5-10 gold particles in a viewed field; poor cell morphology and reasonable basal lamina structure

\+ Less than 5 gold particles in a viewed field; poor cell morphology and indistinct basal lamina structure

Results: Better morphology of epithelial cells and basal laminae was observed in specimens fixed in glutaraldehyde rather than in paraformaldehyde or paraformaldehyde-glutaraldehyde mixture. Tissues fixed in 1% glutaraldehyde showed better contrast of cell membranes and better basal lamina morphology compared to those specimens fixed in 0.5% glutaraldehyde. However, some loss of cytoplasmic detail was seen in tissues fixed in the both glutaraldehyde solutions. Tissues fixed in all fixatives solutions

showed similarly weak label density for laminin and type IV collagen (Table 6) (Figs.3,4)

Conclusions: Rat lingual mucosa morphology is preserved more effectively by glutaraldehyde rather than by paraformaldehyde and paraformaldehyde-glutaraldehyde solutions. The demonstration of laminin and type IV collagen is unsatisfactory using these fixatives at the concentrations specified.

Table 6. Semi-quantitative evaluation of morphology of epithelial cells, basal laminae and label density of laminin and type IV collagen using the different fixatives and concentrations.

Table 6

Fixative	Epithelial cell (Morphology)	Basal lamina (Morphology)	Laminin (Label density)	Type IV collagen (Label density)
0.5% glutaraldehyde	++	++	+	+
1% glutaraldehyde	+++	+++	+	+
4% paraformaldehyde	+	+	+	+
4% paraformaldehyde -0.1%glutaraldehyde	+	+	+	+

Figure 3. Electronmicrographs showing variable tissue morphology and immunolabelling (arrows) for laminin in normal rat tongue mucosa using the following fixatives.

A. Tissue fixed in 0.5% glutaraldehyde
B. Tissue fixed in 1% glutaraldehyde
C. Tissue fixed in 4% paraformaldehyde
D. Tissue fixed in 4% paraformaldehyde-0.1% glutaraldehyde

Specimens fixed in 1% glutaraldehyde (B) exhibit better cytological detail than specimens in the other fixatives described. Weak labelling for laminin was seen in specimens fixed in all fixatives described. E-epithelial cell, B-basal lamina, C-connective tissue (Bar=0.2μm)

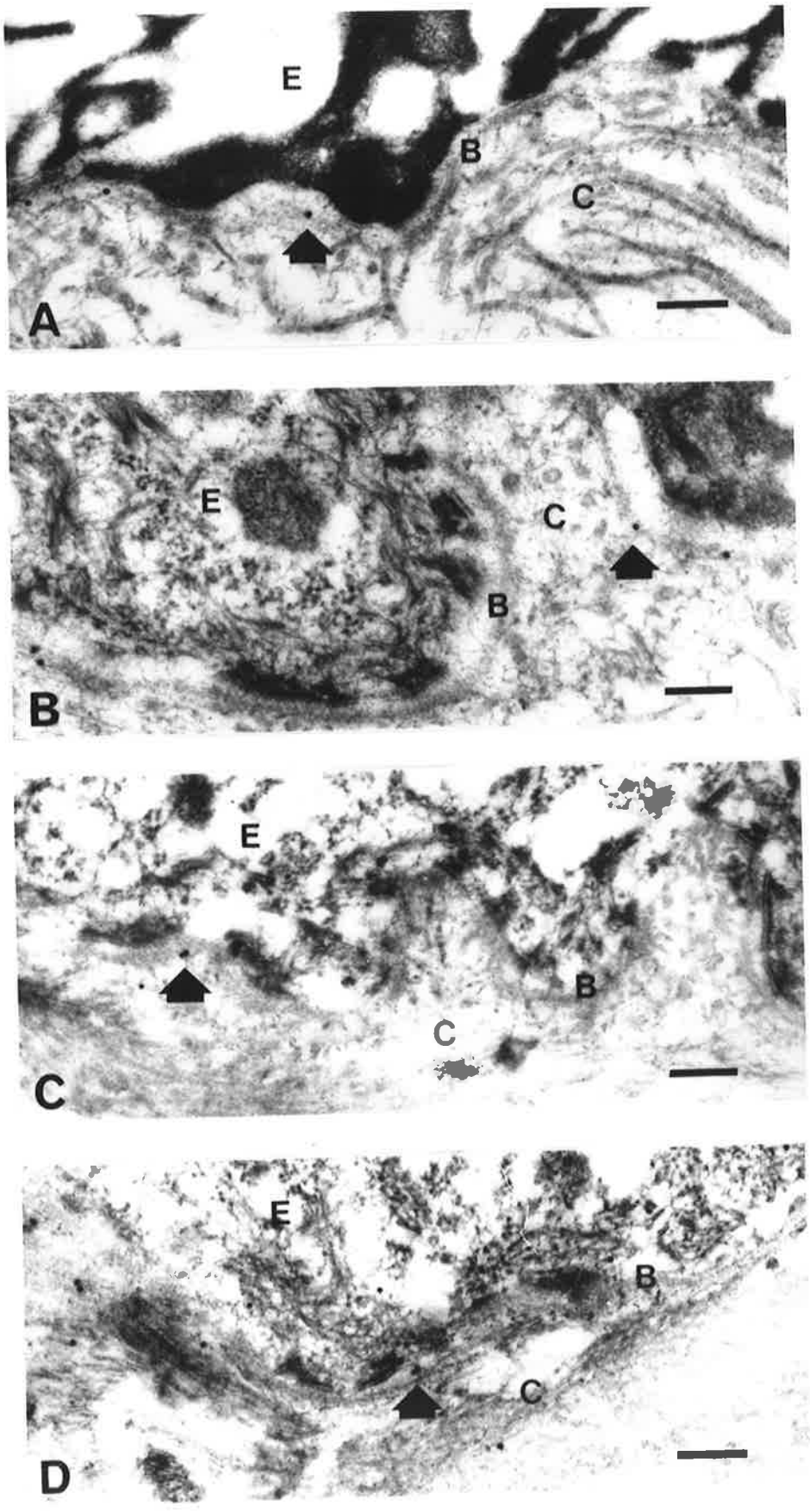


FIG 3

Figure 4. Electronmicrographs demonstrating variable tissue morphology and immunolabelling (arrows) for type IV collagen in normal rat tongue mucosa using the following fixatives.

A. Tissue fixed in 0.5% glutaraldehyde

B. Tissue fixed in 1% glutaraldehyde

C. Tissue fixed in 4% paraformaldehyde

D. Tissue fixed in 4% paraformaldehyde-0.1% glutaraldehyde

Minimal labelling (arrows) is demonstrated in all specimens. E=epithelial cell, B=basal lamina, C=connective tissue (Bar=0.2μm)

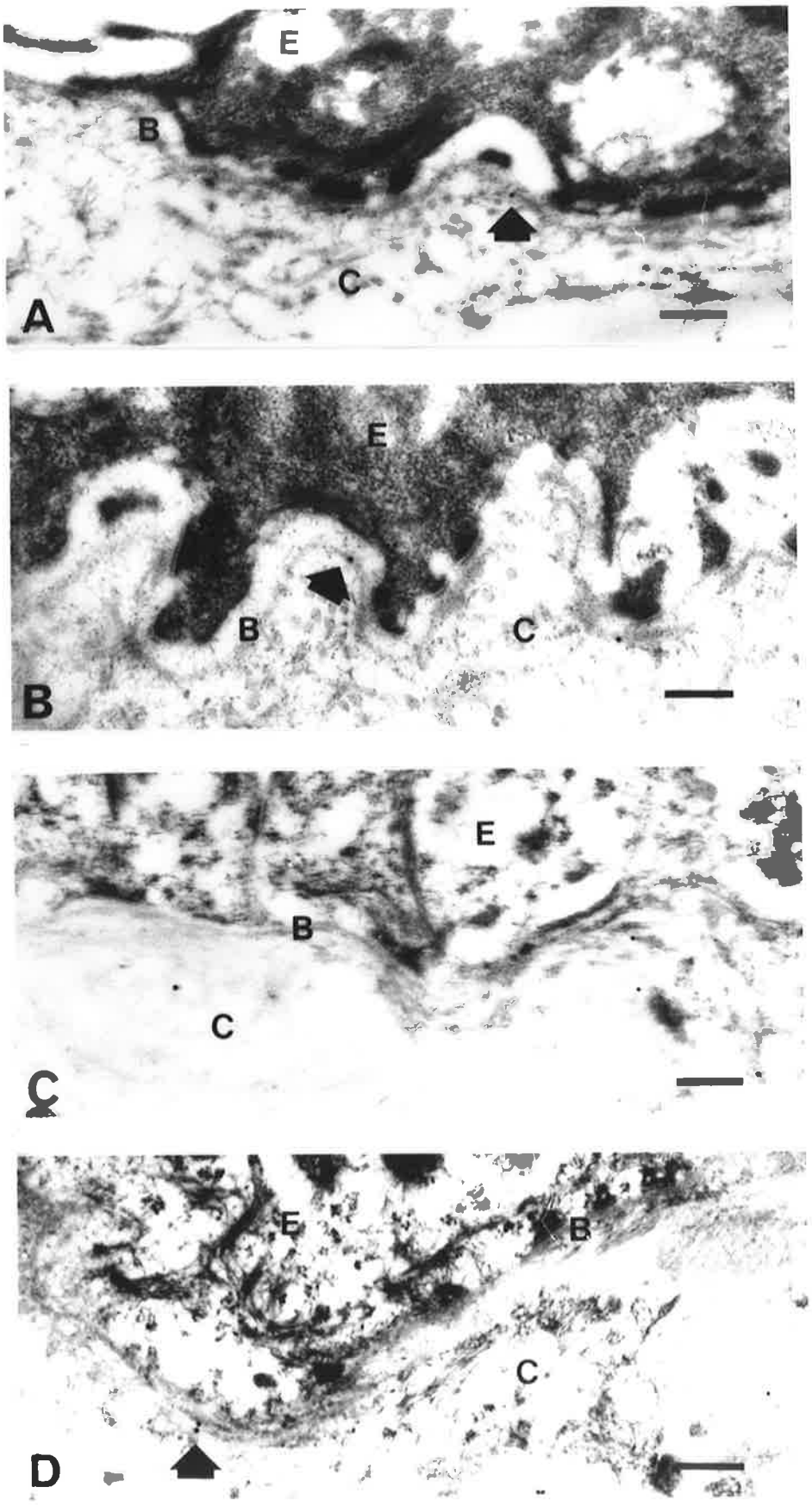


FIG 4

3.4. Effect of fixative concentration on the morphology of lingual mucosa and immunolabelling of laminin and type IV collagen.

The results of the work described in Section 3.3 in addition to demonstrating poor labelling of basal lamina antigens with the fixatives used also indicated that fixative concentration appeared to affect cytological morphology. An experiment was therefore designed to test the effect of variable fixative concentration on morphology and immunolabelling.

Methods: Following animal sacrifice and dissection tongue tissue specimens were immediately fixed by immersion in different concentrations of several fixatives as follows:

1). 0.25% glutaraldehyde in 0.05M PB, PH 7.2.
2). 1% glutaraldehyde in 0.05M PB, PH 7.2.
3). 1% paraformaldehyde in 0.05M PB, PH 7.2.
4). 4% paraformaldehyde in 0.05M PB, PH 7.2.
5). 1% paraformaldehyde-0.1% glutaraldehyde in 0.05M PB, PH 7.2.
6). 4% paraformaldehyde-0.1% glutaraldehyde in 0.05M PB, PH 7.2.

Specimens were processed, cut and immunostained following the protocols described in Sections 3.2.4.1., 3.2.4.2. and 3.2.4.3. Semi-quantitative evaluations (Section 3.3) of the morphology of epithelial cells and basal laminae and label density of laminin and type IV collagen were carried out.

Results: Better morphology of epithelial cells was observed in specimens fixed in 1% glutaraldehyde than those fixed in 0.25% glutaraldehyde, 1% and 4% paraformaldehyde, 1% paraformaldehyde-0.1% glutaraldehyde and 4% paraformaldehyde-0.1% glutaraldehyde. Better morphology of basal laminae was seen in tissues fixed in 0.25% and 1% glutaraldehyde solutions. Tissues fixed in 0.25% glutaraldehyde and 1% paraformaldehyde exhibited better immunolabelling for laminin than specimens fixed in the other fixatives. Weak label density for type IV collagen was evident in tissues fixed in all fixatives (Table 7) (Fig. 5).

Conclusions: The concentration of glutaraldehyde or paraformaldehyde appears to affect the immunolocalization of laminin, but not type IV collagen. The morphological preservation of rat lingual mucosa is associated with the concentration of glutaraldehyde fixative.

Table 7. Semi-quantitative evaluation of morphology of epithelial cells, basal laminae and label density of laminin and type IV collagen using the different fixation protocols described.

Table 7

Fixative	Epithelial cell (Morphology)	Basal lamina (Morphology)	Laminin (label density)	Type IV collagen (Label density)
0.25% glutaraldehyde	+	++	++	+
1% glutaraldehyde	+++	+++	+	+
1% paraformaldehyde	+	+	++	+
4% paraformaldehyde	+	+	+	+
1% paraformaldehyde -0.1% glutaraldehyde	+	+	+	+
4% paraformaldehyde -0.1% glutaraldehyde	+	+	+	+

Figure 5. Electronmicrographs demonstrating morphology and immunolabelling (arrows) for laminin in normal rat tongue mucosa using the different fixative concentrations.

A. Tissue fixed in 0.25% glutaraldehyde

B. Tissue fixed in 1% glutaraldehyde

C. Tissue fixed in 1% paraformaldehyde

D. Tissue fixed in 4% paraformaldehyde

Relatively more gold particles indicating laminin (arrows) were evident in specimens fixed in 0.25% glutaraldehyde (A) and 1% paraformaldehyde (C) compared with those in 1% glutaraldehyde (B) and 4% paraformaldehyde (D). E-epithelial cell, B-basal lamina, C-connective tissue. (Bar=0.2µm).

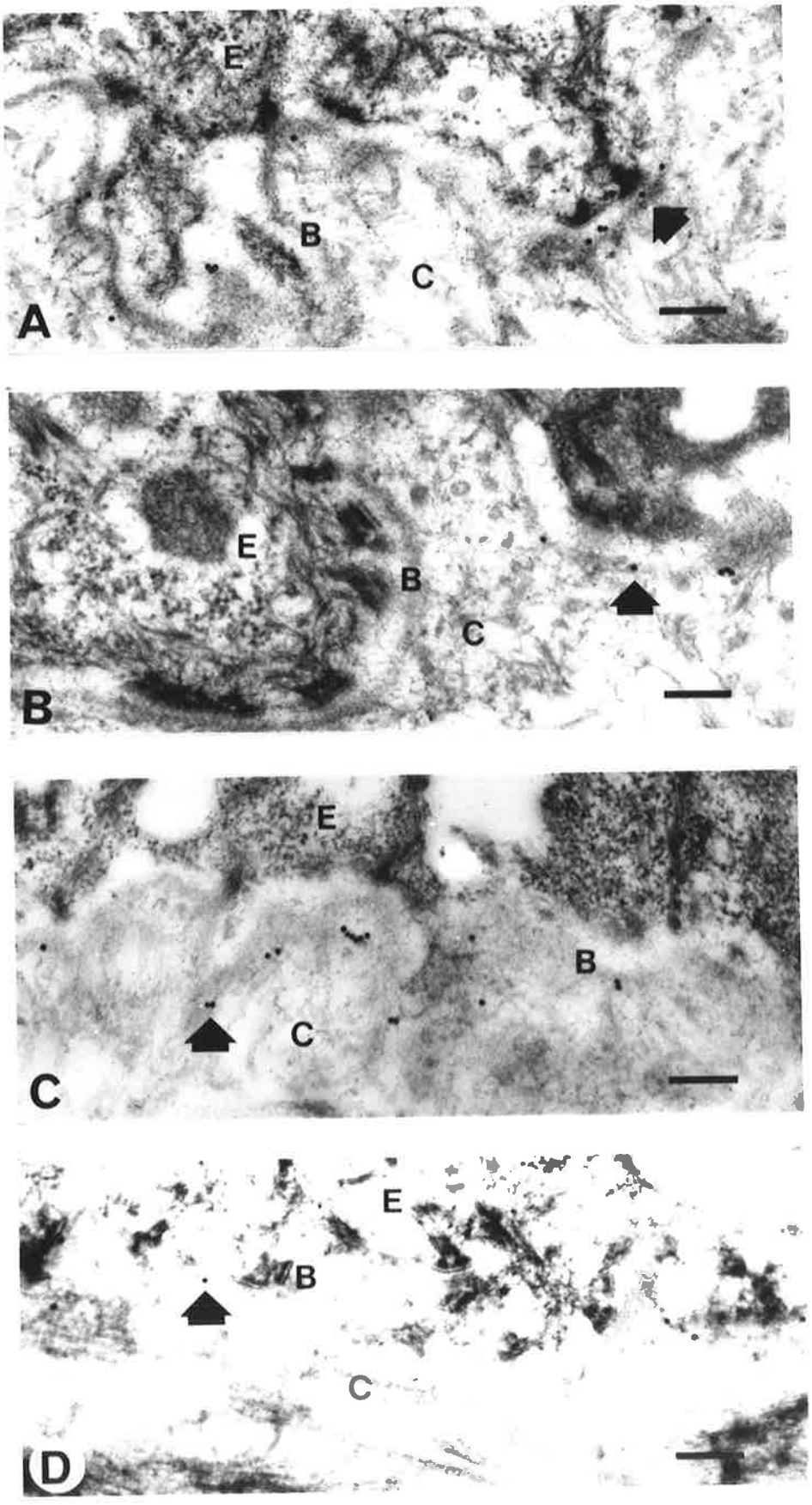


FIG5

3.5. Effect of fixation additive on the morphology of lingual mucosa and immunolabelling of laminin and type IV collagen.

The results of Section 3.4 indicated that the reduction of fixative concentration improved some labelling. However label density was not very satisfactory and cytological detail was not effectively preserved by the mild fixatives described. It has been described by Stirling (1990) that some fixation additives could affect antigen retention and morphological preservation. An experiment was therefore designed to test the effect of fixation additives on immunolabelling and morphology.

Methods: Following animal sacrifice and dissection tongue tissue specimens were immersed in a variety of fixatives containing three different agents namely picric acid, PVP and sucrose (Table 8). Specimens were processed, cut and stained using the protocols described in Sections 3.2.4.1., 3.2.4.2. and 3.2.4.3. Semi-quantitative evaluations of the morphology of epithelial cells and basal laminae and label density of laminin and type IV collagen were performed using the method described in Section 3.3.

Table 8 Various combinations of fixative with additive

Fixation	PB	Sucrose	Picric acid	PVP
0.25% glutaraldehyde	0.05M (PH7.2)	1.5%		
0.25% glutaraldehyde	0.05M (PH7.2)	1.5%		4%
0.25% glutaraldehyde	0.1M (PH7.2)	5%		
0.25% glutaraldehyde	0.1M (PH7.2)	5%	0.15%	
0.25% glutaraldehyde	0.1M (PH7.2)	4%	0.15%	4%

Results: Tissues fixed in the following fixatives:

0.25% glutaraldehyde-5% sucrose in 0.1M PB

0.25% glutaraldehyde-5% sucrose-0.15% picric acid in 0.1M PB

0.25% glutaraldehyde-1.5% sucrose-4% PVP in 0.05M PB

0.25% glutaraldehyde-4% sucrose-0.15% picric acid-4% PVP in 0.1M PB

exhibited better epithelial cell morphology than those fixed in 0.25% glutaraldehyde-1.5% sucrose in 0.05M PB. The best contrast of cell membrane, desmosomes and hemidesmosomes was seen in specimens fixed in 0.25% glutaraldehyde-5% sucrose-0.15% picric acid in 0.1M PB. Basal lamina morphology appeared similar in tissues fixed in all fixative solutions. Better immunolabelling of laminin was observed in tissues fixed in fixatives containing picric acid or PVP. Reasonable immunolabelling of type IV collagen was only seen in 0.25% glutaraldehyde-5% sucrose-0.15%

picric acid in 0.1M PB and 0.25% glutaraldehyde-4% sucrose-0.15% picric acid-4% PVP in 0.1M PB fixed specimens (Table 9) (Figs. 6,7,8,).

Conclusions: The addition of picric acid to fixative solutions appears to affect the immunolabelling of laminin and type IV collagen and to some degree the preservation of tissue morphology. It appears that PVP in fixatives is associated with the preservation of laminin antigen and tissue morphology, but does not enhance the immunolabelling of type IV collagen. The addition of sucrose appears to be related to the preservation of tissue morphology, but does not enhance the immunolabelling of laminin or type IV collagen. Fixatives containing picric acid and PVP together produce the same effect on immunolabelling as those with picric acid or PVP alone.

Table 9. Semi-quantitative evaluation of morphology of epithelial cells, basal laminae and label density of laminin and type IV collagen using the different fixation additives.

Table 9

Fixative	Additive	Epithelial cell (Morphology)	Basal lamina (Morphology)	Laminin (Label density)	Type IV collagen (Label density)
0.25% glutaraldehyde	1.5% sucrose	+	++	++	+
0.25% glutaraldehyde	1.5% sucrose 4% PVP	++	++	+++	+
0.25% glutaraldehyde	5% sucrose	++	++	++	+
0.25% glutaraldehyde	5% sucrose 0.15% picric acid	+++	++	+++	++
0.25% glutaraldehyde	4% sucrose 0.15% picric acid 4% PVP	++	++	+++	++

Figure 6. Electronmicrographs showing morphology of normal epithelial cells using the following fixative-additive solutions:

A. Tissue fixed in 0.25% glutaraldehyde-1.5% sucrose

B. Tissue fixed in 0.25% glutaraldehyde-1.5% sucrose-4% PVP

C. Tissue fixed in 0.25% glutaraldehyde-5% sucrose

D. Tissue fixed in 0.25% glutaraldehyde-5% sucrose-0.15% picric acid

Poor cytological morphology was evident in specimens fixed in 0.25% glutaraldehyde-1.5% sucrose (A) compared with those fixed in the other fixative-additive solutions (B,C,D). The best cytological detail was seen in specimens fixed in 0.25% glutaraldehyde-5% sucrose-0.15% picric acid (D). D-desmosome, F-tonofilament. (Bar=0.2μm).

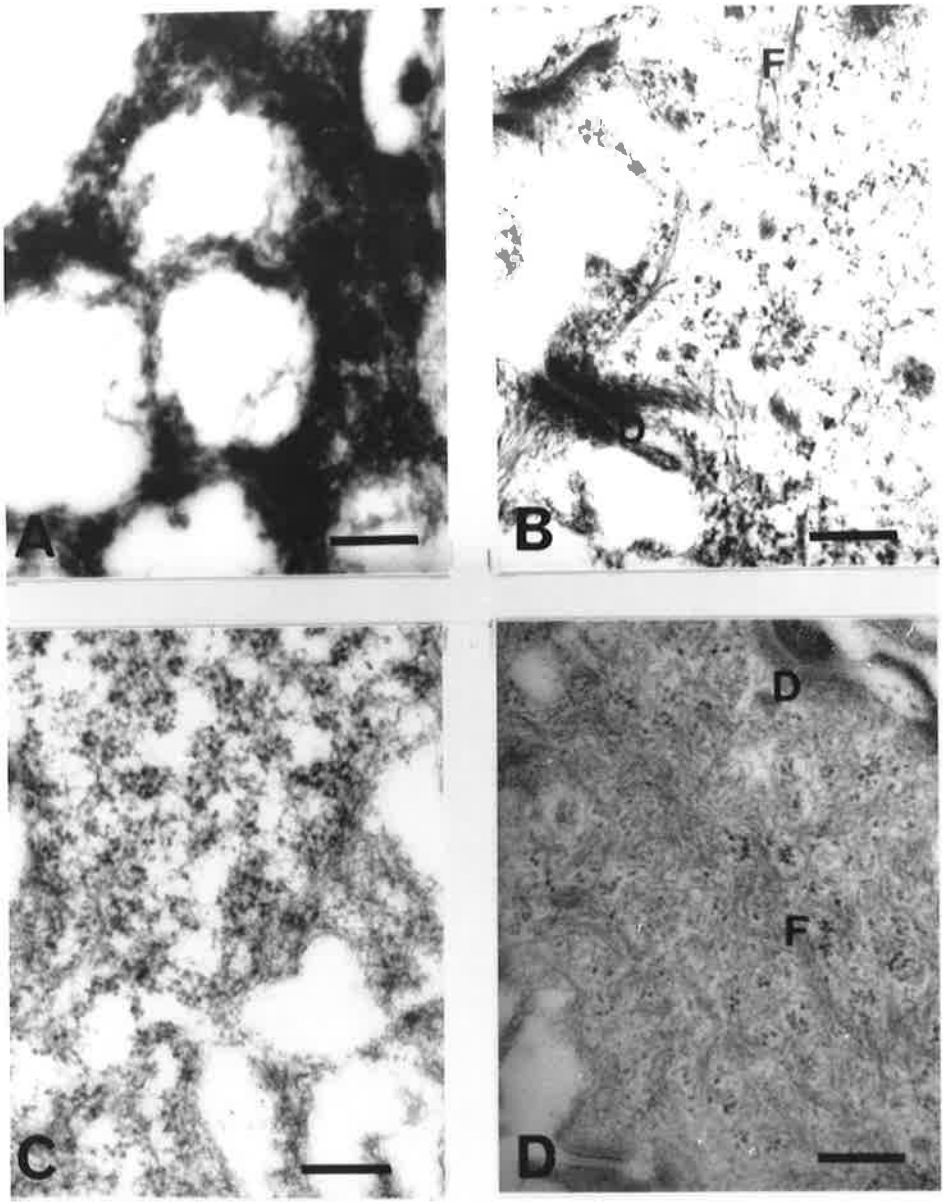


FIG6

Figure 7. Electronmicrographs showing immunolabelling (arrows) for laminin in normal rat tongue mucosa using the following fixative-additive solutions:

A. Tissue fixed in 0.25% glutaraldehyde-1.5% sucrose

B. Tissue fixed in 0.25% glutaraldehyde-1.5% sucrose-4% PVP

C. Tissue fixed in 0.25% glutaraldehyde-5% sucrose

D. Tissue fixed in 0.25% glutaraldehyde-5% sucrose-0.15% picric acid

More gold particles indicating laminin were evident in specimens fixed in fixatives containing PVP (B) or picric acid (D) compared with those fixed in fixatives without PVP or picric acid (A, C). E-epithelial cell, B-basal lamina, C-connective tissue. (Bar=0.2μm).

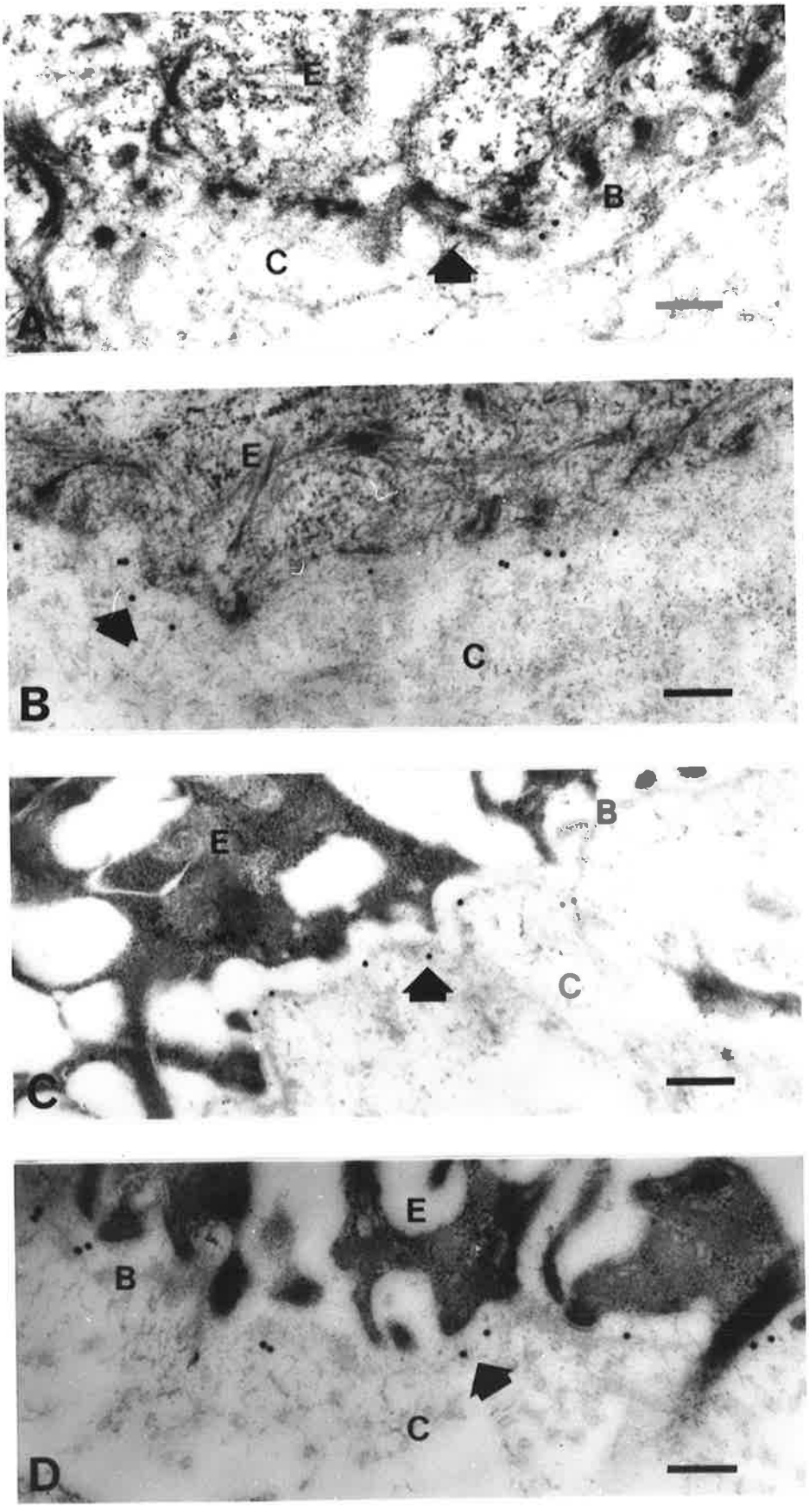


FIG 7

Figure 8. Electronmicrographs showing immunolabelling (arrows) for type IV collagen in normal rat tongue mucosa using the following fixative-additive solutions:

A. Tissue fixed in 0.25% glutaraldehyde-1.5% sucrose

B. Tissue fixed in 0.25% glutaraldehyde-1.5% sucrose-4% PVP

C. Tissue fixed in 0.25% glutaraldehyde-5% sucrose

D. Tissue fixed in 0.25% glutaraldehyde-5% sucrose-0.15% picric acid

More gold particles indicating type IV collagen were evident in specimens fixed in 0.25% glutaraldehyde-5% sucrose-0.15% picric acid solution (D) compared with those fixed in the other fixative-additive solutions (A,B,C). E-epithelial cell, B-basal lamina, C-connective tissue. (Bar=0.2μm).

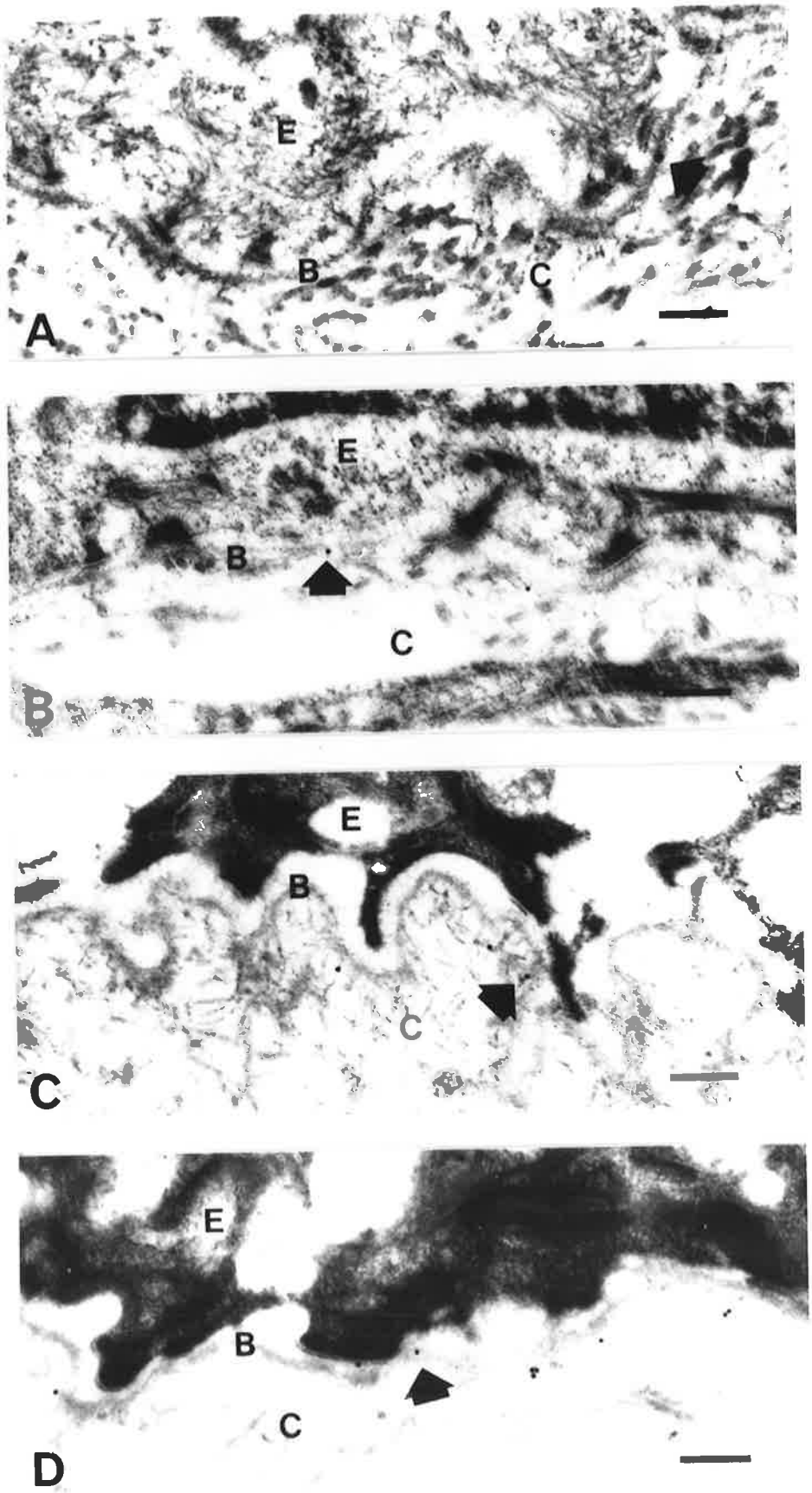


FIG 8

3.6. Effect of buffer system on the morphology of lingual mucosa and immunolabelling of laminin and type IV collagen

The results of Section 3.5. indicated that fixation additives affected epithelial morphology and immunolabelling of basal lamina laminin and type IV collagen. Apart from fixation additives, buffer system is also an important constituent (fixative vehicle) in fixative solution and can affect morphological preservation (Hayat, 1989). However the effect of buffer system on immunolabelling is poorly understood. An experiment was therefore designed to test the effect of buffer systems (two common used phosphate buffer and phosphate buffered saline) on morphology and immunolabelling.

Methods: Following animal sacrifice and dissection tongue tissue specimens were immediately fixed by immersion in the fixatives described (Table 10) with the addition of two different buffer systems namely phosphate buffer (PB) and phosphate buffered saline (PBS). Specimens were processed, cut and immunostained using the protocols described in Sections 3.2.4.1., 3.2.4.2. and 3.2.4.3. A scoring system similar to that described in Section 3.3. was used for semi-quantitative evaluations of the morphology of epithelial cells, basal laminae and label density for laminin and type IV collagen.

Table 10 Various combinations of fixative with buffer system

Fixative	Buffer system
0.25% glutaraldehyde, 5% sucrose, PH 7.2	0.1M PB
0.25% glutaraldehyde, PH 7.2	0.01M PBS
0.5% glutaraldehyde, 5% sucrose, PH 7.2	0.1M PB
0.5% glutaraldehyde, PH 7.2	0.01M PBS

Results and conclusion: Tissues fixed in similar fixatives, but with two different buffer systems exhibited essentially similar tissue morphology preservation and immunolabelling for laminin and type IV collagen (Table 11)(Fig. 9).

Table 11. Semi-quantitative evaluation of morphology of epithelial cells, basal laminae and label density of laminin and type IV collagen using the different buffer systems.

Table 11

Fixative	Buffer system	Epithelial cell (Morphology)	Basal lamina (Morphology)	Laminin (Label density)	Type IV collagen (Label density)
0.25% glutaraldehyde 5% sucrose	0.1M PB	++	++	++	+
0.5% glutaraldehyde 5% sucrose	0.1M PB	+++	++	+	+
0.25% glutaraldehyde	0.01M PBS	++	++	++	+
0.5% glutaraldehyde	0.01M PBS	+++	++	+	+

Figure 9. Electronmicrographs showing morphology and immunolabelling (arrows) for laminin in normal rat tongue mucosa using the different buffer systems.

A. Tissue fixed in 0.25% glutaraldehyde-5% sucrose in 0.1M PB

B. Tissue fixed in 0.25% glutaraldehyde in 0.01 M PBS

C. Tissue fixed in 0.5% glutaraldehyde-5% sucrose in 0.1M PB

D. Tissue fixed in 0.5% glutaraldehyde in 0.01 M PBS

Essentially similar labelling for laminin was evident in specimens fixed in fixatives with the two buffer systems (compare A with B; C with D). E-epithelial cell, B-basal lamina, C-connective tissue. (Bar=0.2μm). (Immunolabelling for type IV collagen was similar to laminin)

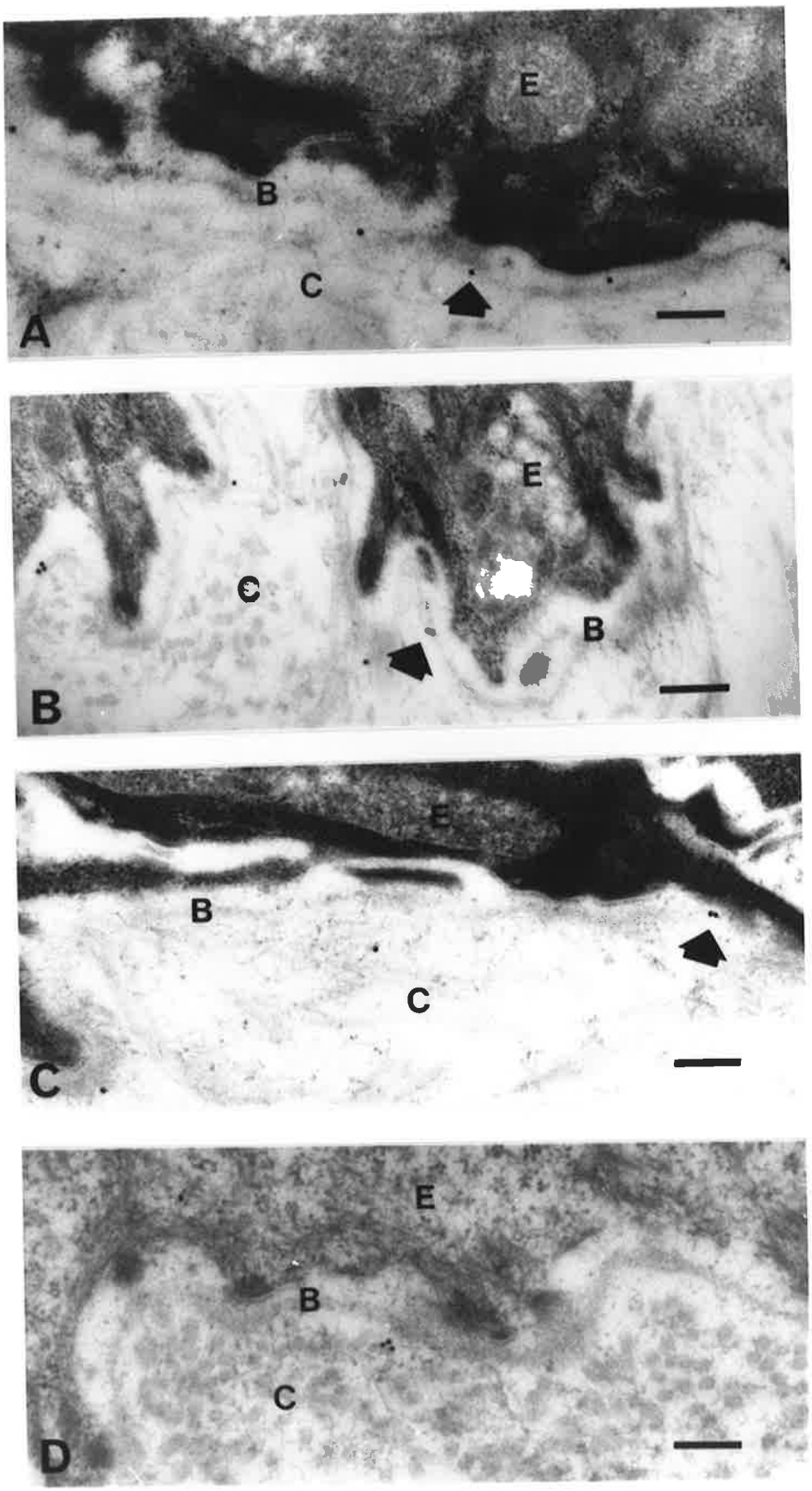


FIG 9

3.7. Effect of fixation osmolarity on the morphology of lingual mucosa and immunolabelling of laminin and type IV collagen.

The results of Section 3.5 indicated that fixative additives affected morphological preservation and immunolabelling. It is known that some additives (i.e sucrose and PVP) can contribute to fixation osmolarity which affects morphology (Hayat, 1989). However the effect of fixation osmolarity on immunolabelling is poorly understood. An experiment was therefore designed to test the effect of fixation osmolarity on morphology and immunolabelling.

Methods: Following animal sacrifice and dissection tongue tissue specimens were immersed in a range of fixatives with a variety of osmolarities which were adjusted using the different buffer solutions and additives such as sucrose and PVP. For paraformaldehyde and paraformaldehyde-glutaraldehyde mixture fixatives, the osmolarities were adjusted only by changing the concentration of fixatives. All fixation osmolarities were measured using a Halbmikro-Osmometer (DHA, Anax PTY, LTD)(Table 12). Tissue preparation, sectioning, staining and semi-quantitative evaluations of epithelial cell and basal lamina morphology and label density of laminin and type IV collagen were carried out using the protocols described in sections 3.2.4.1., 3.2.4.2., 3.2.4.3. and 3.3.

Results: Tissues fixed in the following fixatives:

1). 0.5% glutaraldehyde-5% sucrose in 0.1M PB, PH7.2, **375 mosm**;

2). 0.5% glutaraldehyde-3% sucrose-4% PVP in 0.1M PB, PH7.2, **375 mosm** and

3). 0.5% glutaraldehyde in 0.01M PBS, PH7.2, **380 mosm**

showed better morphology of epithelial cells than those fixed in

4). 0.25%glutaraldehyde-1.5%sucrose in 0.05M PB, PH7.2, **193 mosm**;

5). 0.5% glutaraldehyde-3% sucrose in 0.05M PB, PH7.2, **262 mosm**;

6,7). 1% or 4% paraformaldehyde in 0.05M PB, PH7.2, **335 mosm** or **1140 mosm**;

8,9). 1% or 4% paraformaldehyde-0.1% glutaraldehyde in 0.05M PB, PH7.2, **340 mosm** and **1100 mosm**.

A similar morphology of basal laminae was seen in tissues fixed in all glutaraldehyde solutions with osmolarities ranging from **193 mosm** to **380 mosm**. Poor morphology of basal laminae was observed in tissues fixed in paraformaldehyde and paraformaldehyde-glutaraldehyde mixture in which the osmolarities of solutions ranged from **335 mosm** to **1256 mosm**. Immunolabelling of laminin and type IV collagen did not exhibit a marked difference when fixatives having the different fixation osmolarities were compared (Table 12) (Fig.10).

Conclusions: Fixation osmolarity varies according to the buffer system used, additives and the concentration of paraformaldehyde, but not the concentration of glutaraldehyde.

With the glutaraldehyde fixatives, fixation osmolarity appears to be associated with the preservation of epithelial cell morphology, but not the preservation of basal lamina morphology and tissue antigen. The addition of sucrose to fixatives increases fixation osmolarity and appears to affect the preservation of tissue morphology, but not to improve immunolabelling. It appears that PVP in fixative solutions improves the preservation of laminin antigen possibly by mechanisms other than adjusting fixation osmolarity. With paraformaldehyde fixatives, fixation osmolarity appears not to be associated with the preservation of tissue morphology and tissue antigen.

Table 12. Semi-quantitative evaluation of morphology of epithelial cells, basal lamina and label density of laminin and type IV collagen using the different fixation osmolarities indicated.

Table 12

Fixative	Osmolality (mosm)	Epithelial cell (Morphology)	Basal lamina (Morphology)	Laminin (Label density)	Type IV collagen (Label density)
0.25% glutaraldehyde 1.5%sucrose,0.05MPB	193	+	++	++	+
0.25% glutaraldehyde 5% sucrose, 0.1M PB	340	++	++	++	+
0.25% glutaraldehyde 3% sucrose, 4% PVP, 0.1M PB	345	++	++	+++	+
0.25%glutaraldehyde, 0.01M PBS	370	++	++	++	+
0.5% glutaraldehyde 3% sucrose, 0.05M PB	262	++	++	+	+
0.5% glutaraldehyde 5% sucrose, 0.1M PB	375	+++	++	+	+
0.5% glutaraldehye 3% sucrose, 4%PVP, 0.1MPB	375	+++	++	++	+
0.5% glutaraldehyde, 0.01M PBS	380	+++	++	+	+
4%paraformaldehyde, 0.05M PB	1140	+	+	+	+
4% paraformaldehyde 4% PVP, 0.05M PB	1256	+	+	++	+
1%paraformaldehyde, 0.05M PB	335	+	+	++	+
4%paraformaldehyde-0.1% glutaraldehyde, 0.05M PB	1100	+	+	+	+
1%paraformaldehyde-0.1% glutaraldehyde, 0.05M PB	340	+	+	+	+

Figure10. Electronmicrographs showing morphology of normal epithelial cells using the following the different fixation osmolarities.

A. Tissue fixed in 0.25% glutaraldehyde-1.5% sucrose,**193 mosm**

B. Tissue fixed in 0.25% glutaraldehyde-5% sucrose, **340 mosm**

C. Tissue fixed in 0.5% glutaraldehyde-3% sucrose, **262 mosm**

D. Tissue fixed in 0.5% glutaraldehyde-5% sucrose, **375 mosm**

Better cytological detail was evident in specimens fixed in 0.25% glutaraldehyde-5% sucrose, **340 mosm** (B) and 0.5% glutaraldehyde-5% sucrose, **375 mosm** (D) compared with those fixed in 0.25% glutaraldehyde-1.5% sucrose,**193 mosm** (A) and 0.5% glutaraldehyde-3% sucrose, **262 mosm** (C) (Compare A with B, C with D). D-desmosome, E-epithelial cell. (Bar=0.2μm).

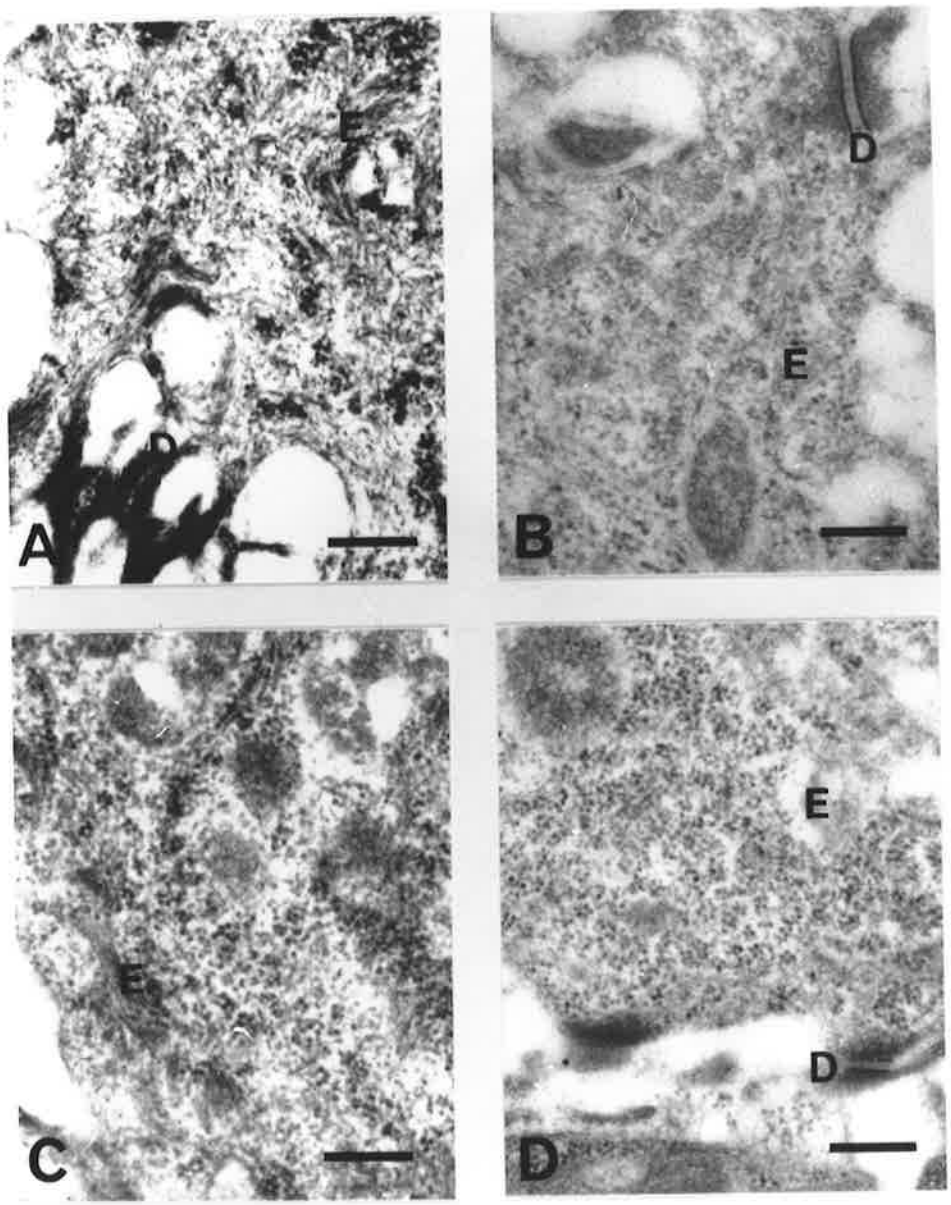


FIG 10

3.8. Effect of dehydration condition on the morphology of lingual mucosa and immunolabelling of laminin and type IV collagen.

Two dehydration protocols for L.R.White resin have been used in previous studies (Newman and Hobot, 1987; Lin and Essener, 1990). However there has been no information on the effect of both dehydration conditions on morphological preservation and immunolabelling of basal lamina antigens. An experiment was therefore designed to test the effect of dehydration condition on morphology and immunolabelling.

Methods: Following animal sacrifice and dissection tongue tissue specimens were fixed by immersion in 0.5% glutaraldehyde-3% sucrose in 0.05M PB, PH 7.2 and 0.5% glutaraldehyde-3% sucrose-0.15% picric acid in 0.05M PB, PH 7.2. and processed using the protocol described in Section 3.2.4.1. An exception was that some specimens were dehydrated only in 70% ethanol. Sectioning, immunostaining and semi-quantitative evaluations of epithelial cell and basal lamina morphology and label density of laminin and type IV collagen were undertaken using the protocols described in Section 3.2.4.2, 3.2.4.3 and 3.3.

Results: Tissues cycled through complete dehydration sequences (70%, 90%, 100% ethanols) exhibited better morphology of epithelial cells than those exposed to partial dehydration (70% ethanol) (Fig.11). There was no difference observed in the morphology of basal laminae or in the intensity of

immunolabelling of laminin and type IV collagen between the two dehydration procedures (Table 13).

Conclusions: Dehydration procedures appear to be associated with the preservation of ultrastructural tissue morphology, but not the expression of laminin and type IV collagen antigens.

Table 13. Semi-quantitative evaluation of morphology of epithelial cells, basal lamina and label density of laminin and type IV collagen using two dehydration procedures

Table 13

Fixative	Dehydration	Epithelial cell (Morphology)	Basal lamina (Morphology)	Laminin (Label density)	Tyep IV collagen (Label density)
0.5% glutaraldehyde 3% sucrose, 0.05M PB	Complete	++	++	+	+
0.5% glutaraldehyde 3% sucrose, 0.05M PB	Partial	+	++	+	+
0.5% glutaraldehyde 3% sucrose, 0.15% picric acid, 0.05M PB	Complete	++++	++	++	+
0.5% glutaraldehyde 3% sucrose, 0.15% picric acid, 0.05M PB	Partial	++	++	++	+

Figure 11. Electronmicrographs showing morphology of normal epithelial cells using two dehydration procedures

A. Tissue fixed in 0.5% glutaraldehyde-3% sucrose and processed in complete dehydration

B. Tissue fixed in 0.5% glutaraldehyde-3% sucrose and processed in partial dehydration

C. Tissue fixed in 0.5% glutaraldehyde-3% sucrose-0.15% picric acid and processed in complete dehydration

D. Tissue fixed in 0.5% glutaraldehyde-3% sucrose-0.15% picric acid and processed in partial dehydration

Better cytological detail was evident in specimens processed in complete dehydration (A, C) compared with those in partial dehydration (B,D). D-desmosome, F-tonofilament. (Bar=0.2μm).

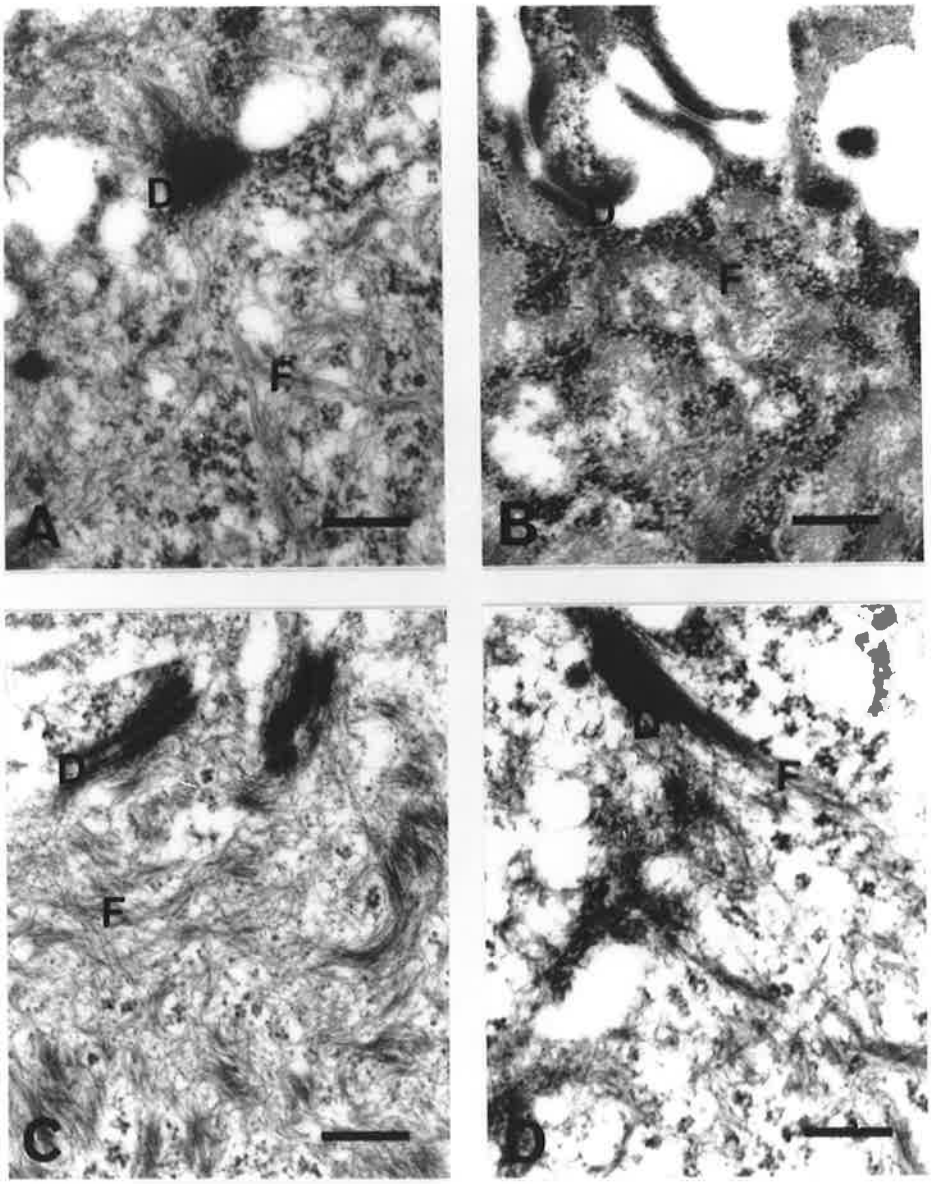


FIG 11

3.9. Effect of polymerization temperature for L.R.White resin on the morphology of lingual mucosa and immunolabelling of laminin and type IV collagen.

In the use of L.R.White resin, two different temperatures for polymerization have been used in immunocytochemical localization of tissue antigens (Newman and Hobot, 1987). However there is little information on the effect of both polymerization temperatures on epithelial morphology and immunolabelling of basal lamina antigens. An experiment was therefore designed to test the effect of polymerization temperature for L.R.White resin on morphology and immunolabelling.

Methods: Following animal sacrifice and dissection tongue tissue specimens were immersed in one of the following fixatives for 1 hour.

1). 0.25% glutaraldehyde-5% sucrose in 0.1M PB, PH 7.2
2). 0.25% glutaraldehyde-5% sucrose-0.15% picric acid in 0.1M PB, PH 7.2
3). 0.25% glutaraldehyde-3% sucrose-4%PVP in 0.1M PB,PH 7.2
4). 0.25% glutaraldehyde-4% sucrose-0.15% picric acid-4% PVP in 0.1M PB, PH 7.2
5). 0.5% glutaraldehyde-5% sucrose in 0.1M PB, PH 7.2
6). 0.5% glutaraldehyde-5% sucrose-0.15% picric acid in 0.1M PB, PH 7.2
7). 0.5% glutaraldehyde-3% sucrose-4% PVP in 0.1M PB, PH 7.2
8). 0.5% glutaraldehyde-4% sucrose-0.15% picric acid-4% PVP in 0.1M PB, PH 7.2

Subsequently tissues were dehydrated and infiltrated using the protocols described in Section 3.2.4.1. For embedding, two different polymerization temperatures for L.R.White resin were used namely room temperature (RT) and 50°C.

At room temperature, a mixture of pure, hard-grade L.R.White resin and accelerator in the proportion of 1µl accelerator to 1ml L.R.White resin was thoroughly stirred in a nitrogen atmosphere. Half of the specimens fixed in each fixative were embedded in a mixture of L.R.White resin and accelerator in gelatin capsules in a continuous flow nitrogen atmosphere at room temperature until polymerization was complete (2-3 hr). The other half of the specimens were embedded in pure, hard-grade L.R.White resin polymerized at 50°C for 24 hours. Sectioning, immunostaining and semi-quantitative evaluations of epithelial cell and basal lamina morphology and label density of laminin and type IV collagen were carried out using the protocols described in Sections 3.2.4.2., 3.2.4.3. and 3.3.

Results: Tissues embedded with the L.R.White resin polymerized at room temperature exhibited a higher label density for laminin and type IV collagen than the specimens embedded with L.R.White resin polymerized at 50°C. Some specimens embedded at 50°C exhibited better epithelial cell morphology compared to those embedded at room temperature (Table 14)(Figs.12,13,14).

Conclusions: The polymerization temperature for L.R.White resin appears to affect the expression of laminin and type IV collagen under the experimental conditions employed. The polymerization

temperature for L.R.White resin also possibly affects the ultrastructural morphology of epithelium.

Table 14. Semi-quantitative evaluation of morphology of epithelial cells, basal laminae and label density of laminin and type IV collagen using two different polymerization temperatures for L.R.White resin

Table 14

Fixative	Temperature (polymerization)	Epithelial cell (Morphology)	Basal lamina (Morphology)	Laminin (Label density)	Type IV collagen (Label density)
0.25% glutaraldehyde, 5% sucrose	50	++	++	++	+
	RT	++	+	+++	++
0.25% glutaraldehyde, 5% sucrose, 0.15% picric acid	50	+++	++	+++	++
	RT	++	++	++++	+++
0.25% glutaraldehyde, 3% sucrose, 4% PVP	50	++	++	+++	+
	RT	++	++	++++	++
0.25% glutaraldehye, 4% sucrose, 0.15% picric acid, 4% PVP	50	++	++	+++	++
	RT	++	+	++++	+++
0.5% glutaraldehyde, 5% sucrose	50	+++	++	+	+
	RT	++	+	++	++
0.5% glutaraldehyde, 5% sucrose, 0.15% picric acid	50	++++	++	++	++
	RT	++	++	+++	++
0.5% glutaraldehyde, 3% sucrose, 4% PVP	50	++	++	++	+
	RT	++	++	+++	++
0.5% glutaraldehyde, 4% sucrose, 0.15% picric acid, 4% PVP	50	+++	++	++	++
	RT	+++	++	+++	++

Figure 12. Electronmicrographs illustrating morphology of normal epithelial cells using two different polymerization temperatures for L.R.White resin.

A. Tissue fixed in 0.25% glutaraldehyde-5% sucrose-0.15% picric acid and embedded at 50°C

B. Tissue fixed in 0.25% glutaraldehyde-5% sucrose-0.15% picric acid and embedded at room temperature

C. Tissue fixed in 0.5% glutaraldehyde-5% sucrose-0.15% picric acid and embedded at 50°C

D. Tissue fixed in 0.5% glutaraldehyde-5% sucrose-0.15% picric acid and embedded at room temperature

Better cytological detail was evident in specimens embedded with L.R.White resin polymerized at 50°C (A,C) compared with those embedded with L.R.White resin polymerized at room temperature (B,D). D-desmosome, F-tonofilament. (Bar=0.2µm)

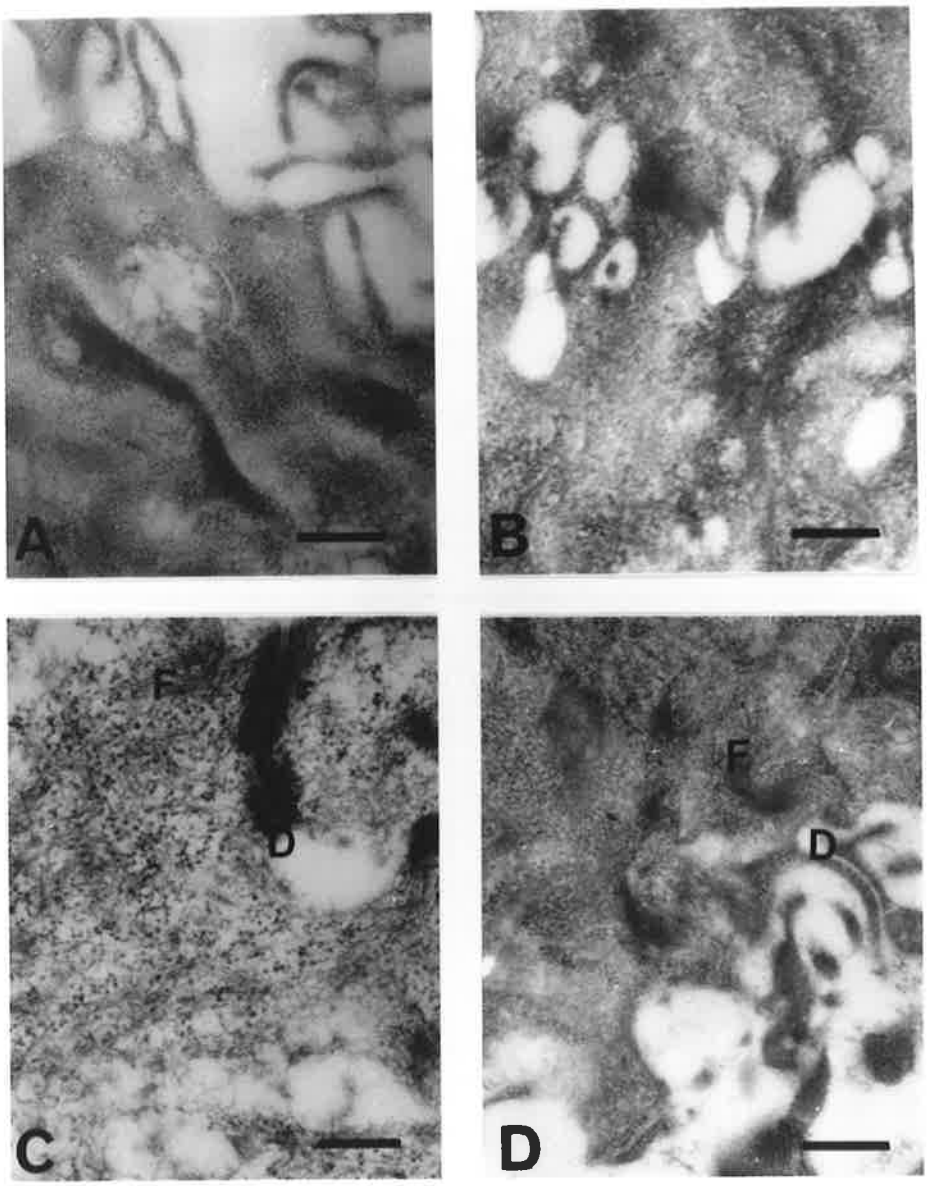


FIG 12

Figure 13. Electronmicrographs showing immunolabelling (arrows) for laminin in normal rat tongue mucosa using two different polymerization temperatures for L.R.White resin.

A. Tissue fixed in 0.25% glutaraldehyde-5% sucrose-0.15% picric acid and embedded at 50°C

B. Tissue fixed in 0.25% glutaraldehyde-5% sucrose-0.15% picric acid and embedded at room temperature

C. Tissue fixed in 0.25% glutaraldehyde-3% sucrose-4% PVP and embedded at 50°C

D. Tissue fixed in 0.25% glutaraldehyde-3% sucrose-4% PVP and embedded at room temperature

More gold particles indicating laminin was evident in specimens embedded at room temperature (B,D) compared with those embedded at 50°C (A,C).

E-epithelial cell, B-basal lamina, C-connective tissue. (Bar=0.2μm).

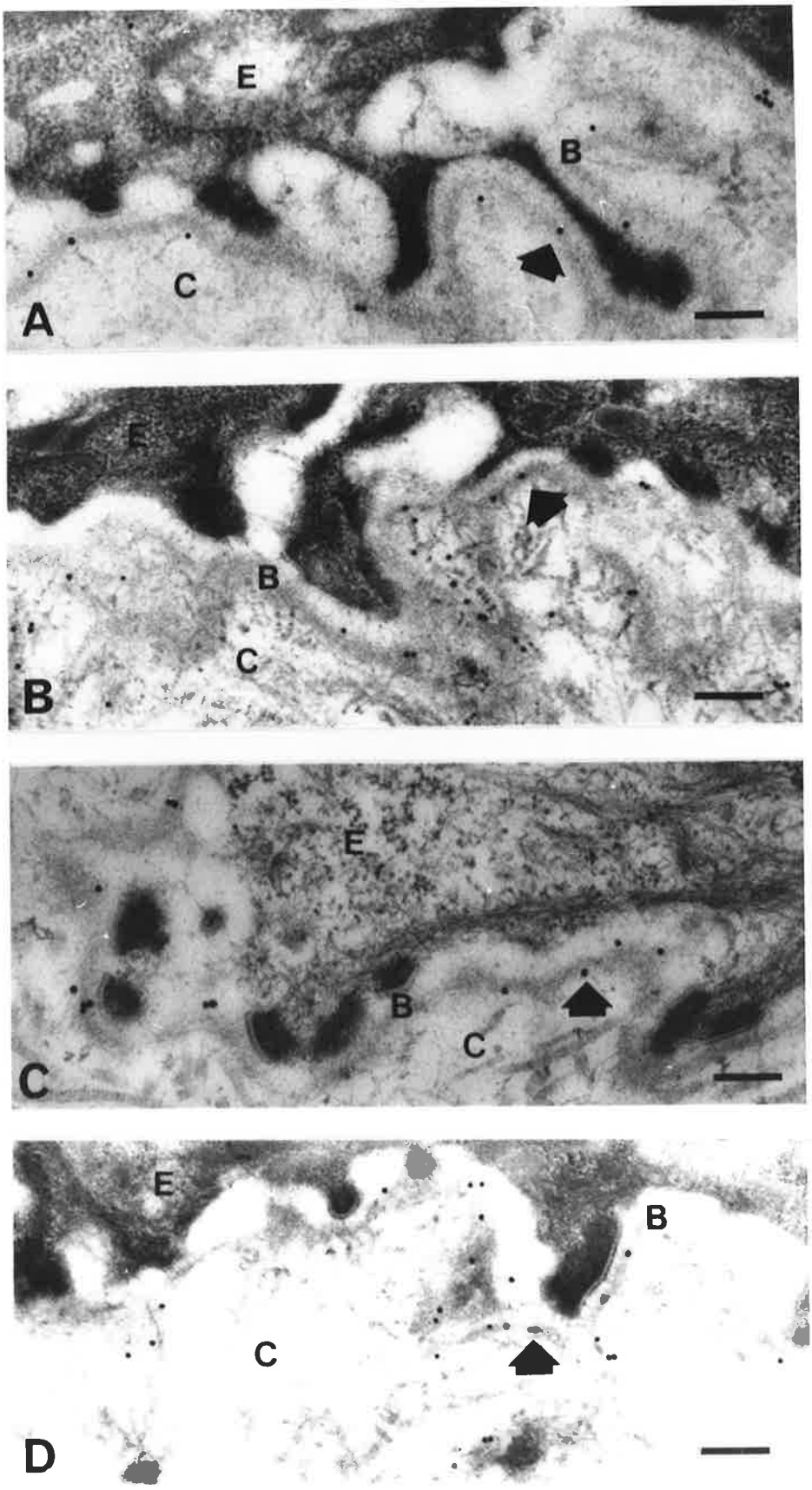


FIG 13

Figure 14. Electronmicrographs showing immunolabelling (arrows) for type IV collagen in normal rat tongue mucosa using two different polymerization temperatures for L.R.White resin.

A. Tissue fixed in 0.25% glutaraldehyde- 5% sucrose-0.15% picric acid and embedded at 50°C

B. Tissue fixed in 0.25% glutaraldehyde-5% sucrose-0.15% picric acid and embedded at room temperature

C. Tissue fixed in 0.25% glutaraldehyde-3% sucrose-4% PVP and embedded at 50°C

D. Tissue fixed in 0.25% glutaraldehyde-3% sucrose-4% PVP and embedded at room temperature

More gold particles indicating type IV collagen were evident in specimens embedded at room temperature (B,D) compared with those embedded at 50°C (A,C).
E-epithelial cell, B-basal lamina, C-connective tissue. (Bar=0.2μm).

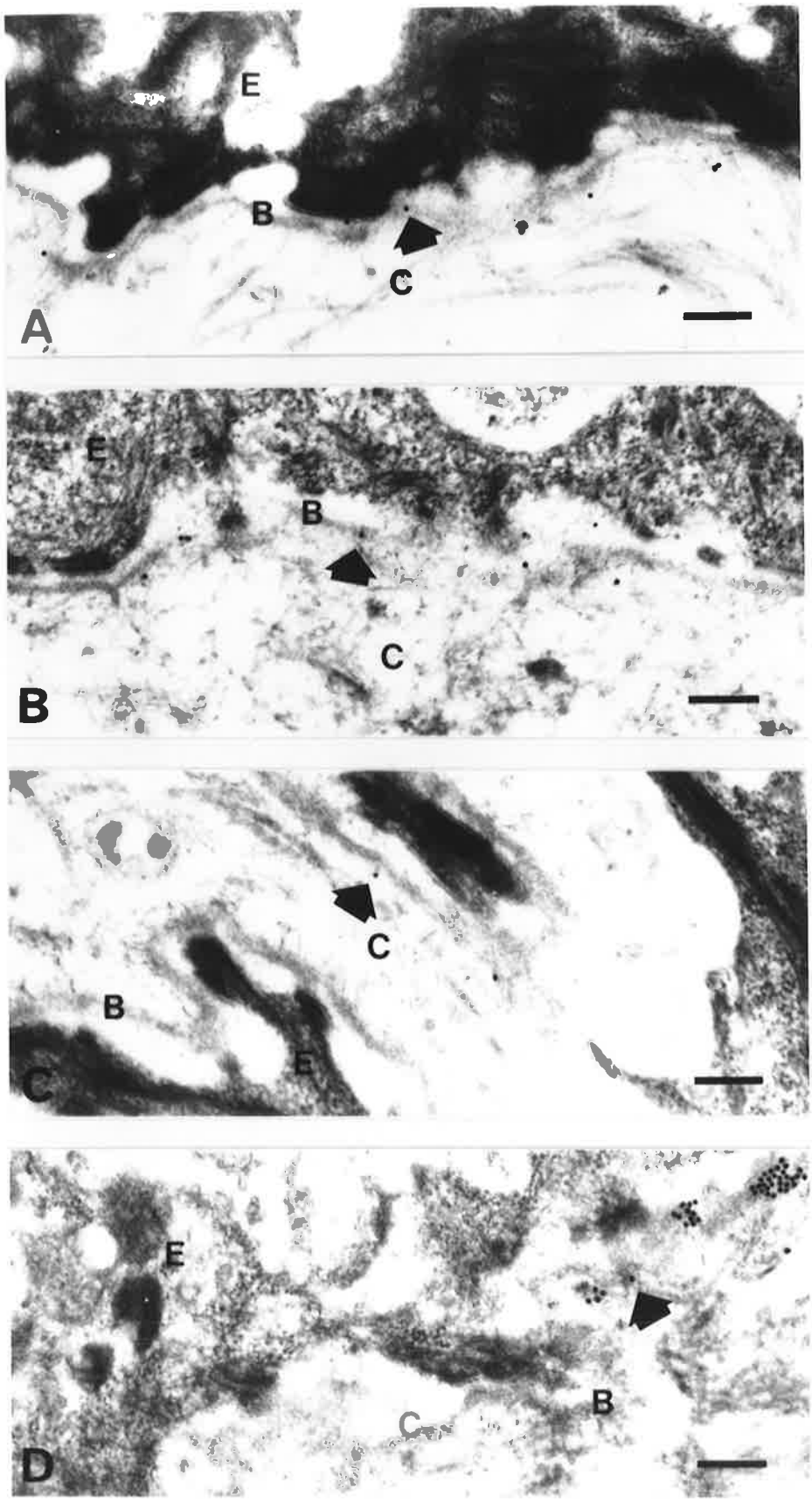


FIG 14

3.10. Investigation of causes of poor labelling and high non-specific background staining in immunolabelling for type IV collagen.

The immunogold demonstration of tissue antigens is dependent on the labelling of exposed antigen epitopes on the sections. This may be affected by tissue preparation techniques. In the experiments so far described immunolabelling for type IV collagen was consistently poorer than that observed for laminin. One possibility accounting for this observation is that the type IV collagen antigen epitopes were masked by the tissue preparation techniques employed. In order to test this possibility, an investigation of the effect of enzyme digestion of ultrathin sections prior to immunolabelling for type IV collagen was undertaken.

In addition to a poor intensity of labelling a high level of non-specific background staining was observed in the pilot experiment where the one type IV collagen antibody was used. Previous investigators including Roth, Taatjes and Warhol (1989) and Stirling (1990) have suggested that the use of inappropriate concentrations of antibody, inappropriate markers and blocking agents can contribute to poor level of immunolabelling and high level of background staining. To examine these possibilities as causes of the poor type IV collagen labelling observed and background staining, investigation of different antibody dilutions, blocking agents and primary antibody-gold complex combinations for immunolabelling of type IV collagen was carried out in a series of experiment as described in Sections 3.10.1 to 3.10.5.

3.10.1. Use of different sources of antibody for immunolabelling of type IV collagen.

Methods: Ultrathin sections were cut from specimens fixed in several fixatives (glutaraldehyde, paraformaldehyde and glutaraldehyde-paraformaldehyde mixture) and embedded with L.R.White resin at room temperature or at 50°C. Immunostaining was carried out using the protocol described in Section 3.2.4.3. Several types of antibody were examined as primary antibody namely:

1). rabbit anti-human type IV collagen polyclonal antibody (Eurodiagnostics, Netherlands),

2). rabbit anti-human type IV collagen polyclonal antibody, purified rabbit anti-human type IV collagen polyclonal antibody (Mrs. B. Reinboth, Department of Pathology, University of Adelaide),

3). rabbit anti-mouse type IV collagen polyclonal antibody (Chemicon, CA,USA) and

4). purified goat anti-human and bovine type IV collagen polyclonal antibody (Southen Biotechnology, Birmingham).

Results and conclusion: Sections incubated with all rabbit anti-human type IV collagen polyclonal antibodies exhibited poor labelling. Good labelling was observed on sections incubated with rabbit anti-mouse type IV collagen polyclonal antibody and the purified goat anti-human and bovine type IV collagen polyclonal antibody. However, sections incubated with rabbit anti-mouse type IV collagen polyclonal antibody showed high non-specific background staining (Fig. 15).

Figure 15. Electronmicrographs showing immunolabelling (arrows) for type IV collagen in normal rat tongue mucosa using the different types of antibody.

A. Rabbit anti-human type IV collagen polyclonal antibody (Eurodiagnostics, Netherlands)

B. Rabbit anti-mouse type IV collagen polyclonal antibody

C. Rabbit anti-human and bovine type IV collagen polyclonal antibody

More gold particles were evident in specimens incubated with rabbit anti-mouse type IV collagen polyclonal antibody (B) and rabbit anti-human and bovine type IV collagen polyclonal antibody (C) compared with those incubated with rabbit anti-human type IV collagen polyclonal antibody (A). A number of gold particles indicating non-specific background staining (arrow heads) were seen within epithelial cell and connective tissue in specimens incubated with rabbit anti-mouse type IV collagen polyclonal antibody (B). E-epithelial cell, B-basal lamina, C-connective tissue. (Bar=0.2μm).

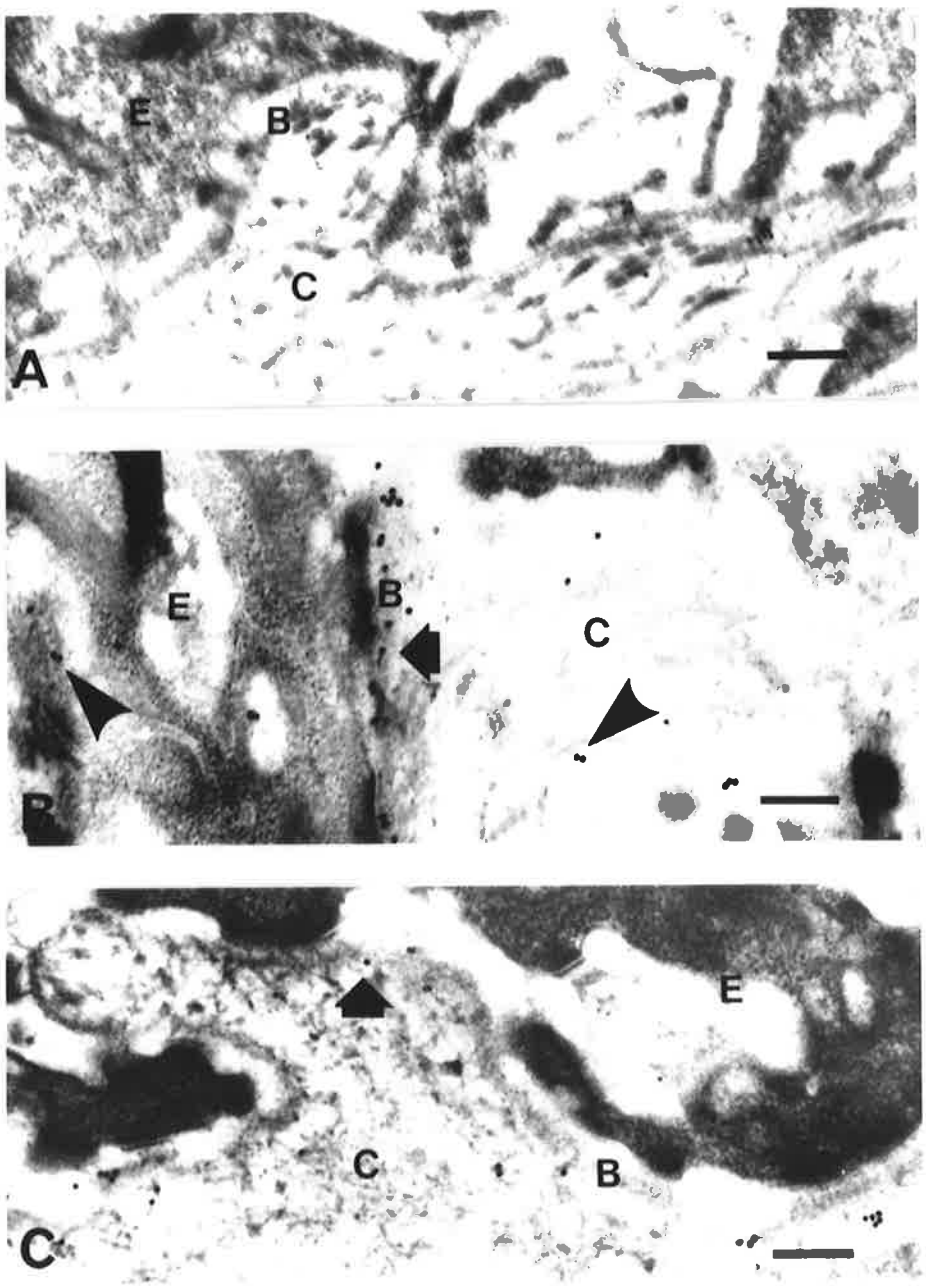


FIG 15

3.10.2. Predigestion of sections with enzyme for immunolabelling of type IV collagen.

Methods: Ultrathin sections were cut from tissue blocks which had previously shown good labelling for laminin and inverted on drops of 0.1% trypsin at 37^{0}C for 30 minutes (Hayat, 1989). Subsequently enzyme treated sections were incubated with rabbit anti-human type IV collagen polyclonal antibodies (Eurodiagnostics, Netherlands and Mrs. B. Reinboth, Department of Pathology, University of Adelaide) and stained using the protocol described in Section 3.2.4.3.

Results: Sections predigested with 0.1% trypsin showed poor immunolabelling of type IV collagen (Fig.16).

Conclusions: It appears that the predigestion of sections with 0.1% trypsin dose not improve immunolabelling of basal lamina type IV collagen in the tissue investigated in this study.

Figure 16. Electronmicrograph showing the absence of labelling of basal lamina type IV collagen on 0.1% trypsin pretreated sections. E-epithelial cell, B-basal lamina, C-connective tissue (Bar=0.2µm).

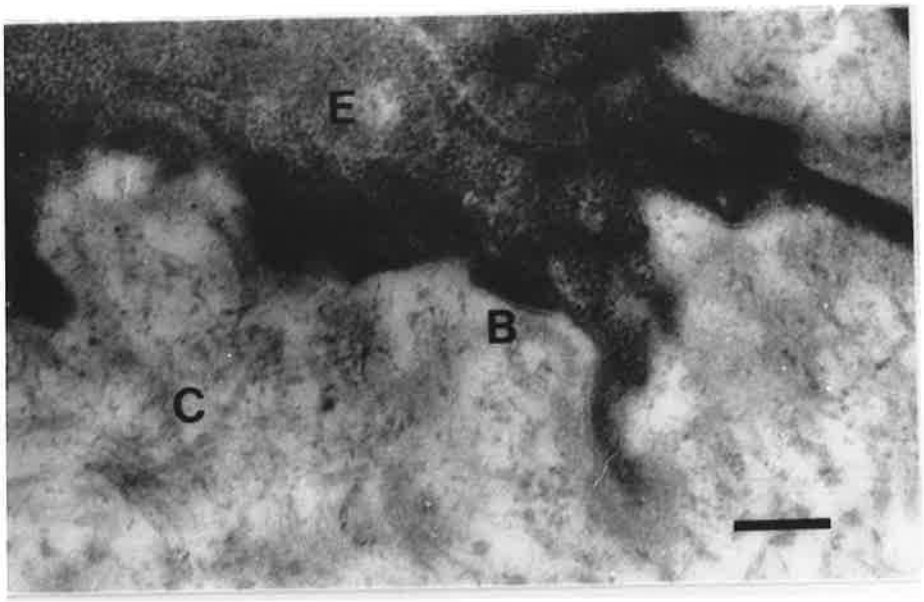


FIG 16

3.10.3. Selection of different antibody dilutions for immunolabelling of type IV collagen.

Methods: Ultrathin sections were cut from tissue blocks which had previously shown good labelling for laminin and immunoincubated using the protocol described in Section 3.2.4.3. In this experiment incubations at 1:20, 1:40, 1:80 and 1:160 dilutions of rabbit anti-mouse type IV collagen polyclonal antibody were used.

Results: Good labelling of type IV collagen was only seen on sections incubated with 1:20 and 1:40 diluted primary antibody. However, a high level of non-specific background staining was also observed on these specimens (Fig. 17).

Conclusion: High non-specific background staining in immunolabelling for type IV collagen does not appear to result from the use of inappropriate antibody dilution.

Figure 17. Electronmicrographs showing immunolabelling (arrows) for type IV collagen in normal rat tongue mucosa using the different dilutions of antibody.

A. High level of non-specific background staining (arrow heads) on section incubated with 1:40 diluted antibody

B. High level of non-specific background staining (arrow heads) on section incubated with 1:80 diluted antibody

C. Poor labelling of type IV collagen on section incubated with 1:160 diluted antibody

E-epithelial cell, B-basal lamina, C-connective tissue. (Bar=0.2μm).

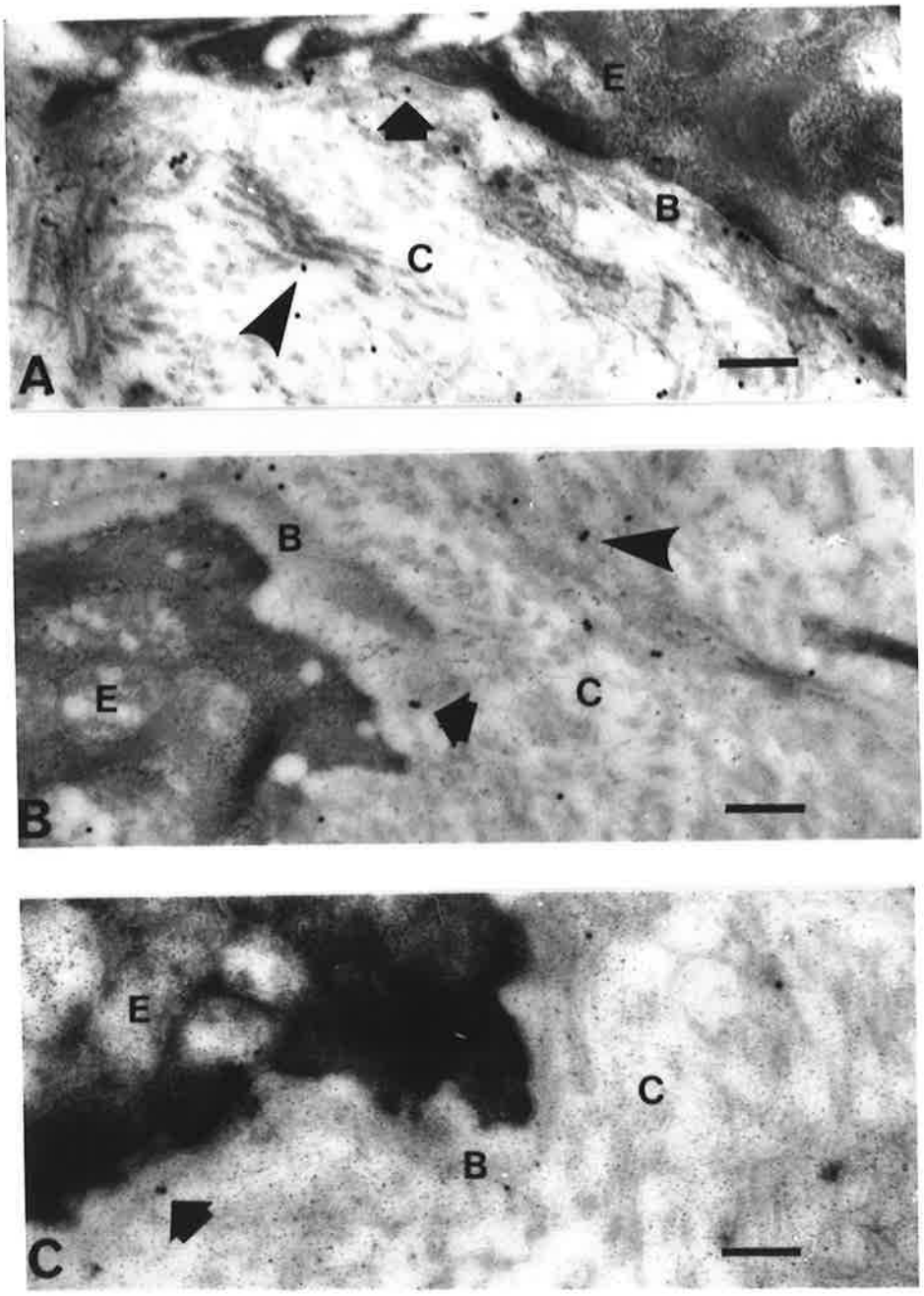


FIG 17

3.10.4. Effect of different blocking agents on immunolabelling for type IV collagen.

Methods: Ultrathin sections were selected from specimen blocks which previously showed good labelling for laminin. In this experiment, rabbit anti-mouse type IV collagen polyclonal antibody was used. 1% bovine albumin serum (BAS), 1% ovalbumin with 0.02M glycine and 1% fish gelatin with 0.02M glycine (Fanning et al, 1990) were used as blocking agents.

Results: A similarly high level of non-specific background staining was observed on sections, when the three different blocking agents were used (immunolabelling feature was similar to that shown in Fig 17A).

Conclusion: The presence of non-specific background staining in rat tongue tissues immunostained for type IV collagen does not appear to be related to the use of the blocking agents described.

3.10.5. Effect of different primary antibody-gold complex combinations on immunolabelling for type IV collagen.

Two main types of gold complex have frequently been used in immunogold techniques namely protein A-gold and IgG-gold. It has been described that the use of primary antibody combined with the different types of gold complex can affect immunolabelling (Grant and Leblond, 1988; Stirling, 1990). An experiment was therefore designed to test the effect of different primary antibody-gold complex combinations on immunolabelling for type IV collagen.

Methods: Ultrathin sections were cut from tissue blocks which had previously shown good labelling for laminin and immunoincubated using the protocol described in Section 3.4.2.3. In the incubation with primary antibody and gold complex, several primary antibodies combined with different gold complexes were used as follows:

1). Rabbit anti-human type IV collagen antibody---protein A-gold.
2). Rabbit anti-human type IV collagen antibody---IgG-gold
3). Rabbit anti-mouse type IV collagen antibody---protein A-gold.
4). Rabbit anti-mouse type IV collagen antibody---IgG-gold
5). Goat anti human and bovine type IV collagen antibody---proteinA-gold
6). Goat anti-human and bovine type IV collagen antibody---IgG-gold

Results: Sections incubated with rabbit anti-human type IV collagen antibody combined with protein A-gold or IgG-gold and goat anti-human and bovine type IV collagen antibody combined with protein A-gold showed poor basal lamina labelling. Good labelling for type IV collagen was seen on sections incubated with rabbit anti-mouse type IV collagen antibody combined with protein A-gold or IgG-gold and goat anti-human and bovine type IV collagen antibody combined with IgG-gold. There was a similar, high level of non-specific background staining on sections incubated with the rabbit anti-mouse type IV collagen antibodies combined with protein A-gold or IgG-gold (Table 15) (Figure 18).

Table 15 Demonstration of immunolabelling for type IV collagen using the different primary antibody-gold complex combinations

Antibody(Ab)-gold complex	Specific staining	Background staining
Rabbit anti-human Ab-protein A-gold	+ or -	-
Rabbit anti-human Ab-IgG-gold	+ or -	-
Rabbit anti-mouse Ab-protein A-gold	+++	+++
Rabbit anti-mouse Ab-IgG-gold	+++	+++
Goat anti-human & bovine Ab-protein A-gold	+ or -	-
Goat anti-human & bovine Ab-IgG-gold	+++	-

Conclusions: Protein A-gold and IgG-gold have a similar affinity for immunoglubin G (IgG) from rabbit. But IgG-gold has a high affinity for IgG from goat, in contrast, protein A-gold has a low affinity for goat IgG.

Figure 18. Electronmicrographs showing immunolabelling (arrows) for type IV collagen using different antibody-gold complex combinations as follows:

A. Section incubated with rabbit anti-human type IV collagen antibody followed by protein A-gold

B. Section incubated with rabbit anti-human type IV collagen antibody followed by IgG-gold.

C. Section incubated with rabbit anti-mouse type IV collagen antibody followed by protein A-gold

D. Section incubated with rabbit anti-mouse type IV collagen antibody followed by IgG-gold

E. Section incubated with goat anti-human & bovine type IV collagen antibody followed by protein A-gold

F. Section incubated with goat anti-human & bovine type IV collagen antibody followed by IgG-gold

Labelling was absent in specimens incubated with rabbit anti-human type IV collagen antibody followed by protein A-gold (A) and IgG-gold (B). Good labelling and a high level of background staining is evident in specimens incubated with rabbit anti-mouse type IV collagen antibody followed by protein A-gold (C) and IgG-gold (D). Good labelling is evident in specimens incubated with goat anti-human and bovine type IV collagen antibody followed by IgG-gold (F), but basal lamina is devoid of gold particles in specimens incubated with goat anti-human & bovine type IV collagen antibody followed by protein A-gold (E). E-epithelial cell, B-basal lamina, C-connective tissue. (Bar=0.2μm).

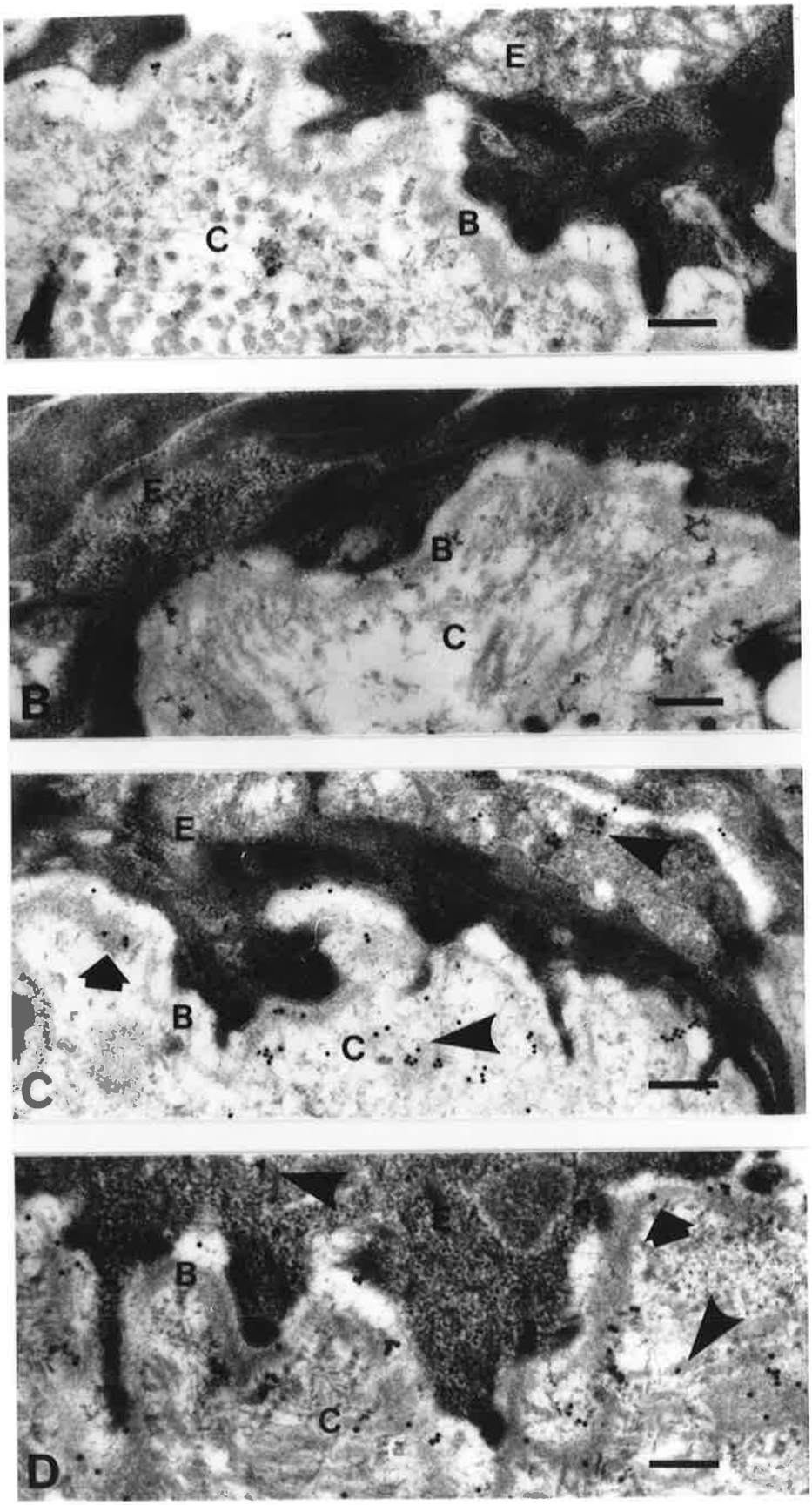


FIG 18

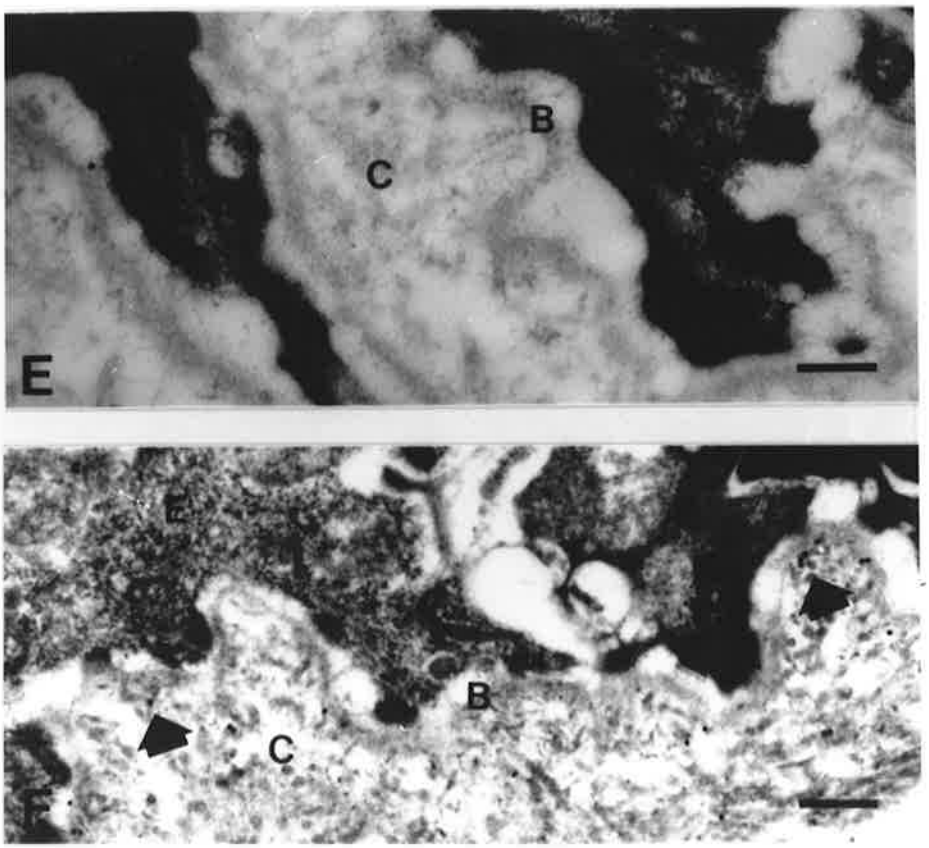


FIG 18

3.11. Establishment of controls for immunolabelling of laminin and type IV collagen.

In immunocytochemistry, it is possible that gold complex may bind to endogenous immunoglobulin in tissues and that antibody solution may contain contaminating antibody with the result to produce non-specific labelling. In order to assess the specificity of the immunolabelling appropriate controls should be established. An experiment was therefore designed to confirm the specificity of immunolabelling observed.

Methods: Ultrathin sections were selected from tissues which had shown good labelling for laminin and type IV collagen and incubated following the protocol described in Section 3.2.4.3 except that in the incubation with primary antibody the primary antibody was replaced by antibody-antigen absorption solution or PBS. For the type IV collagen antibody-antigen absorption control, the type IV collagen antibody was preabsorbed with an excess of corresponding antigen (2mg/ml). Three forms of antigen were used namely purified human type IV collagen, purified bovine type IV collagen and human and bovine type IV collagen combination. For the laminin antibody-antigen absorption control, laminin antibody was preabsorbed with an excess of purified laminin (1mg/ml).

Results: All sections immunoincubated for laminin after using control methods exhibited negative labelling. However, sections stained for type IV collagen showed different appearances in that sections incubated with antibody preabsorbed with the human

type IV collagen or bovine type IV collagen showed positive labelling; negative labelling was seen only on sections incubated with a solution of antibody preabsorbed with the human and bovine type IV collagen antigen combination. Observation of PBS type IV collagen antibody control sections demonstrated negative labelling (Figs. 19, 20).

Conclusions: The control staining protocols selected for laminin in this study appear to be appropriate. For type IV collagen it is necessary to use human and bovine type IV collagen antigens together in the absorption control.

Figure 19. Electronmicrographs demonstrating negative labelling for basal lamina laminin on control sections.

A. Antibody-antigen absorption control

B. Antibody replaced with PB control

Labelling was absent in basal lamina (A,B). E-epithelial cell, B-basal lamina, C-connective tissue. (Bar=0.2µm).

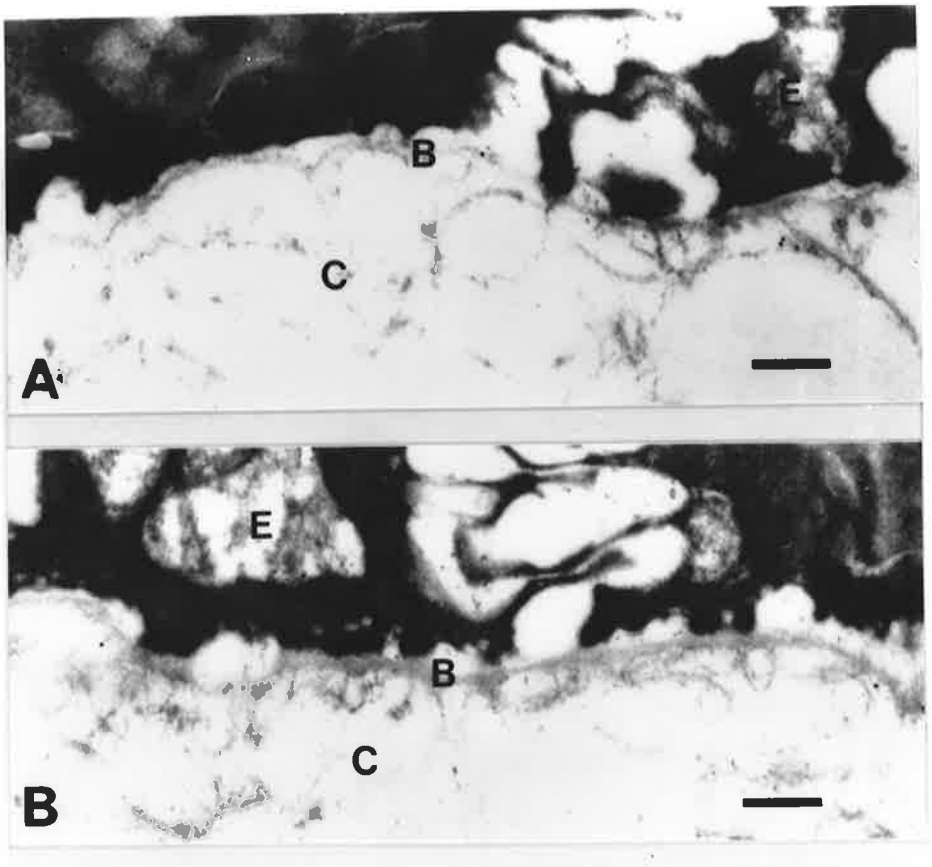


FIG 19

Figure 20. Electronmicrographs demonstrating some negative labelling and some positive labelling of type IV collagen on control sections investigated.

A. Antibody absorbed by human type IV collagen control (positive labelling of basal lamina (arrows))

B. Antibody absorbed by bovine type IV collagen control (positive labelling of basal lamina (arrows))

C. Antibody absorbed by human and bovine type IV collagen control (negative labelling of basal lamina)

D. Antibody replaced with PB (negative labelling of basal lamina)

E-epithelial cell, B-basal lamina, C-connective tissue. (Bar=0.2μm)

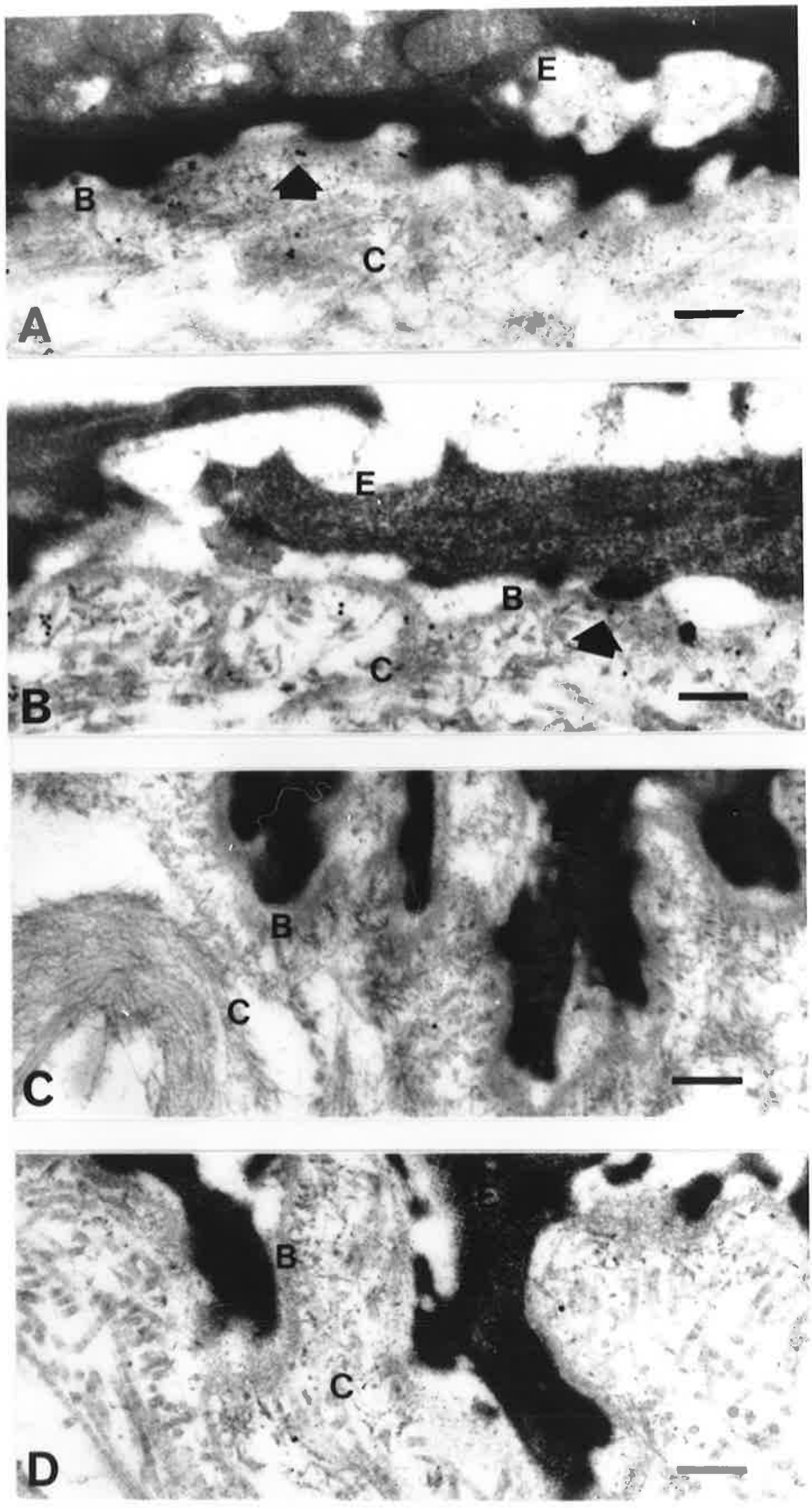


FIG 20

3.12. Discussion

Antigen retention and expression are associated with the tissue preparation and immunostaining techniques employed (Roth, 1986; Bendayan, Nanci and Kan, 1987; Stirling, 1990). In this phase of study, the effect of several in tissue preparation and immunostaining variables on ultrastructural morphological preservation and immunoreactivity of laminin and type IV collagen antigens in basal lamina of rat oral mucosa were investigated.

In the present study, investigations in relation to the choice of optimum fixation conditions were initiated using two commonly employed fixatives namely glutaraldehyde and (para)formaldehyde. In the first experiment, it was apparent that a low level of immunolabelling was observed in tissues fixed in those fixatives which have apparently been successfully used in previous studies by others (Bendayan, 1985; Lin and Essner, 1990; Smith, 1990; Herken and Miosge, 1991). The reason for this discrepancy is not clear. However, on the basis of the characteristics of glutaraldehyde and paraformaldehyde, it is possible that several factors may have to be considered.

First, there may be variations in the sensitivity of tissue antigens to glutaraldehyde in oral mucosa tissue compared with other tissue antigens. Stirling (1990) emphasized that the sensitivity of tissue antigens to glutaraldehyde was variable. Further, Kraehenbuhl and Jamieson (1973) recommended that 0.5% glutaraldehyde was the optimal mild, fixative for the successful

immunolocalization of tissue antigens. Nakane (1973) reported that epithelial basal lamina antigens were lost when 1% or 2% glutaraldehyde fixation was used. In the present study, two mild (0.5% and 1%) glutaraldehyde solutions were employed, but these glutaraldehyde fixatives still resulted in an unacceptably low level of immunolabelling. In this context Stirling (1990) pointed out that fixation time was also critical for antigen retention in tissues and recommended that a short period of fixation was compatible with sensitive epitopes expression. In the present study, a relatively short one hour fixation time was used.

It is well known that glutaraldehyde can effectively preserve ultrastructural morphology in conventional electron microscopy (Hayat, 1989). However, it has also been recognized that glutaraldehyde fixative, especially mild fixative, omitting osmication in immunocytochemistry, does lead to more extraction of tissue proteins following tissue processing procedures such as dehydration and embedding (Hopwood, 1973; Newman and Hobot, 1987). In the present study, epithelial cells of oral mucosa tissue fixed in the mild glutaraldehyde solutions exhibited obvious extraction. This finding agrees with those described in previous studies (Hopwood, 1973). However, an interesting observation in the present study was that an acceptable morphology of basal lamina was seen in those tissues fixed in the mild glutaraldehyde solutions. This phenomenon might be related to the structural characteristics of the basal lamina.

Some investigators (Eldred et al, 1983; Tokuyasu, 1984; Stirling, 1990) have suggested that paraformaldehyde and glutaraldehyde-

paraformaldehyde fixatives are better for antigen preservation than glutaraldehyde. Tokuyasu (1984) and Stirling (1990) suggested that the effective antigen preservation in tissues fixed in paraformaldehyde or paraformaldehyde-glutaraldehyde mixtures might be attributed to their rapid penetration and "light" denaturation of tissue proteins. However, in the present study (Sections 3.3 and 3.4) a low level of immunolabelling and unsatisfactory preservation of tissue morphology were seen in tissues fixed in these solutions. The exact reason for this remains unclear. However several possible explanations may exist. One possibility is that certain tissue antigens such as laminin and type IV collagen may be sensitive to these fixatives. Other is that poor preservation of morphology may result in the loss of tissue antigens. No obvious improvement in immunolabelling when the fixation concentration was reduced in the present study tends to rule out the first possibility.

Gosselin (1986) and Fox and Benton (1987) described that paraformaldehyde was characterized by its slow reactivity with tissue proteins and that several factors were related to the preservation of tissue morphology namely concentration of fixative, period of fixation, PH value and fixation osmolarity. It was suggested that a prolonged fixation period was generally required for morphological preservation. In the present study, a much shorter period of fixation was employed compared with the 48 hours recommended by Fox and Benton (1987) in order to reduce the loss of tissue antigen during fixation. It was thought that this shorter fixation time should enhance antigen retention, but it was found that poor preservation of epithelial cells and

basal lamina and low immunolabelling were observed in tissues fixed using the protocols employed. The explanation for these observations probably resides in the facts that the fixation time was too short for adequate preservation of tissue constituents resulting in diffusion, activity loss and displacement of antigens.

Although it has been reported that the use of (para)formaldehyde-glutaraldehyde fixative mixture may improve the preservation of tissue morphology and antigen retention (Karnosky, 1965; Tokuyasu, 1984), no difference in the preservation of tissue morphology and immunolabelling was seen in this study when paraformaldehyde alone and paraformaldehyde-glutaraldehyde mixture were compared. The reasons for this remain obscure. However, a short fixation time in paraformaldehyde and very low concentration of glutaraldehyde may be one of factors resulting in poor morphology. In addition, the type of tissue studied may be of significance. Rat tongue tissues are relatively "tough" or "dense" compared with some tissues (i.e. kidney, retina) used by others in previous studies (Lin and Essner, 1990; Herken and Miosge, 1991). For rat tissue, it was realized that a relatively prolonged period of fixation might be required for effectively preserving morphology. Further investigations using prolonged fixation times may provide more information on the use of these fixatives in relation to morphology preservation in rat tongue tissues. However, further work of this type was not carried out in the present study because it was considered that prolonged tissue fixation would result in significant loss of tissue antigens. Additionally, owing to the demonstration of relatively satisfactory basal lamina morphology in tissues fixed by the

glutaraldehyde fixatives used in the present study, it was considered that further work on the improvement of tissue antigens was more appropriately undertaken by modifying the glutaraldehyde fixative rather than further exploring (para)formaldehyde and (para)formaldehyde-glutaraldehyde fixatives.

Following the initial exercise (Section 3.3) aimed at studying the effect of fixative types, more attention was focused on the modification of glutaraldehyde fixatives in order to improve antigen retention. In the present study, several variables were investigated. Firstly, the concentration of glutaraldehyde used in the preliminary study was further reduced (Section 3.4.). Results showed that a very low concentration of glutaraldehyde improved the level of immunolabelling. However, this improvement was only shown in relation to laminin, not type IV collagen. In the context of tissue morphology preservation, no marked change of basal lamina morphology was seen in specimens fixed in this very mild glutaraldehyde solution compared with those studies in the preliminary study (Section 3.3). These findings appeared that laminin antigen was sensitive to glutaraldehyde fixation.

A number of investigators have proposed the use of additives to fixative solutions to improve ultrastructural morphology and antigen retention (Zamboni and Martino, 1967; Mclean and Nakane, 1974; Kuhlman and Krischan, 1981; Hermanns et al, 1986). In the present study (Section 3.5) two additives (picric acid, PVP) were investigated. Results indicated that a modified glutaraldehyde-picric acid fixative improved the preservation of not only laminin

antigen but also type IV collagen antigen. A significant difference between this modified glutaraldehyde-picric acid fixative and that used by others (Newman, Jasani and Williams, 1983; Newman and Hobot, 1987; Berryman and Rodewald, 1990) is the use in the present study of a very low concentration of glutaraldehyde and the addition of sucrose. The rationale for the addition of sucrose to this modified fixative was to adjust fixation osmolarity to improve the ultrastructural preservation.

In the present study, it was found that tissues fixed in this modified fixative showed an improvement in ultrastructural preservation of epithelium, especially cellular membranes, desmosomes and hemidesmosomes compared with specimens fixed in glutaraldehyde alone. This observed improvement of cellular membrane and hemidesmosome detail proved very beneficial for the morphometric studies described later.

In summary the findings indicate that the addition of picric acid to low concentration glutaraldehyde fixative can effectively preserve tissue antigens and tissue morphology which compensates for the loss of tissue morphology caused by a low concentration of glutaraldehyde.

The mechanism for picric acid enhancement of morphological preservation and antigen retention has been not well established. Newman (1989) explained that the precipitation of picric acid with tissue proteins prevented damage to tissue antigens by dehydration and embedding processes. Moreover, it was suggested that tissues should be directly dehydrated without any buffer

washing after fixation ensuring the prevention of loss of picric acid during buffer washing. In the present study all specimens were washed in 0.1M PB with 50 mM NH_4Cl in order to quench free aldehyde groups after glutaraldehyde fixation. The subsequent level of immunolabelling was still satisfactory following washing. Consequently, it is concluded that the reaction of picric acid with tissue antigens mainly occurs during tissue fixation and that buffer washing, which may reduce non-specific background staining, will in some cases not affect the protective role of picric acid in antigen retention. It is suggested that picric acid possibly improves ultrastructural preservation and antigen retention in two ways. It may accelerate the penetration of fixative allowing rapid morphological preservation, and/or it may independently react with tissue proteins by "coagulation or precipitation".

The other additive investigated in this study was polyvinlpyrrolidone (PVP). Initially PVP was used to adjust fixation osmolarity to improve the ultrastructural preservation. It was found that the addition of PVP improved not only epithelial morphology but also the immunolabelling of laminin. Although no marked effect of PVP on the preservation of type IV collagen antigen was demonstrated. It is known that PVP, as a non-electroyte, contributes to fixation osmolarity which is associated with morphological preservation (Hayat, 1989). The improvement of tissue morphology in the present study is probably ascribed to the adjustment of the fixation osmolarity by PVP.

The mechanism accounting for the improvement of antigen retention by PVP is poorly understood. One explanation for PVP enhanced antigen retention is that proposed by Kuhlman and Krischan (1981) who suggested that PVP preserves tissue antigen by micellar formation with protein. On the basis of the structural features and chemical properties of PVP, It can also be theorized that antigen retention may be associated with the molecular reaction of PVP with tissue proteins by the formation of reverse hydrogen bonding between PVP and laminin during tissue fixation which may protect laminin antigen epitopes from the damage of fixatives or subsequent dehydration and embedding; whereas the possible absence of this bonding between PVP and type IV collagen which may be related to the observed lack of improvement in type IV collagen immunolabelling.

Notwithstanding these possibilities, another possibility accounting for the improvement of antigen retention by PVP may exist. That is, PVP improves morphology by adjusting fixation osmolarity with the result that tissue antigen is also effectively retained. To verify this, in this study sucrose was employed without PVP to adjust fixation osmolarity to a similar level as fixatives containing PVP (Sections 3.6 and 3.7). The results indicated that fixation only with sucrose additive did not improve immunolabelling. It is thus concluded that tissue antigen is retained effectively by PVP itself, rather than by fixation osmolarity.

In view of the observed enhancing effect of picric acid and PVP on antigen retention, an attempt to further improve immunolabelling

was made in the present study by combining both additives in the same fixative. However, the efficiency of picric acid and PVP in antigen retention was not enhanced by combination.

Aside from the effect of additives, it is also recognized that fixation osmolarity is related to ultrastructrual morphological preservation (Bone and Ryan, 1972, Hayat, 1989). In the present study (Section 3.7), it was found that fixation osmolarity varied with the amount of added PVP and sucrose. Whether or not fixation osmolarity per se affects antigen retention remains unknown. In the present study the effect of fixation osmolarity on the immunolabelling of laminin and type IV collagen and tissue morphology were investigated. In these investigations, a variety of fixation osmolarities were produced by adding different amounts of additives into various fixatives and by changing buffer systems. It was found that the preservation of tissue antigens (laminin and type IV collagen) was not affected by fixative osmolarity and that the ultrastructural preservation of epithelium was affected by the osmolarity of glutaraldehyde fixation, but not by the osmolarity of paraformaldehyde or paraformaldehyde-glutaraldehyde mixture fixatives.

Hayat (1989) suggested that a physiological range of osmolarity was 300-400 mosm. In the present study, it was demonstrated that good preservation of tissue morphology was obtained from tissues fixed in glutaraldehyde fixatives in which the osmolarity was appropriately 350 mosm. Accordingly, it appears that an optimum fixation osmolarity for the ultrastructural preservation of rat tongue tissue (or oral mucosa) is approximately 350 mosm

which is in the " physiological range " recommended by Hayat (1989).

Hayat (1989) described that the response of tissue to osmolarity was dependent on tissue types in that the "more dense" the tissue was the less effect osmolarity had. In the present study, basal lamina appeared to be relatively "condensed" compared with epithelial cell structure. Thus it is possible to suggest that the features of basal lamina structure might tolerate a wide range of fixation osmolarity such that tissue antigens in the basal lamina are not greatly affected by fixation osmolarity.

As described by some authors (Bullock, 1984, Hayat, 1989), fixative vehicles contribute to fixation osmolarity and the ultrastructural preservation. However, information on the effect of fixative vehicles on antigen retention in immunocytochemistry is scare. Stirling (1990) mentioned that some buffer systems (eg. Tris buffer, Zwitterionic buffer) might be associated with antigen retention.

In immunocytochemistry, two commonly used fixative vehicles are phosphate buffer (PB) and phosphate buffered saline (PBS). However, there is a paucity of information describing their possible effects on both morphology and antigen retention. In the present study, the measurement of the osmolarities of these buffer systems showed that the osmolarity of the usually used 0.01M PBS was within the " physiological range " recommended by Hayat (1989); whereas the osmolarity of the usually used 0.05M PB and 0.1M PB was lower than the recommended " physiological

level ". Moreover, glutaraldehyde in 0.05M PB or 0.1M PB showed a lower level of osmolarity than those in 0.01M PBS. If additives (eg. sucrose or PVP) were added to fixatives in 0.05M PB or 0.1M PB, the fixation osmolarity tended toward a level similar to that of 0.01M PBS. These findings indicate that PBS and PB contribute differently to fixation osmolarity.

In this investigation, it was found that glutaraldehyde in 0.05 M or 0.1M PB vehicle without the addition of additives resulted in poor preservation of epithelial cell morphology; while glutaraldehyde in 0.05M or 0.1M PB vehicle with the osmolarity adjusted by sucrose or PVP and glutaraldehyde in 0.01M PBS vehicle produced satisfactory preservation of epithelial cell morphology. However, the level of immunolabelling and basal lamina morphology did not vary with buffer systems. Thus, it appears that PB and PBS buffer systems in tissue processing may play a role in contributing to fixation osmolarity, but may not exert any effect on antigen retention. Furthermore, it is suggested that fixation osmolarity should be measured and adjusted if PB is used as a fixative vehicle. For instance, PB was preferably used as the fixative vehicle in the study (Sections 3.5 and 3.9). PB was selected because additives (eg. PVP) which increased fixation osmolarity were added to the fixative solution in order to improve antigen retention. If PBS had been used as a fixative vehicle, fixation osmolarity with the addition of PVP would have exceeded the "physiological level".

It is known that dehydration procedures in tissue processing may result in the loss of tissue antigens due to tissue extraction and

denaturation of tissue proteins (Stirling, 1990). To reduce the loss of tissue antigens during dehydration in immunocytochemistry, the choice of an appropriate dehydration protocol compatible with a resin system is important. Previous studies have shown that two types of ethanol dehydration are used for L.R.White resin namely partial dehydration and complete dehydration (Bendayan, Nanci and Kan, 1987; Newman and Hobot, 1987). Partial dehydration (up to 70% ethanol) is strongly recommended by Newman and Hobot (1987).

The rationale for using partial dehydration is that L.R.White resin is water miscible and may tolerate 12% (by volume) water during polymerization. Moreover, a lower concentration of ethanol for dehydration may reduce the deleterious influence of dehydration on tissue antigen (Newman and Hobot, 1987). Some authors have reported that tissue antigens are satisfactorily retained using a partial dehydration procedure and L.R.White resin (Newman and Hobot, 1987). However, others report that antigen retention can still be demonstrated using more than 70% ethanol dehydration (Lin and Essener, 1990; Herken and Miosge, 1991).

Herken and Miosge (1991) reported the results of using three different concentrations of ethanol dehydration to identify laminin in kidney specimens embedded with L.R.White resin. These investigators described that up to 100% ethanol complete dehydration resulted in better preservation of tissue morphology, but loss of tissue antigen; while up to 70% partial ethanol dehydration produced good antigen retention, but poor preservation of tissue morphology. It was suggested that optimum

dehydration for ultrastructural preservation and antigen retention should involve the use of up to 90% partial ethanol dehydration. In the present study (Section 3.8), two different ethanol dehydration protocols were used namely up to 70% ethanol and up to 100% ethanol. Results indicated that there was no marked difference in immunolabelling for laminin and type IV collagen when partially dehydrated tissues and completely dehydrated tissues were compared. However, better preservation of tissue morphology was observed from completely dehydrated tissues than from partially dehydrated tissues.

Two main types of resin system are used in immunocytochemistry (Causton, 1984). The literature suggested that acrylic resins are more superior to epoxy resins in antigen preservation and that L.R.White resin has become more popular (Hayat, 1989). L.R.White resin can be polymerized at low temperature, room temperature and in an oven. Newman and Hobot (1987) suggested that good immunolabelling can be obtained by the use of heat cure polymerization of L.R.White resin. The explanation for this was that limited cross-linking of the molecules in the resin and the formation of linerarity in the molecular arrangement by slow heat allowed easy penetration of immunoreagents. However, heat cure polymerization often needs a relatively long period such that the prolonged exposure of tissue to plastic monomer could result in tissue extraction during tissue infiltration (Newman,1987).

An alternative to the heat cure polymerization is catalytic cure polymerization (at room temperature) or UV-light polymerization (at low temperature). However, UV-light polymerization for

L.R.White resin is a rarely used technique due to the existence of Lowicryl resins which are more adaptable to low temperature techniques (Newman, 1989). Catalytic cure polymerization of L.R.White resin has been used by some investigators (Yoshimura et al, 1986; Newman and Hobot, 1989) and it has been shown that rapid embedding can be obtained by this method.

Newman and Hobot (1987) reported that the two different polymerization conditions (heat cure polymerization and catalytic cure polymerization) did not significantly affect immunolabelling when immunogold techniques were used. In the present study (Section 3.9), comparison on antigen retention and the ultrastructural preservation using the two types of polymerization conditions was made. The results indicated that catalytic cure polymerization (at room temperature) produced better immunolabelling; while the heat cure polymerization produced better ultrastructural preservation. Although Newman (1987) pointed out that tissue antigen might be damaged by excessive accelerator in the catalytic cure polymerization process, a small quantity of accelerator than that used by Newman was used in the present study. The finding that antigen expression for laminin and type IV collagen using catalytic cured L.R.White resin was better than in heat cured specimens could indicate that basal lamina laminin and type IV collagen antigens are sensitive to temperature.

In the present study, it was also found that a different tissue morphology was apparent under the two polymerization conditions. The reason for this is not clear. While a comparison of

the both polymerization conditions was made, it was found that the difference, apart from temperature, lay in the period of tissue infiltration. From the point of view of tissue infiltration, it is thought that the better ultrastructural preservation observed in the heat cure polymerization specimens may be attributable to better adequate tissue infiltration during a relatively prolonged embedding time. In addition, the relatively high temperature may accelerate tissue infiltration. In contrast, the short embedding period under catalytic cure polymerization conditions at the room temperature may lead to inadequate tissue infiltration with the result that poor morphology is obtained. In this context some investigators have suggested that tissue infiltration under catalytic cure polymerization conditions might be accelerated by increasing the temperature (Yoshimura et al, 1986; Newman and Hobot, 1989). However, this method was not applied to the present study due to concerns regarding the effect of temperature on antigen retention. Accordingly, a more prolonged period of tissue infiltration during the catalytic cure polymerization process was used in the present study compared with that in previous studies (Newman and Hobot, 1989).

Stirling (1990) emphasized that a satisfactory immunolabelling of tissue antigens in addition to relying on factors already discussed also relies on careful selection of antibody, blocking agents and marker. In the preliminary work, a problem with the selection of an appropriate type IV collagen antibody was encountered. An anti-human type IV collagen polyclonal antibody which had successfully been used on paraffin tissue sections at the light microscope level (Wilson, Jiang et al, 1993) was

employed to identify type IV collagen on plastic tissue sections at the electron microscope level. However, poor ultrastructural immunolabelling was observed using this antibody. To account for this phenomenon, two possibilities were considered. First, tissue preparation techniques could have damaged tissue antigen so that insufficient antigen epitopes were retained. Secondly, the use of an inappropriate immunostaining method could have failed to detect antigen epitopes. When subsequent investigations (Section 3.10) related to immunolabelling of type IV collagen were carried out using different sources of primary antibody it appeared that poor ultrastructrural immunolabelling of type IV collagen was mainly associated with the source of antibody.

In the present study, alternative sources of antibody for successful identification of basal lamina type IV collagen included one which was raised against mouse type IV collagen, and another which was against bovine type IV collagen and human type IV collagen. Whether antigen epitopes of type IV collagen are more homogeneous among animals than between animals and humans remains unknown. It has been described that there is heterogeneity in the molecular structure of type IV collagen among tissues (Foellmer, Madri and Furthmayr, 1983). In the control experiments used in the present investigation, it was interesting to note that the use of human type IV collagen or bovine type IV collagen alone preabsorbed with primary antibody solution failed to block the immunoreactivity of antibody with antigen epitopes of rat type IV collagen on ultrathin tissue sections. Only when both human type IV collagen and bovine type IV collagen were preabsorbed with primary antibody solution the

immunoreactivity of the respective primary antibody was blocked. Thus it appears that antigen epitopes of type IV collagen in rat tissues may share some similarity with those in human tissues and in bovine tissues. Furthermore, the findings may imply that there is heterogeneity in molecular structure of type IV collagen among species. Furthermore the findings may provide an interpretation for the observation that while the anti-human type IV collagen antibody stained positively on paraffin tissue sections (Wilson, Jiang et al, 1993) it did not stain at the immunoelectron microscopic level. The explanation could reside in the fact that anti-human type IV collagen antibody could have bound to antigen epitopes of rat tongue type IV collagen similar to those of human type IV collagen. But, the degree of antigen-antibody binding may have been so slight that it was only detected by ABC peroxidase techniques with a relatively higher sensitivity.

Lin and Essner (1989) and Stirling (1990) described that the use of different gold complexes might in some cases, affect immunolabelling. In many situations, protein A-gold is more frequently used for immunocytochemistry. However, it is recognized that protein A-gold has some limitations in application due to a low affinity between protein A and IgG from some species such as rat, mouse, sheep and goat (Bendayan and Garzon, 1988, Stirling, 1990). For instance, Lin and Essner (1989) reported that a lower level of immunolabelling of type IV collagen was seen in tissues incubated with goat anti-type IV collagen antibody in combination with protein A-gold complex compared with those incubated with similar antibody and IgG-gold complex.

In the present study (Section 3.10.5), the results were shown to be consistent with those described by Lin and Essner (1989).

Although it is known that protein A has a high affinity for IgG from rabbit whether or not there is a difference in the affinity for rabbit IgG (primary antibody) between protein A-gold complex and IgG-gold complex is unknown. In the present study, the immunolabelling of type IV collagen using rabbit anti-human type IV collagen antibody in combination with either protein A-gold or IgG-gold was investigated. The results indicated that a similar level of immunolabelling occurred. From these findings, it is suggested that both protein A-gold and IgG-gold complexes can be used in immunogold techniques when primary antibody raised from rabbit is used. However, primary antibody raised from goat is compatible with IgG-gold complex.

Geoghegan (1988) and Kramarcy and Sealock (1991) reported that several phenomena might be observed in the use of IgG-gold complex namely IgG-gold clusters and free active antibodies. Lin and Essner (1989) reported that clumped gold particles appeared on tissue sections, when IgG-gold complex was used for the demonstration of type IV collagen. A similar finding of clumped gold particles was also observed in the present study. Stirling (1990) stated that this phenomenon was a consequence of a high level of immunolabelling due to the amplification of gold probes. However, other investigators have suggested that the presence of clumped gold particle is associated with IgG-gold complex preparation techniques (Geoghegan, 1988; Lin and Essner, 1989). Geoghegan (1988) described that the $F_{a,b}$ and F_c ends of IgG were

able to simultaneously absorb to colloidal gold. The absorption of IgG to more than one colloidal gold particles would thus produce IgG-gold clusters.

In the context of qualitative evaluation, clumped gold particles could enhance the level of immunolabelling. However, in a quantitative study, the appearance of clumped gold particles would likely result in estimation errors. Accordingly, an attempt to reduce the occurrence of clumped gold particles was made in the present study by centrifuging the IgG-gold solution. As a result, obvious reduction of clumped gold particles on tissue sections was obtained. This finding supports the suggestion that clumped gold particles are related to the source of the antibody used (Lin and Essner, 1989).

Roth, Taatjes and Warhol (1989) stated that the use of hydrophilic resins (i.e. L.R.White resin) in immunogold techniques could efficiently reduced the level of non-specific background staining due to the characteristics of their low attraction to immunoreagents. In the present study (Section 3.10.1), unacceptable levels of non-specific background staining were observed when anti-mouse type IV collagen antibody was used. In exploring the causes of the high level of non-specific background staining, several aspects of investigation (Sections 3.10.3; 3.10.4 and 3.10.5) had been undertaken namely the concentration of antibody, blocking agents and gold complex. The results indicated that the high level of non-specific background staining was not successfully reduced by changing the concentration of antibody, using other blocking agents and changing gold complex. After

these studies, it was considered that the high level of non-specific background staining observed was probably due to impure antibody. Although the purification of this antibody by biochemical methods may prove of consequence, such work has been not done in the present study due to the lack of availability of biochemical facilities in our laboratories and the very small volume of antibody remaining.

To sum up, the experiments described in this section were designed to establish optimum fixation, embedding, processing and immunocytochemical techniques for the ultrastructural localization of basal lamina laminin and type IV collagen in normal and neoplastic rat tongue tissues. As described in the review of literature an increasing body of knowledge has accumulated which clearly indicates the need for careful evaluation of all of these variables as no single protocol has yet been described which is universally applicable to the identification of all possible antigens in tissues.

As detailed earlier, this study in its broader context aims to identify and quantitate the expression of laminin and type IV collagen in normal rat tongue epithelial basal lamina and in the basal lamina associated with induced carcinomas. The results of the experiments described in this section (Sections 3.3 to 3.11) establish

1). The effect of fixatives on tissue morphology and tissue antigens is at variant among species, tissues and antigens. A mild glutaraldehyde fixation is more adaptable to the ultrastructural localization of basal lamina laminin and type IV collagen in rat

tongue tissues compared to paraformaldehyde and paraformaldehyde-glutaraldehyde fixatives.

2). The use of additives to fixatives may improve ultrastructural preservation and antigen retention.

3). Fixation osmolarity is associated with the ultrastructural preservation of some tissues, not with the antigen retention of basal lamina laminin and type IV collagen. PB and PBS as fixative vehicles mainly contribute to fixation osmolarity and are interchangeably used in immunocytochemistry.

4). Complete dehydration procedures for L.R.White resin is more appropriate for the ultrastructural localization of basal lamina laminin and type IV collagen in rat tongue tissues than partial dehydration procedure.

5). Room polymerization temperature for L.R.Withe resin is better for the expression of laminin and type IV collagen in rat tongue tissue compared to heat polymerization temperature.

6). The use of primary antibody and gold complex appears to affect immunolabelling.

7). It appears that morphological preservation and antigen retention in tissue preparation are exclusive. After a compromise between the ultrastructural preservation and antigen retention is reached, optimum tissue preparation and immunostaining methods are determined as follows:

(1). Fixation: 0.25% glutaraldehyde-0.15% picric acid-5% sucrose in 0.1M PB, PH 7.2, 365 moms for 1 hr.

(2). Buffer Washing: 0.1M PB with 50 mM NH_4CL and 3% sucrose, 375 moms for 1 hr.

(3). Dehydration: 70% ethanol for 5 min, 90% ethanol for 5 min, 100% ethanols twice for 50 min.

(4). Infiltration: pure, hard-grade L.R.White resin thrice for 3 hr at room temperature.

(5). Embedding: pure, hard-grade L.R.White resin in the proportion of 1μl accelerator of 1ml L.R.White resin under anaerobic condition at room temperature.

In immunostaining, rabbit anti-human laminin polyclonal antibody (primary antibody) in combination with protein A-gold complex is the preferred combination for labelling laminin, and goat anti-human and bovine type IV collagen polyclonal antibody with rabbit anti-goat IgG-gold complex is the appropriate combination for labelling type IV collagen.

CHAPTER FOUR: ULTRASTRUCTURAL IMMUNOLOCALIZATION OF BASAL LAMINA LAMININ AND TYPE IV COLLAGEN IN NORMAL RAT TONGUE MUCOSA AND INDUCED ORAL CARCINOMAS

4.1. Introduction

Basal lamina is a form of specialized extracellular matrix separating epithelial cells and other cell types from adjacent stroma. A number of components have been identified in the basal laminae of various tissues namely laminin, type IV collagen, heparan sulfate proteoglycan, fibronectin, entactin and tenascin. Among these, laminin and type IV collagen constitute two major constituents of basal lamina. As described by previous authors (Kleinmam et al, 1982; 1985; Campbell and Terranova, 1988; Raymond and Leong, 1991; Buck et al, 1992), basal lamina play important homoeostatic roles and have significant roles in pathological situations including neoplasms.

The role of basal lamina in relation to the behaviour of malignant neoplasms of epithelial origin is not well understood. However, it is believed that the properties of carcinoma invasion and metastasis are linked to basal lamina changes. A variety of pathological changes in basal lamina have been reported in a number of epithelial neoplasms including those of skin, cervix, breast and oral mucosa using electron microscopy and immunohistochemistry (Steven and Robert, 1978; Raymond and Leong, 1991; Jiang, Wilson and Wiebkin, 1993; Wilson et al, 1993). It is considered that these changes of basal lamina in epithelial neoplasms are related to tumour differentiation and behaviour, and the further investigation of such changes may provide more information on the biology of tumours and may lead to improved means of diagnosis of these tumours.

The ultrastructural localization of basal lamina constituents has been described in some normal tissues (Grant and Leblond, 1988; Lin and Essner, 1990). Despite this, information on the ultrastructural immunolocalization of basal lamina laminin and type IV collagen in normal oral mucosa and in neoplasms including oral carcinoma is scanty. It is felt that the elucidation of the ultrastructural distributive features of basal lamina laminin and type IV collagen in normal oral mucosa and induced oral carcinomas may provide further understanding of the molecular organization of normal basal lamina and the pathobiological properties of tumour basal lamina.

In this chapter, the immunogold techniques developed and described in the previous section were used to localize basal lamina laminin and type IV collagen in normal rat tongue mucosa and experimentally induced oral carcinomas. The distribution of laminin and type IV collagen in these tissues is described.

4.2. Materials and methods

Induction of tongue carcinomas using 4 Nitroquinoline 1-oxide (4NQO)

4.2.1. Materials

4.2.1.1. Animals

Thirty four Albino Wistar rats were housed in standard cages and fed with standard pellets and tap water *ad libitum* in standard cages in the Animal House, The University of Adelaide. The age of rats was 13 weeks and the average weight of rats was 180 gm at the beginning of tumour induction. Rats were divided into three groups: (1). Untreated control group containing 11 rats. (2). Propylene glycol-treated control group comprising 11 rats. (3). 4 Nitroquinoline1-oxide(4NQO)-treated experimental group consisting of 12 rats.

4.2.1.2. Chemicals

4-Nitroquinoline1-Oxide (4NQO) was purchased from Sigma, USA. Propylene glycol was bought from Ajax Chemicals, Sydney. The sources of glutaraldehyde, picric acid, ammonium chloride, L.R.White resin, accelerator and immunoreagents (except antibodies and gold complexes) were those described in Sections 3.2.2. and 3.2.3. The antibodies and gold complexes used were as follows:

Rabbit anti-human laminin polyclonal antibody (EY Labs, CA, USA).

Purified goat-anti-human and bovine type IV collagen polyclonal antibody (Southen Biotechnology, Birmingham).
Protein A-gold (20nm) (kindly donated by Dr. P. Smith, The Institute of Medical and Veterinary Science, Adelaide).
Rabbit anti-goat IgG-gold (20nm)(ICN Biochemicals, USA).

4.2.2. Methods

4.2.2.1. Carcinoma induction

Rats in all three groups were sedated by introducing a mixture of nitrous oxide-oxygen-halothane into a closed perspex box. After sedation, rats in the 4NQO-treated experimental group were treated with 10 microliters of 0.5% 4-Nitroquinoline1-oxide (4NQO)(Sigma, USA) in propylene glycol vehicle (AJAX CHEMICALS, Sydney) applied to the dorsum of the tongue thrice a week for 24 weeks. Rats in the propylene glycol-treated group received only propylene glycol applied to the dorsum of the tongue thrice a week for 24 weeks. Rats in the untreated control group were sedated only (Fig.21).

4.2.2.2. Sacrifice and dissection of animals

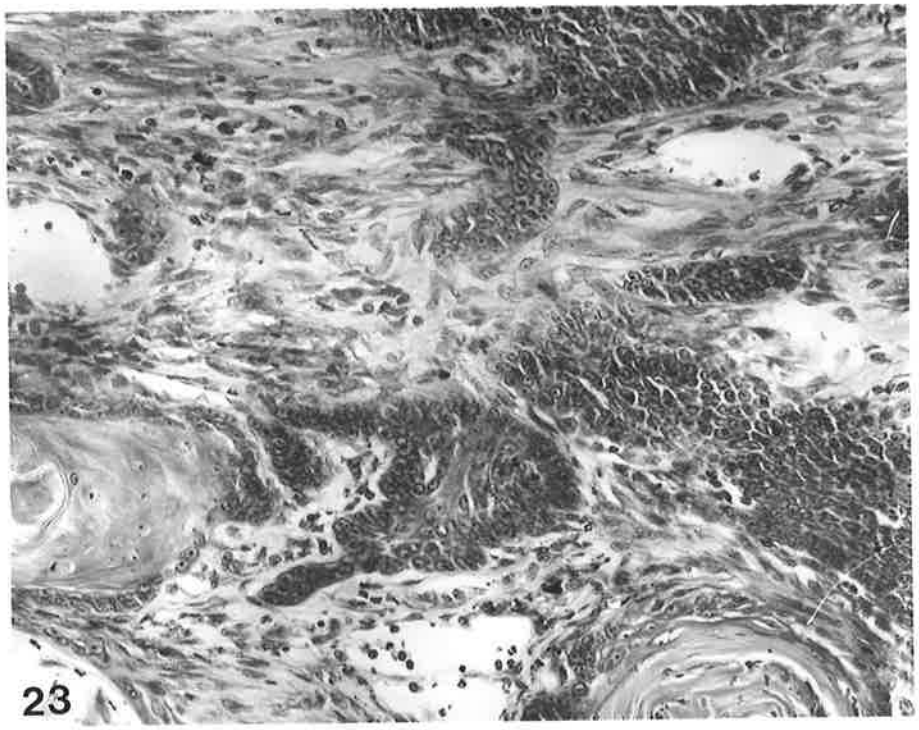
All animals were killed at 24 weeks. By this time all animals in the 4 NQO-treated group exhibited the presence of at least one tongue carcinoma (Figs. 22, 23).

Figure 21. Application of carcinogen on the surface of rat tongue

Figure 22. Induced carcinoma (arrows) on the dorsum of the tongue

Figure 23. Histological features of induced carcinoma (Haematoxylin & eosin staining, original magnification 20X)

22



23

Animals were killed by an overdose of nitrous oxide-halothane and the whole tongue was excised from the rat mouth. Sagittal tongue slices containing epithelium, connective tissue and muscle were cut from the posterior dorsal two thirds of the control tongues and induced carcinoma specimens and then approximate $1mm^3$ tissue blocks were cut perpendicularly to the epithelial surface of each tongue slice in a few drops of PBS.

4.2.2.3. Tissue processing

The harvested tissue specimens were immediately immersed in 0.25% glutaraldehyde-5% sucrose-0.15% picric acid in 0.1M PB, PH 7.2 for 1 hour. After fixation, tissues were washed in 0.1M PB with 50mM NH_4Cl and 3% sucrose for 1 hour and dehydrated in 70% ethanol for 5 minutes, 90% ethanol for 5 minutes, 100% ethanols twice for 50 minutes. Specimens were then infiltrated in pure, hard-grade L.R.White resin thrice for 3 hours and then embedded in gelatin capsules with a mixture of pure, hard-grade L.R.White resin with the manufacture's accelerator in the proportion of 1µl accelerator of 1ml L.R.White resin under anaerobic conditions. The latter was produced by the introduction of nitrogen at room temperature.

4.2.2.4. Sectioning:

Semithin sections (1 micrometer) were cut from all tissue blocks with a Reichert-Jung ultramicrotome and stained with toluidine blue for histological examination and orientation adjustment to ensure the mucosal surface sectioning perpendicular. After

surveying semithin sections, silver or gold ultrathin sections (60-90nanometers thick) were cut with a diamond knife and mounted on formvar-coated 150 mesh parallel lined nickel grids.

4.2.2.5. Immunostaining:

Grids mounted with ultrathin sections were inverted on to drops of each of the following solutions (Table 16) on paraffin film for immunolabelling.

Table 16 . The protocol for immunolabelling

1). 1% BSA in PBS	5min
2). Primary antibody at the dilution of 1:10 (for anti-laminin antibody) or 1:5 (for anti-type IV collagen antibody) with PBS containing 1% BSA and 1% Tween 20	3 hr
3). 3 X PBS wash	15 min
4). 1% BSA in PBS	5 min
5). Gold-complex at the dilution of 1:200 (Protein A-gold, for laminin) or 1:20 (IgG-gold, for type IV collagen) with PBS containing 1% BSA and 1% Tween 20	30 min
6). 3 X PBS wash	15 min
7). Distilled water wash	5 min
8). 2% aqueous uranyl acetate	2 min
9). Reynold's lead citrate	30 sec

4.3. Ultrastructural observation of basal lamina and the distribution of laminin and type IV collagen in normal rat tongue mucosa and induced oral carcinomas.

Methods: Ultrathin sections were selected from normal rat tongues and 4NQO-induced experimental oral carcinomas and stained immunohistochemically following the protocol described in Section 4.2.2.5. Sections were examined under a JEOL 100S transmission electron microscope at an original magnification of 20,000 times.

Results:

Normal rat tongue mucosa

Basal lamina showed distinct lamina densa and lamina lucida layers. A number of hemidesmosomes were distributed along the basal lamina. Distinct anchoring fibrils in some areas were seen to attach to the lamina densa. Collagen fibrils, blood vessels and nerve were seen in underlying connective tissue.

Gold particles indicating laminin were irregularly distributed on the lamina densa of epithelial basal lamina. A few gold particles were observed within the lamina lucida of the epithelial basal lamina (Fig.24A). Very few gold particles appeared within epithelial cells or in connective tissue. Basal laminae in blood vessels and nerves exhibited good immunolabelling (Fig.24B). Immunogold labelling for laminin was also seen around the nuclei of fibroblasts in the connective tissue (Fig. 24C).

Figure 24. Electronmicrographs showing immunolabelling (arrows) for laminin on the lamina densa of basal lamina in normal rat tongue mucosa (A), on nerve basal lamina (B) and within stromal fibroblast (C). E-epithelial cell, C-connective tissue, B-basal lamina, F-fibroblast. (Bar=0.2μm).

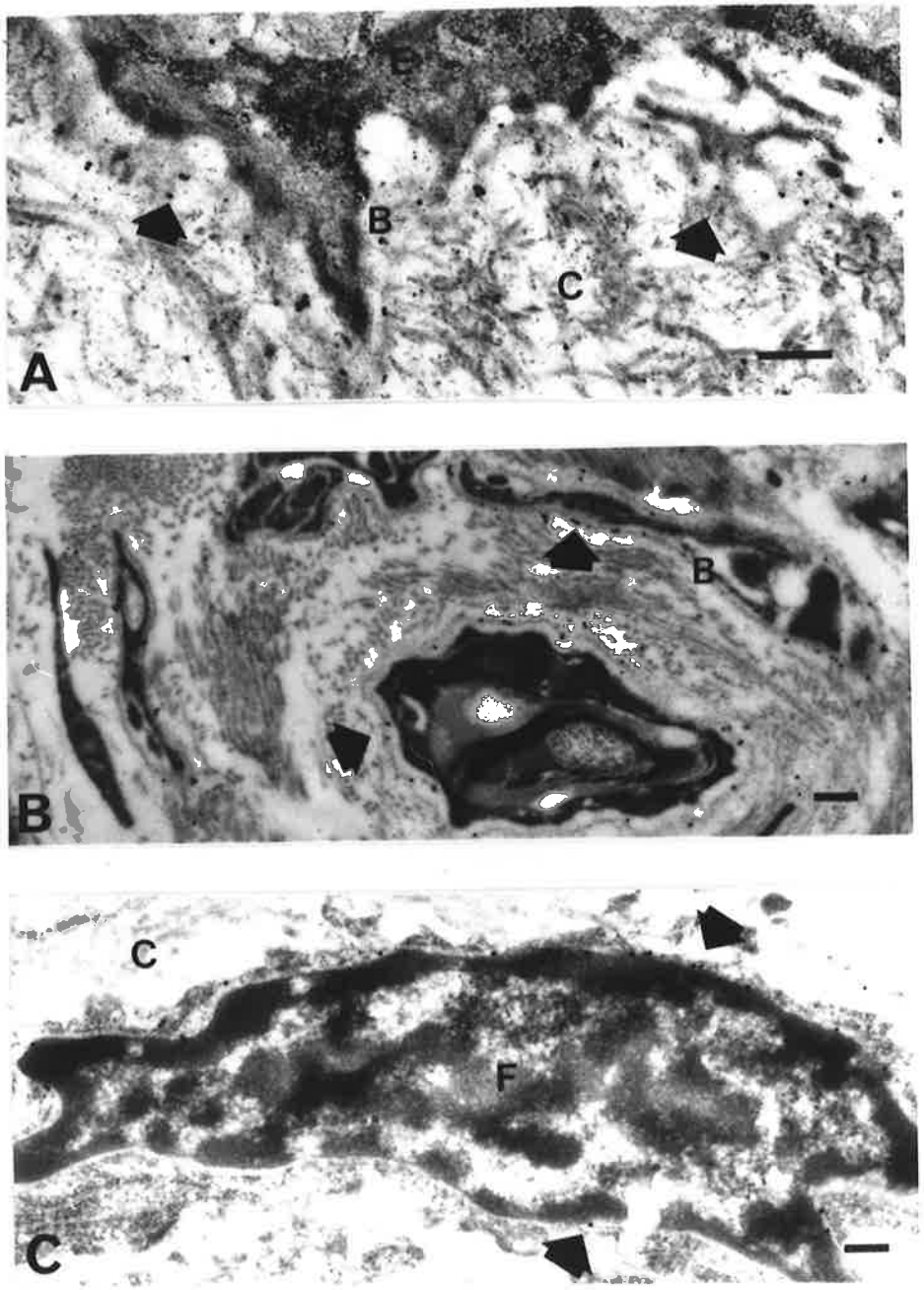


FIG 24

Immunogold labelling for type IV collagen was observed on the lamina densa of continuous basal lamina (Fig.25A). Blood vessel and nerve basal laminae showed good immunolabelling (Fig. 25B). Epithelial cells and connective tissue displayed very few gold particles. No gold particles were seen within stromal fibroblasts.

Induced carcinomas

The epithelial basal lamina showed focal discontinuities, duplication and thickening. Neoplastic islands surrounded by basal laminae invaded into the underlying connective tissue. Fragmented collagen fibrils and more abundant blood vessels were seen within the stroma of carcinomas.

Compared with normal rat tongue mucosa, neoplastic specimens exhibited more gold particles indicating laminin on the lamina densa of basal laminae (Fig.26A). Moreover, many gold particles were dispersed within the stroma of carcinoma (Fig.26B). Some epithelial cells had immunogold labelling for laminin within the cytoplasm and around the nuclei. Some gold particles were seen within fibroblasts.

Gold particles indicating type IV collagen were still distributed on the lamina densa of basal lamina. It was observed that some carcinoma specimens showed variable expression of type IV collagen (Fig.26C). Epithelial cells displayed very few gold particles. Some gold particles were observed in the connective tissue adjacent to the neoplastic basal lamina (Fig.26C).

Figure 25. Electronmicrographs showing immunolabeling (arrows) for type IV collagen on the lamina densa of basal lamina in normal rat tongue mucosa (A) and on blood vessel basal lamina (B). E-epithelial cell, C-connective tissue, B-basal lamina. (Bar=0.2μm).

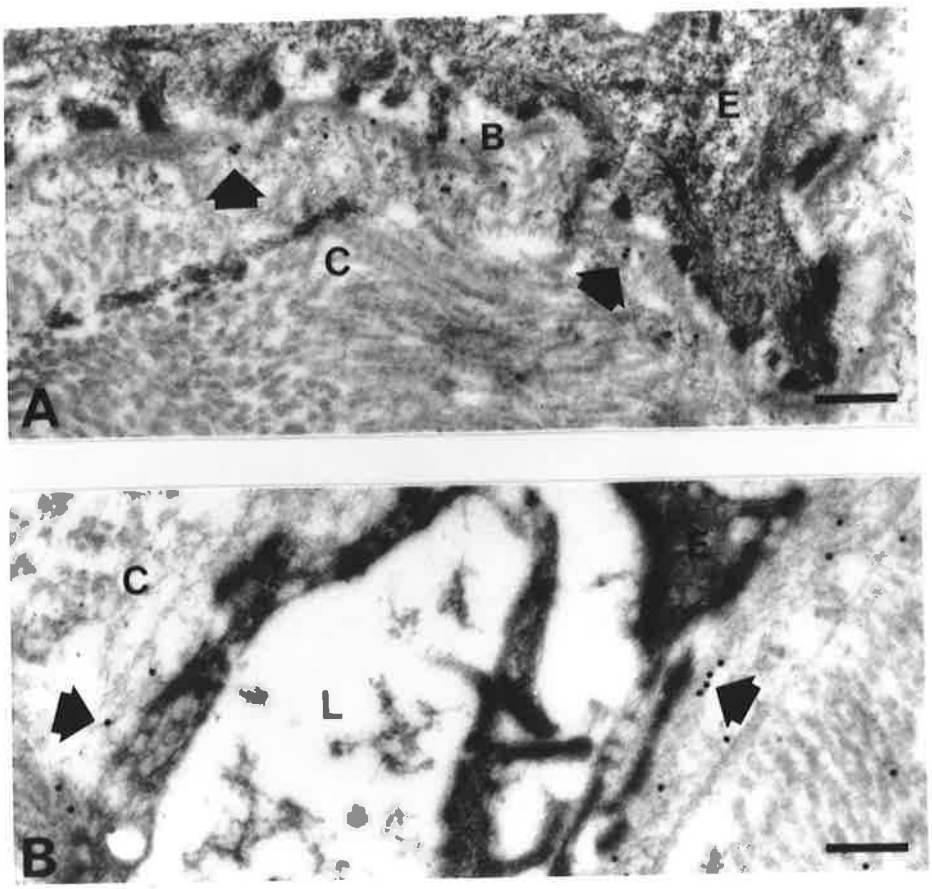


FIG 25

Figure 26. Electronmicrographs demonstrating immunolabelling (arrows) for laminin (A,B) and type IV collagen (C) on the basal lamina of carcinoma and within the stroma of carcinoma. E-epithelial cell, C-connective tissue, B-basal lamina. (Bar=0.2μm).

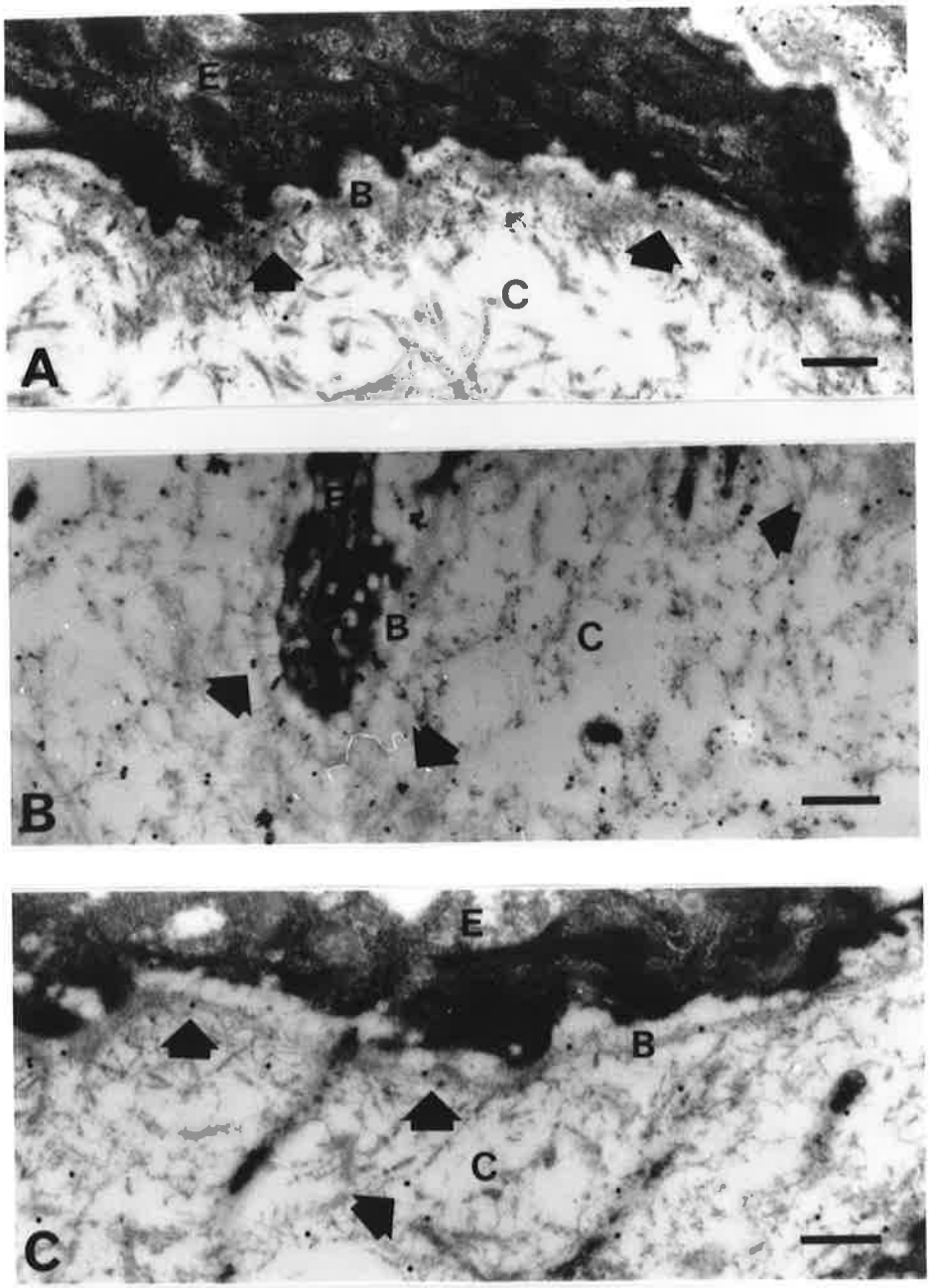


FIG 26

4.4. Immunohistochemical controls.

Methods: Two ultrathin sections were respectively selected from each of the untreated control group, propylene glycol-treated group and experimental oral carcinoma groups. Two types of control experiments for each antigen (laminin or type IV collagen) were undertaken. Firstly the primary antibody was replaced with antibody-antigen absorption solution, secondly the primary antibody was replaced with PBS. In the antibody-antigen absorption controls, anti-laminin antibody was preabsorbed with an excess of purified laminin (1mg/ml) and anti-type IV collagen antibody was preabsorbed with an excess of purified human and bovine type IV collagen mixture (2mg/ml). All sections for control experiments were stained following the protocol described in Section 4.2.2.5 except for the incubation with primary antibody.

Results: All control sections for the laminin and type IV collagen exhibited negative labelling (Fig. 27).

Figure 27. Electronmicrographs showing negative labelling for laminin and type IV collagen in control staining experiments.

(A). Laminin antibody-antigen absorption control (carcinoma tissue)

(B). Type IV collagen antibody-antigen absorption control (carcinoma tissue)

E-epithelial cell, C-connective tissue, B-basal lamina. (Bar=0.2μm).

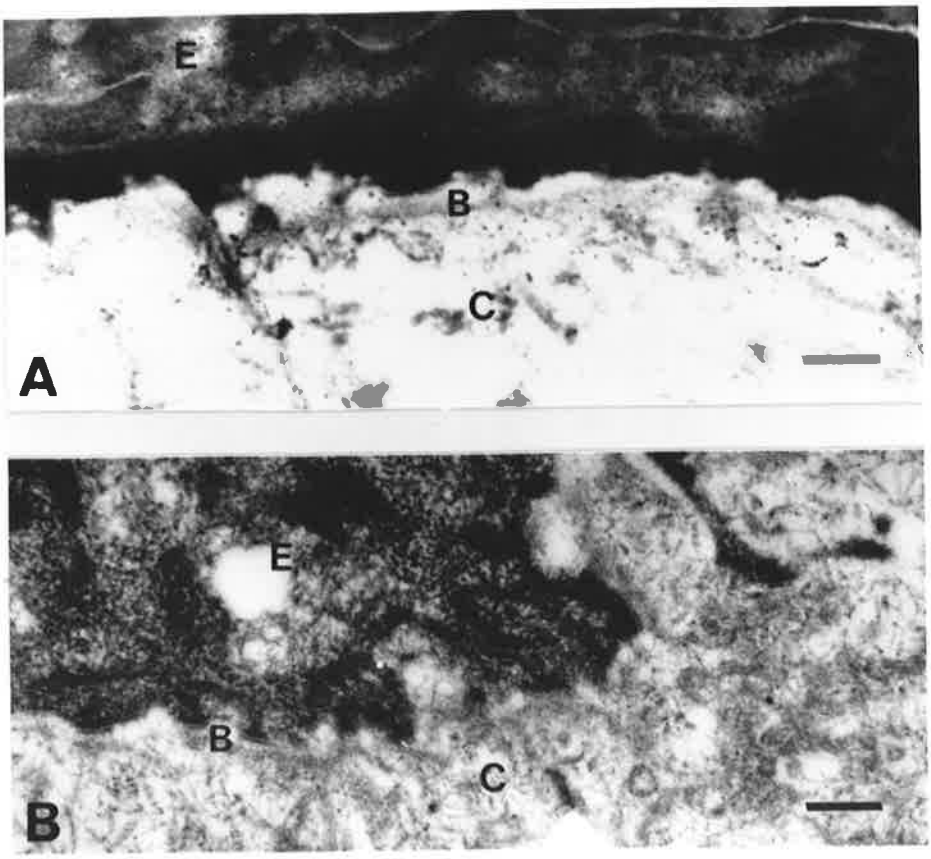


FIG 27

4.5. Discussion

Using immunohistochemistry, basement membrane (basal lamina) can be easily visualized by various markers detecting the reaction of antibody-antigen. Using optical immunohistochemistry, the distribution of basement membrane laminin and type IV collagen have been characterized in normal oral mucosa tissues and oral neoplastic tissues by a number of investigators (Carter et al, 1985; Jungell, 1990; Hirota et al, 1990; Cheng et al, 1992; Wilson et al, 1993). Although these studies have provided valuable information towards understanding the biological properties of normal oral tissues and pathological properties of oral neoplasms, the structural detail of basement membrane and the location of basement membrane macromolecules are poorly understood due to the low resolution of the light microscope.

In the present study, immunogold techniques were employed to ultrastructurally identify basal lamina laminin and type IV collagen in normal rat tongue mucosa and induced oral carcinomas. With the use of high density of gold particles, the location sites of both basal lamina components were easily identified and specific staining could be assessed by correlating staining patterns with the morphological detail.

In previous studies, the distribution of basal lamina laminin has been reported in a number of tissues. For instance, it has been described that laminin is distributed in the lamina densa and the lamina lucida of glomeruli, muscle, intestine and human oral mucosa and in the lamina lucida of skin and esophagus using

immunoperoxidase electron microscopic techniques (Foidart et al,1980; Martinez-Hernandez and Chung, 1984; Jungell, 1990). Using immunogold electron microscopic techniques, a number of studies have demonstrated that laminin is localized in the lamina densa and the lamina lucida of basal laminae in glomeruli, colon mucosa and enamel organ (Abrahamson, 1986; Grant and Leblond, 1988; Desjardins and Bendayan, 1989). In the present study, it was observed that laminin was confined to the lamina densa of oral mucosa epithelial basal lamina. By comparison, the location site of laminin in rat oral mucosa was different from that in other tissues such as kidney, skin, intestine, muscle, human oral mucosa (site not stated) and retinal vessels (Martinez-Hernandez and Chung, 1984; Abrahamson, 1986; Grant and Leblond, 1988; Jungell, 1990; Lin and Essner, 1990). The reasons for the different location sites of basal lamina laminin among tissues is not well known. There has been a view suggested by some authors (Woodley, 1987; Desjardins and Bendayan, 1989) that the different location sites may be attributed to "intrinsic" factors (ie. heterogeneity of the molecular structure of laminin in tissues or in species).

Aside from " intrinsic factors ", the different location sites of laminin may also be related to extrinsic factors (tissue preparation and immunostaining techniques). For instance, it is known that antigen retention varies with the different tissue preparation conditions. It is possible that insufficient retention of laminin antigenity after tissue preparation may result in negative labelling in tissues. In immunocytochemical techniques, it is recognized that the specificity of antibody may affect the

localization of basal lamina antigens. For example, Herken and Miosge (1991) employed three types of antibody raised against the different fragments of laminin molecule for the demonstration of basal lamina laminin. It was demonstrated that the immunolocalization of laminin varied with antibody types.

In the present study, basal lamina laminin was identified by the cross-reactivity of anti-human laminin polyclonal antibody. It is possible that some antigen epitopes of basal lamina laminin in rat tissue are not recognized by this antibody due to the heterogeneity of molecular structure of laminin between human tissue and rat tissue.

Jungell (1990) claimed that laminin was identified in the lamina densa and the lamina lucida of basal lamina in human oral mucosa using immunoperoxidase electron microscopic techniques. It is possible on the basis of the characteristics of immunoperoxidase technique that diffuse immunoperoxidase staining may have influenced the description of localization of type IV collagen in Jungell's study.

As described by some authors (Martinez-Hernandez and Chung, 1984; Desjardins and Bendayan, 1989; Yurchenco and Schittny, 1990), the distribution of basal lamina components has been related to the functional characteristics of various tissues. Although the exact location site of basal lamina laminin in oral mucosa need to be confirmed by further investigation (eg. using human tissue and monoclonal antibody), it is possible on the basis of the aforementioned view that the apparently different

distribution of basal lamina laminin between rat oral mucosa tissue and other tissues (eg. skin, kidney, intestine) may reflect functional characteristics.

In the present study, the distribution pattern of gold particles indicating laminin was irregular on the lamina densa of oral mucosa basal lamina. Whether or not this staining pattern reflect the real distribution of laminin in basal lamina is not clear. However, from a immunocytochemical point of view, a number of factors could have affected this result, because it is known that label density of tissue antigens depends on many factors such as the number of exposed antigen epitopes, tissue preparation techniques and the sensitivity of the immunostaining method (refer Chapter three).

Although some investigators have reported that laminin is also distributed in the lamina lucida of basal lamina in some tissues (i.e. glomeruli, skin) (Campbell and Terranova, 1988; Grant and Leblond, 1988), very few gold particles indicating laminin were observed in the lamina lucida of oral mucosa epithelial basal lamina in the present study. The appearance of some gold particles in the lamina lucida of oral mucosa basal lamina could be due to two possibilities. Firstly, a certain amount of laminin may actually be located in the lamina lucida of rat mucosa basal lamina. Secondly, the location of these gold particles could be due to artifact. At this stage, it appears that the second possibility is more likely. The reasons to support the second statement are (1). Gold particles in lamina lucida were infrequent and were only observed in a few specimens. (2). Quantitative analysis (see the

next Chapter) indicated that there was a marked change of laminin expression in lamina densa between normal tissues and carcinoma tissues; while no change was seen on laminin expression in lamina lucida. The explanations for artifactal location of gold particles on the lamina lucida are that (1). gold particles located on the edge of lamina densa may appear to be recorded on the lamina lucida at the photographic plane due to the oblique sectioned plane of basal lamina or (2). some antigens may diffuse during tissue preparation or immunostaining.

It was interesting to note from the present study that some gold particles were distributed within fibroblasts in normal tissue and experimental carcinoma. Laurie and colleagues (1982) investigated the biosynthesis of laminin in the endodermal cells of rat parietal yolk sac using anti-laminin antibodies and immunoperoxidase techniques. It was demonstrated that immunostaining was seen on cellular organelles (rough endoplasmic reticulum and Golgi apparatus) and it was suggested that the intracellular expression of laminin was attributed to laminin precursors. Campbell and Terranova (1988) reported that epithelial cells, endothelial cells, muscle cells and some types of fibroblasts were the source of laminin production. Chung (1991) studied the function of fibroblasts in prostatic cancer. It was described that there was a metabolic co-operation between fibroblast and epithelial cells and that fibroblast could produce extracellular matrix including laminin. From this, it is suggested that stromal fibroblasts demonstrating laminin contain antigen epitopes of laminin and may be involved in the production of laminin.

In the present study, the expression of laminin within normal epithelial cells was not seen. Despite this, it can not be concluded that epithelial cells are not involved in the production of laminin, because some epithelial cells producing laminin may not be identified due to the absence of available antigen epitopes or insufficient antigen epitope expression.

In contrast to normal epithelial cells, some neoplastic cells observed in this study demonstrated laminin labelling within the cytoplasm and around neuclei. Intracytoplasmic labelling of laminin within neoplastic cells has also been reported by a number of other investigators (Ormerod et al, 1985; Lee, 1988). This phenomenon has been related to cell differentiation. Lee (1988) suggested that poorly-differentiated neoplasms might demonstrate intracytoplasmic staining of laminin. In the present study, the experimental oral carcinomas were histologically well-differentiated. It seems therefore that the presence of intracytoplasmic laminin in the present study does not support Lee's suggestion. However, one possibility for this is that there is a heterogeneity of cell differentiation within the experimental oral carcinomas investigated.

In the present study a dispersed labelling for laminin was observed within the stroma of tumours. It was found that some labelling was related to detached basal lamina; however, some dispersed labelling of laminin within the stroma did not show any evidence of relationship to the basal lamina. Control experiments confirmed that the dispersed labelling within the stroma was specific for laminin. In previous studies, it has been described

that tumour cells could produce more laminin (Martinez-Hernandez and Amenta, 1983; Campbell and Terranova, 1988).

In previous studies, some investigators have shown that laminin plays important roles in tumour invasion and metastasis (Liotta et al, 1982; Kleinman et al, 1984; Campbell and Terravona, 1988; Sweeney et al, 1991). For instance, Liotta and colleagues (1982) have demonstrated that laminin can enhance the metastatic propensity of tumour cell lines. On the basis of the described characteristics of laminin, it is suggested that the finding of dispersed laminin within the stroma of carcinoma may be associated with the spread of carcinoma cells in the stroma.

In previous studies, the distribution of basal lamina type IV collagen has also been examined in a number of investigators (Bendayan, 1985; Grant and Leblond, 1988; Lin and Essner, 1990). For instance, Grant and Leblond (1988) and Lin and Essner (1990) used immunogold electron microscopic techniques to identify the distribution of type IV collagen in the lamina densa of glomeruli, colon and retinal vessels. In the present study using immunogold electron microscopic techniques, it was found that type IV collagen was distributed in the lamina densa. The results of the present study showed the same location site for type IV collagen in rat oral mucosa basal lamina as that previously described for various tissues such as kidney, skin, retinal vessels and intestine. From a functional point of view, this consistent distribution of type IV collagen in various tissues may indicate a similar role for type IV collagen in all basal laminae.

In the synthesis of type IV collagen, epithelial cells have been considered to be one of the main source (Kleinman et al, 1982), but from the present study, it was shown that no immunolabelling was seen within normal tissue cells or within neoplastic tissue cells. At this stage, the possibility that epithelial cells do not produce type IV collagen can not be ruled, because several factors may also affect the immunolabelling namely insufficient antigen epitopes and weak binding activity of the anti-type IV collagen antibody used.

In the present study, type IV collagen exhibited less label density in oral mucosa basal lamina compared with the labelling intensity of laminin in normal tissues. Previous investigators have suggested that weak labelling of type IV collagen might indicate that (1). basal lamina contains little type IV collagen; (2). the antibody used might have weak binding activity (Grant and Leblond, 1988). Risteli et al (1981) described that type IV collagen was characterized by its weak immunogenicity. As a consequence, antibody raised against type IV collagen might have a weak binding to the corresponding antigen. Timpl and colleagues (1979) reported that laminin was a better immunogen than type IV collagen. From the results of the present study, it was also apparent that the localization of laminin was more easily obtained than that of type IV collagen. The demonstrated differences in label density between basal lamina laminin and type IV collagen in the present study may indicate that there will be some differences in immunological characteristics (i.e. immunogenicity) and quantity between the both basal lamina antigens.

With regard to the expression of type IV collagen in neoplastic tissue, it was found in the present study that a variable expression of type IV collagen occurred among individual carcinomas. In that, some carcinoma specimens appeared to express more labelling of type IV collagen, whereas other carcinoma specimens showed less labelling of type IV collagen.

In neoplastic stroma, type IV collagen showed low density of labelling compared with laminin. The reasons for and implication of this observation remain unclear. It has been described that basal lamina can be destroyed by various enzymes such as type IV collagenases and cathepsin (Liotta et al, 1982; Pauli et al, 1983). Moreover, it has been reported that detached basal laminae often appear in neoplastic stroma (Tsujioka et al, 1991). In the present study, it was difficult to assess whether or not type IV collagen labelling in stroma was related to detached basal laminae as the labelling was very dispersed of low intensity.

CHAPTER FIVE: ULTRASTRUCTURAL MORPHOMETRY OF BASAL LAMINA LAMININ AND TYPE IV COLLAGEN IN NORMAL RAT TONGUE MUCOSA AND INDUCED ORAL CARCINOMAS

5.1. Introduction

It is well known that basal laminae play important roles in relation to malignant epithelial cell invasion from parent epithelium and within tumour stroma (Pauli et al, 1983; Aznavoorian et al, 1993). Several morphological changes in basal lamina (for example, discontinuities, duplication, thickening) have been described in various epithelial neoplasms using electron microscopy (Steven and Robert, 1978; Jiang, Wilson and Wiebkin, 1993). The distribution of basal lamina laminin and type IV collagen in both normal and neoplastic tissues has been described by a number of investigators using immunohistochemistry (Ormerod et al, 1985; Soini, Autio-Harmainen and Miettinen, 1989; Wilson et al, 1993). These studies demonstrated intensive staining of laminin and discontinuous staining of type IV collagen. The distribution of basal lamina laminin and type IV collagen in normal rat tongue oral mucosal and 4 Nitroquinoline1-oxide-induced experimental tongue carcinomas has been defined at both the optical and electron microscopic levels using immunohistochemical techniques. Observations indicate that the optical microscopic and ultrastructural changes in basal lamina morphology seen in experimental oral carcinomas may be related to qualitative and quantitative alterations in basal lamina laminin and type IV collagen (Jiang, Wilson and Wiebkin, 1993; Wilson et al, 1993).

To verify previous observations and further describe the properties of experimental oral carcinomas, the quantitative analysis of basal lamina laminin and type IV collagen in normal

rat tongue mucosa and in the experimentally induced oral carcinoma was undertaken.

In this Chapter, the quantitation of basal lamina laminin and type IV collagen in normal rat tongue mucosa and in 4NQO-induced experimental oral carcinomas was carried out using the established post-embedded immunogold techniques and morphometric methods described by Weibel (1969). In addition, several concommitant studies were also carried out including the determination of optimum magnification and sample size, the establishment of structural criteria and the selection of an appropriate measurement grid.

5.2. Materials and methods

For this experiment the tissue materials used comprised blocks of tongue tissue from each of the 3 animal groups described in Section 4.2.1.1. A total of 30 animals was used (10 animals per group) to provide the samples. Details of the sampling methods used are described in the next section (Section 5.3). Tissue preparation and sectioning techniques employed were similar to that described in Sections 4.2.2.3 and 4.2.2.4. Ultrathin sections for the specific labelling of each antigen (laminin or type IV collagen) were stained following the protocol described in Section 4.2.2.5. and the control staining experiments were similar to those in Section 4.4. All ultrathin sections were examined under a JEOL 100 transmission electron microscope at a voltage of 80 KV. A stereological point-counting method and MOP image analysis system (CARL, ZEISS. Inc.) were used to estimate basal lamina area and length respectively. Programms 3V, 5V and 8V from the BMDP statistical software package (1991 release, UCLA, ed W.J. Dixon) were employed for the statistic analysis of variance.

5.3. Sampling

After sacrifice of the rats, the posterior dorsal two thirds of individual control tongue were cut into 1-2mm sagittal slices. Induced carcinoma specimens were similarly sliced. Every second tissue slice was selected from the sample pool of each animal. All selected tissue slices were then cut perpendicularly to the epithelial surface into approximately 1 mm^3 size tissue blocks.

Thirty representing tissue blocks per animal were selected randomly from each of the three group 10 animals (approximately total 900 tissue blocks). Ninety plastic-embedded blocks (10 tissue pieces in each embedded block) were processed, from which 30 plastic-embedded tissue blocks were then randomly selected. Ultrathin sections were subsequently cut from the 30 selected blocks and mounted on formvar-coated 150 mesh parallel lined nickel grids. A total of 60 grids mounted with ultrathin sections (2 grids per block or per animal) were selected randomly for immunostaining and then 30 grids were randomly selected from within the 60 stored grids for photographic analysis (Table 17).

Electronmicrographs were recorded from each section in a sampling pattern which was designated as a consistent interval (6μm) in the horizontal plane across the section and a variable interval in the vertical plane to cover basal lamina areas (Figs.28, 29). In normal tissues, electronmicrographs consisted of epithelial ridge basal laminae, basal laminae over connective tissue papillae and basal laminae between both regions. In carcinoma tissues, electronmicrographs comprised epithelial basal laminae as well as epithelial island basal laminae.

	Untreated rats	Propylene glycol-treated rats	Experimental carcinoma rats
Number of animals	10	10	10
Tissue blocks ($1 mm^3$)	300 (30 per animal)	300	300
Embedded blocks	30 (3 per animal)	30	30
Sectioning blocks	10 (1 per animal)	10	10
Immunoincubation	20 (2 grids per animal)	20	20
TEM examination	10 (1 grid per animal)	10	10
Electronmicrographs	50 (5 per animal)	50	50

Table 17. Sampling at the different levels

Figure 28. Diagram showing the sampling pattern for selecting electronmicrographs in normal tissue specimens. Areas of basal lamina in which counts were made were selected by taking equi-distant photomicrographs in a horizontal plane across the section. Horizontal interval distance (6 μm) were measured using the micrometre drive of the electronmicroscope. Where a " field " (shaded) did not incorporate the basal lamina, a perpendicular was dropped until basal lamina was reached, and that field was used for photomicrography and counting.

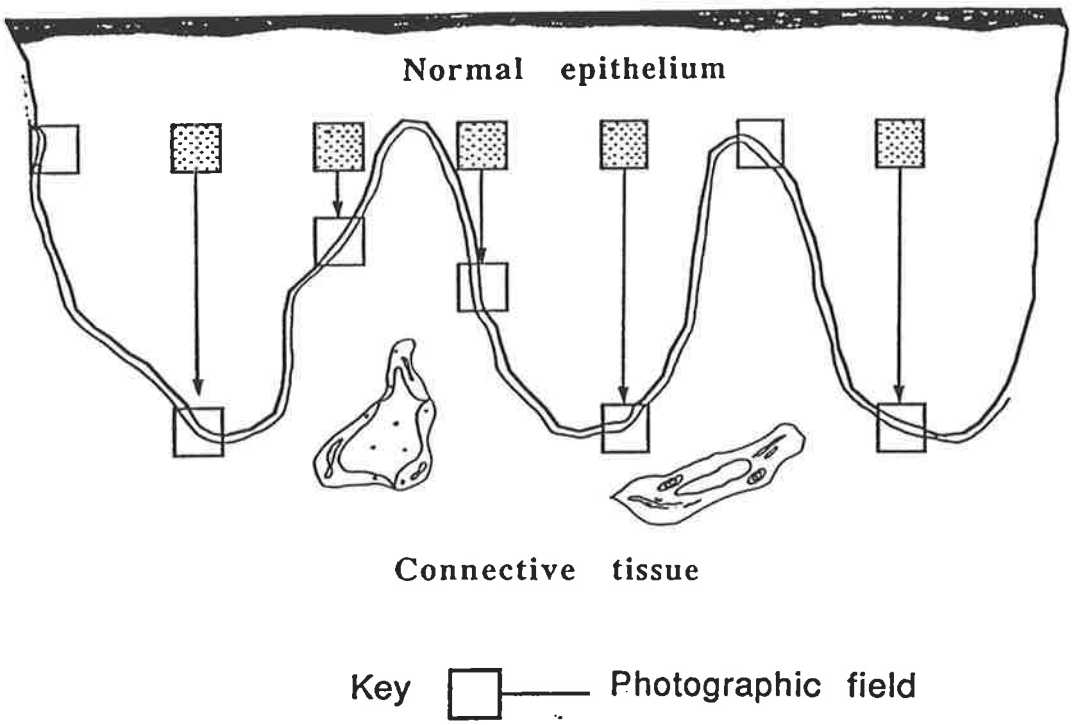

Normal epithelium
Connective tissue
Key
Photographic field

Figure 28

Figure 29. Diagram showing the sampling pattern for selecting electronmicrographs in carcinoma specimens. Areas of basal lamina in which counts were made were selected by taking equi-distant photomicrographs in a horizontal plane across the section. Horizontal interval distance (6 µm) were measured using the micrometre drive of the electronmicroscope. Where a " field " (shaded) did not incorporate the basal lamina a perpendicular was dropped until basal lamina was reached and that field counted. If a field incorporated neoplastic island basal lamina, a perpendicular was dropped and all fields incorporating basal lamina were counted.

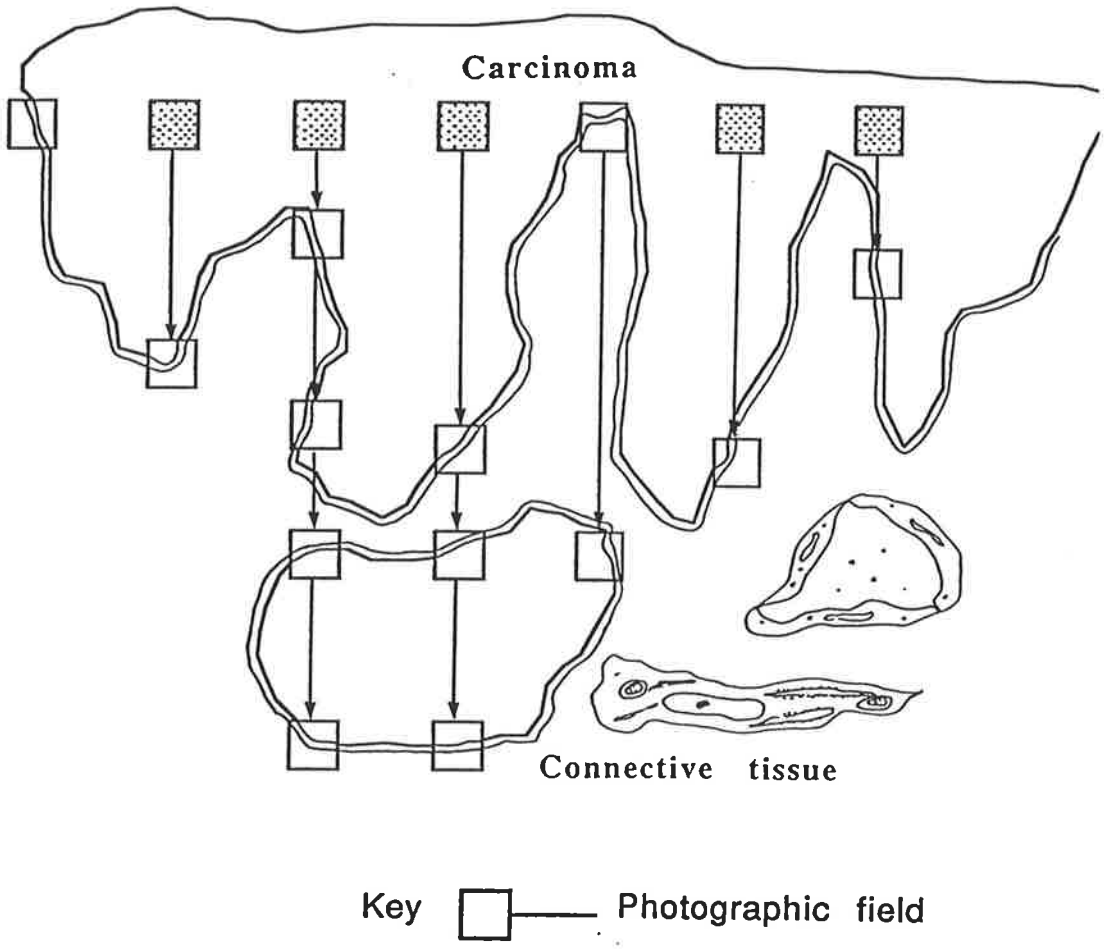


Figure 29

5.4. Determination of an optimum magnification of electronmicrographs.

Methods: Electronmicrographs were recorded from basal lamina regions of normal rat tongue mucosa at original magnifications of 10,000, 15,000 and 20,000 times. Electronmicrographs from a carbon grating replica (SO_3, Probing & Structure, QLD) were also taken at each magnification. Electronmicrographs, standardized with the calibration grating, were printed on 20.3 X 25.4 cm (8 X 10 inches) photographic paper at final magnifications of 30,000; 45,000 and 60,000 times respectively.

Results: Electronmicrographs at a final magnification of 30,000 times (ie. original magnification 10,000) showed the greatest length of basal lamina, but indistinct basal lamina structure. Distinct basal lamina morphology was seen in electronmicrographs at a final magnification of 45,000 times (ie. original magnification 15,000) and 60,000 times (ie. original magnification 20,000). By comparison, electronmicrographs at a final magnification of 45,000 times revealed a greater length of basal lamina areas than those at the final magnification of 60,000 times.

Conclusion: An optimum magnification for the achievement of maximum basal lamina areas with satisfactory resolution is 45,000 times (ie. original magnification 15,000).

5.5. Selection of an appropriate measurement grid for estimating the surface area of basal lamina on sections.

Methods: Twenty electronmicrographs from 1 section of normal tissue and 20 electronmicrographs from 1 section of experimental oral carcinoma were recorded at an original magnification of 15,000 times using the sampling pattern described in Section 5.3. All electronmicrographs, standardized with the calibration grating, were printed on 20.3 X 25.4 cm (8 X 10 inches) photographic paper to produce a final magnification of 45,000 times. The distortion of printing was estimated using a calibration grating. Five millimeter and 10mm lined spacing grids were drawn on transparent paper. The surface area of basal lamina (lamina densa) for each electronmicrograph was estimated using the 5mm and 10mm lined spacing grids respectively in each of three positions (30^0, 60^0 and180^0).

Results: The shrinkage of photographic paper after drying was approximate 1.3%. The distortion in the central area of electronmicrographs (18 X 24 cm) was less than 2.3%. The surface area of basal lamina estimated with the 5mm and 10 mm lined spacing grids in three positions was similar. The surface areas of basal lamina estimated with the 5mm lined spacing grid was a little more consistent than those with 10mm lined spacing grid.

Conclusions: The distortion of measurement of electronmicrographs caused by printing is very small and consistent. The area of less distortion of electronmicrographs is within the central 18 X 24 cm area. The optimum measurement

grid for estimating basal lamina area in this study is a 5mm lined spacing grid.

5.6. Analysis of appearances of basal lamina and hemidesmosomes in different given planes.

Methods: Separate schematic diagrams of basal lamina structure were drawn incorporating the following principles. (1). The thickness of lamina densa is equal to that of lamina lucida (Chen and Squier, 1984). (2). The thickness of the section (60-90nm) is approximately equal to that of the lamina densa and lamina lucida (100nm) (Chen and Squier, 1984). (3). A sub-basal densa plate of hemidesmosomes is localized between the basal plasma membrane and the lamina densa. The possible appearances of basal lamina and hemidesmosomes were analysed in two planes; the perpendicular and the oblique plane.

Results: Basal lamina in the perpendicular plane should show an approximately equal thickness of lamina densa and lamina lucida on the photographic plane. The borders of lamina densa, lamina lucida and basal plasma membrane should be distinct (Fig.30). In the oblique plane, a wider lamina densa with a narrow lamina lucida or only a lamina densa should appear on the photographic plane (Fig.31). In addition, a distinct attachment plaque and a sub-basal densa plate of hemidesmosome should be seen only in the perpendicular plane (Fig.32, 33) (Tidman and Eady, 1984).

Conclusions: Appearances of basal lamina are related to the plane in which the tissue is sectioned. Perpendicularly sectioned basal lamina can be selected according to the appearances of basal lamina in each electronmicrograph.

Figure 30. Diagram showing appearance of basal lamina in a perpendicularly sectioned plane. Approximately equal thickness of lamina densa (LD) and lamina lucida (LL) with distinct borders appear on the photographic plane. BC-basal cell.

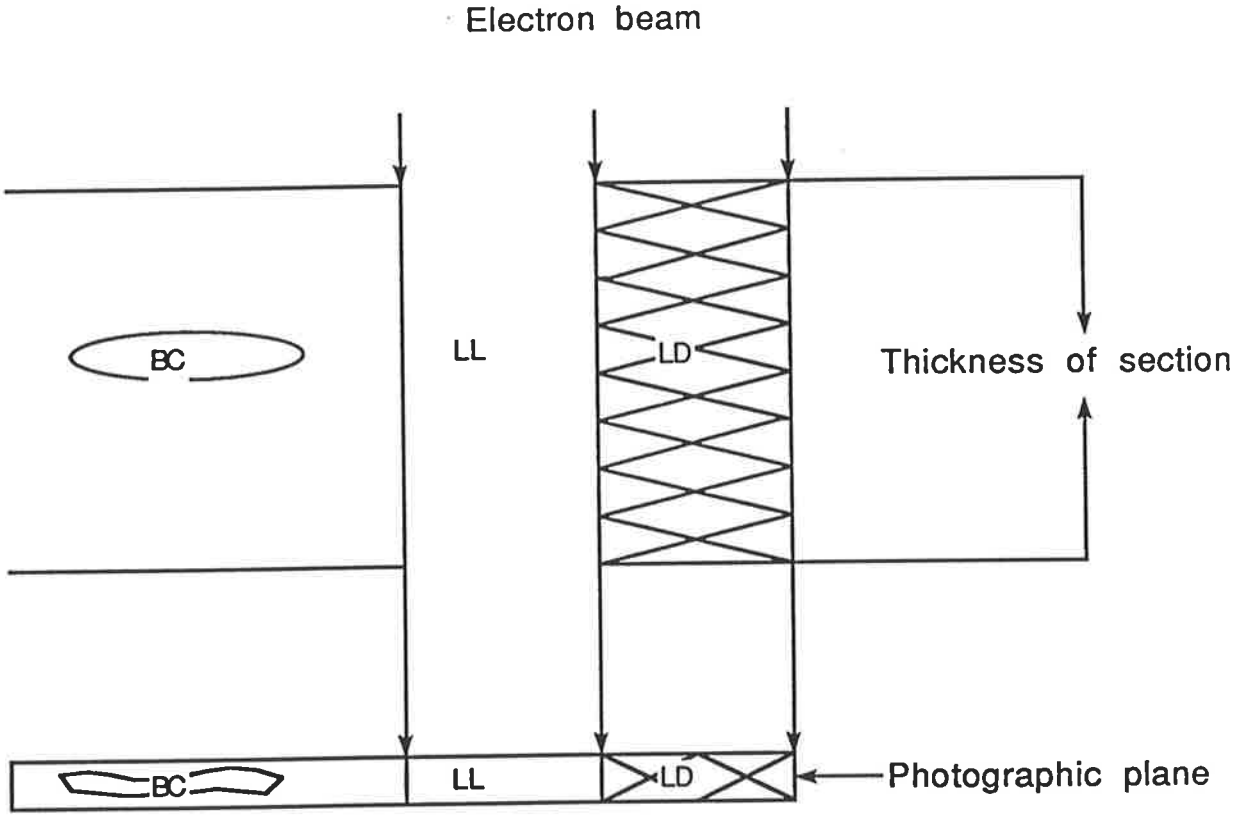

Electron beam
BC
LL
LD
Thickness of section
BC
LL
LD
Photographic plane

Figure 30

Figure 31 Diagram showing appearance of basal lamina in a obliquely sectioned plane. Lamina lucida (LL) is partially obscured by basal cell (BC) and lamina densa (LD). A wider lamina densa with a narrow lamina lucida appear on the photographic plane.

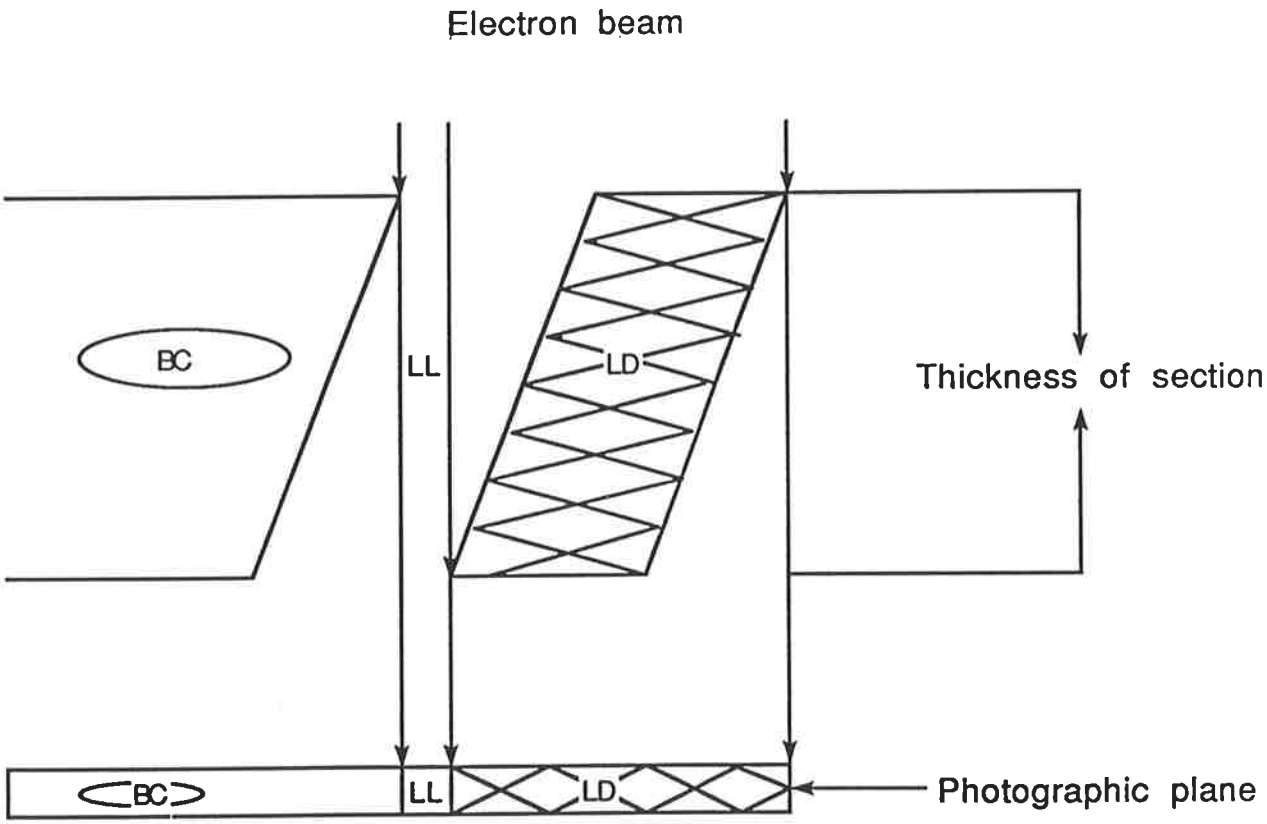

Electron beam
BC
LL
LD
Thickness of section
BC
LL
LD
Photographic plane

Figure 31

Figure 32. Diagram showing appearance of hemidesmosome in a perpendicularly sectioned plane. Sub-basal densa plate (DP) of hemidesmosome clearly appears in the lamina lucida on the photographic plane

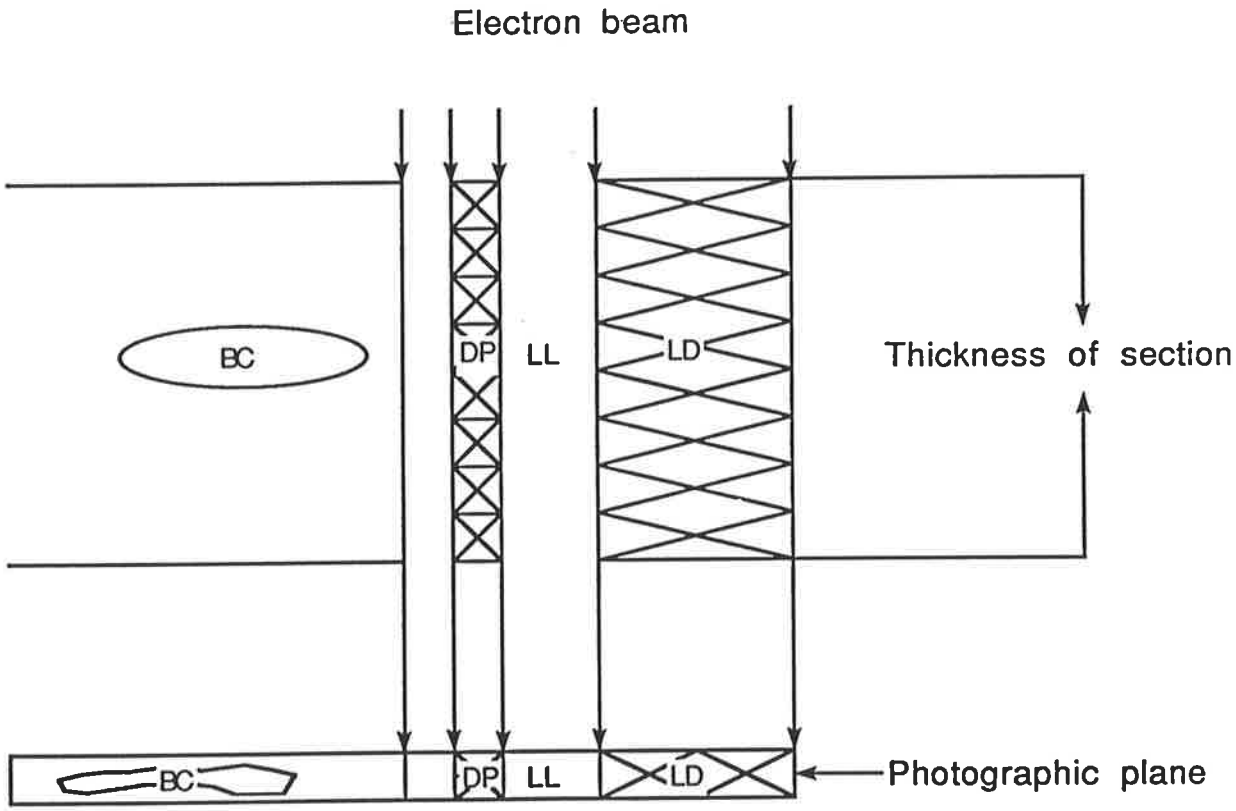

Electron beam
BC
DP
LL
LD
Thickness of section
BC
DP
LL
LD
Photographic plane

Figure 32

Figure 33. Diagram showing appearance of hemidesmosome in a obliquely sectioned plane. Sub-basal densa plate (DP) of hemidesmosome is obscured by basal cell (BC) and lamina densa (LD). The borders of basal plasma membrane, lamina lucida (LL) and lamina densa are indistinct on the photographic plane

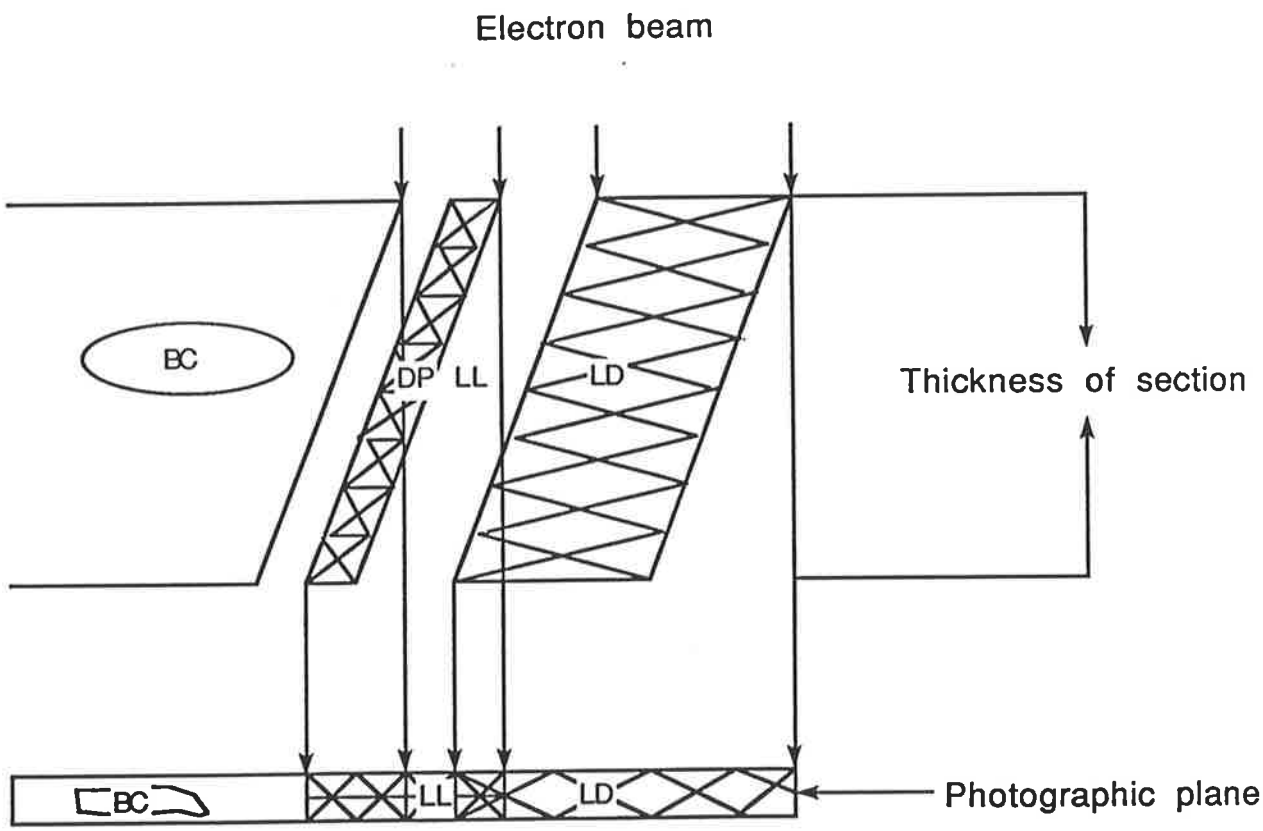

Electron beam
BC
DP
LL
LD
Thickness of section
BC
LL
LD
Photographic plane

Figure 33

5.7. Establishment of structural criteria for the ultrastructural morphometry of basal lamina laminin and type IV collagen in normal rat tongue mucosa and induced oral carcinomas.

Methods: All electronmicrographs recorded for the ultrastructural morphometry of basal lamina laminin and type IV collagen in normal rat tongue mucosa and in 4NQO-induced experimental oral carcinoma were examined. The structural criteria for selecting basal lamina areas to be measured in each electronmicrograph were established as follows:

Results: Inclusions:

(1). The borders of lamina densa and lamina lucida should be well delineated (Fig.34A).

(2). The basal plasma membrane should be intact and well defined (Fig.34A).

(3). An acceptable length of basal lamina (approximately 4cm) should be obtained.

(4). Attachment plaque and sub-basal densa plate of hemidesmosome should be distinct (Fig.34A).

Exclusions:

(1). Only lamina densa or very wider lamina densa with a narrow lamina lucida appearing in basal lamina regions (Fig.34B).

(2). Isolated epithelial cells with lamina densa and lamina lucida in normal tissues (Fig. 34C).

(3). Detached basal lamina in neoplastic tissues (Fig.34D).

Figure 34. Electronmigraphs showing immunolabelling (arrows) for laminin in normal oral tongue mucosa and carcinoma.

A. Gold particles indicating laminin within the distinct layers of basal lamina of normal tissue (Inclusion criterion).

B. Gold particles indicating laminin (arrow heads) appeared on clumped lamina densa of normal tissue (Exclusion criterion).

C. Gold particles (arrow heads) on the basal lamina of isolated normal epithelial cell process (Exclusion criterion).

D. Gold particles (arrow heads) on the detached basal lamina in carcinoma tissue (Exclusion criterion).

E-epithelial cell, C-connective tissue, B-basal lamina, L-lamina lucida of basal lamina, D-lamina densa of basal lamina. (Bar=0.2μm)

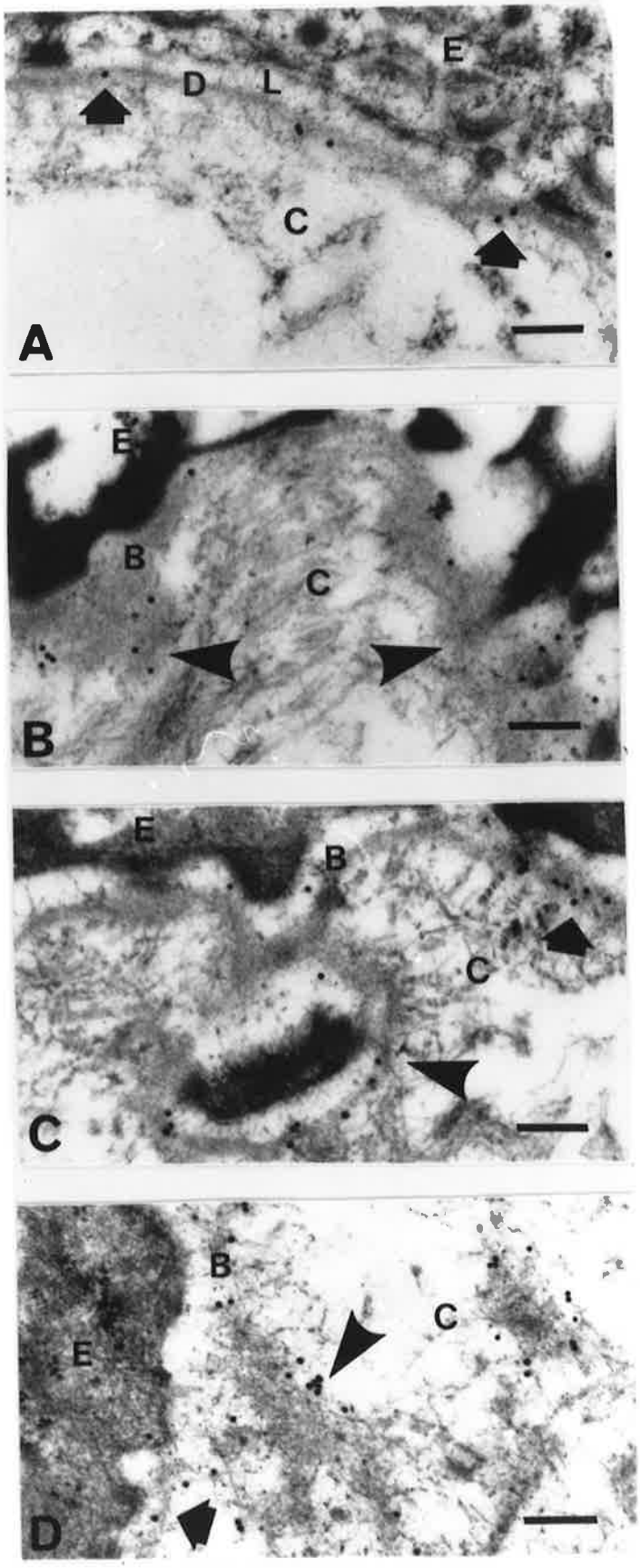


FIG 34

5.8. Determination of the optimum number of electronmicrographs for the ultrastructural morphometry of basal lamina laminin and type IV collagen in normal rat tongue mucosa and induced oral carcinomas.

Methods: 15 electronmicrographs (3 electronmicrographs per animal) from 5 animals in each of the three groups (untreated group, propylene glycol-treated group and carcinomas) were recorded at an original magnification of 15,000 times following the sampling pattern described in Section 5.3. All electronmicrographs, standardized with the calibration grating and restricted to the central 18 X 24 cm area, were printed on 20.3 X 25.4 cm (20.3 X 25.4 cm) photographic paper at a final magnification of 45,000 times. For each of the electronmicrographs, the surface areas of basal lamina (lamina densa) were estimated using a point-counting method (Weibel, 1979) with a 5mm lined transparent spacing grid, superimposed over the micrographs as gold particles in the corresponding basal lamina areas were counted. The label density of each antigen for each electronmicrograph was represented as the number of gold particle/per unit area. The statistical "component of variance" model was used to determine the optimum number of electronmicrographs necessary (Snedecor and Cochran, 1967). In this model, the observations are considered as

$$\textbf{ratio}_{ijk}\ (\text{number of gold particle/unit area}) = \mu_{i} + \mathbf{a}_{ij} + \mathbf{e}_{ijk}$$

where

μ_i = mean of i th group, i = 1, 2, 3 (i.e. control group, carcinoma group)

$\mathbf{a}_{ij}$ = an effect due to j th animal in i th group. j = 1,...5

$\mathbf{e}_{ijk}$ = random measurement error of k th field, j th animal, i th group

It was assumed that $\mathbf{a}_{ij} \sim N(0, \acute{o}_a^2)$ and $\mathbf{e}_{ijk} \sim N(0, \acute{o}^2)$. Therefore, the estimate of mean value (number of gold particle/ per unit area) has variance given by

$$V(\bar{X}_{i..}) = \frac{\acute{o}_a^2}{N} + \frac{\acute{o}^2}{N\,n}$$

$\acute{o}_a^2$ = variance among animals

$\acute{o}^2$ = variance among fields

N = number of animal per group

n = number of field per animal

Results: The pilot study produced estimates of $\acute{o}_a^2$ and $\acute{o}^2$ as follows

for label density of laminin $\acute{o}_a^2 = 381.68$, $\acute{o}^2 = 171.79$

for label density of type IV collagen $\acute{o}_a^2 = 40.30$, $\acute{o}^2 = 166.95$

The variance of mean value of label density of each antigen showed a decrease following an increasing number of selected electronmicrographs (fields)/per animal. As more than 5 electronmicrographs/per animal in a 10 animal/per group or more than 10 electronmicrographs/per animal in a 5 animal/per group were chosen, the variances showed "diminishing return" (Table 18-21 & Fig. 35-38).

Conclusions: The optimum number of electronmicrographs is 5 electronmicrographs per animal if 10 animals/per group are used or the optimum number of electronmicrographs is 10 electronmicrographs per animal if 5 animals/per group are used.

Table 18 & Figure 35. The relationship between the variance ($V(\bar{X})$) of the mean value of label density of laminin and the number (n) of selected electronmicrographs/per animal when 10 animals/per group were used.

Table 18

n	$V(\bar{X})$	$\sqrt{V(\bar{X})}$
2	46.76	6.84
3	43.89	6.62
5	41.60	6.45
10	39.89	6.32
20	39.03	6.25

Figure 35

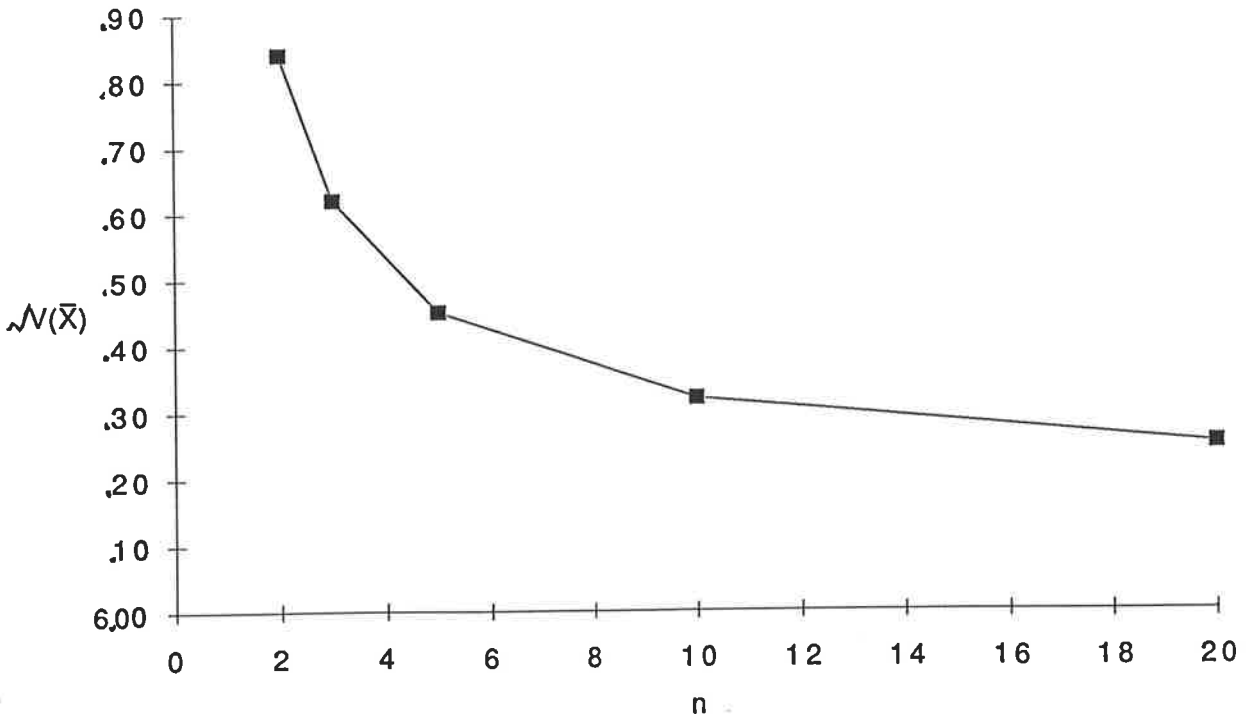


Table 19 & Figure 36. The relationship between the variance ($V(\bar{X})$) of the mean value of label density of laminin and the number (n) of selected electronmicrographs /per animal when 5 animals/per group were used.

Table 19

n	$V(\bar{X})$	$\sqrt{V(\bar{X})}$
2	93.52	9.67
3	87.79	9.37
5	83.21	9.12
10	79.77	8.93
20	78.05	8.83
25	77.71	8.82

Figure 36

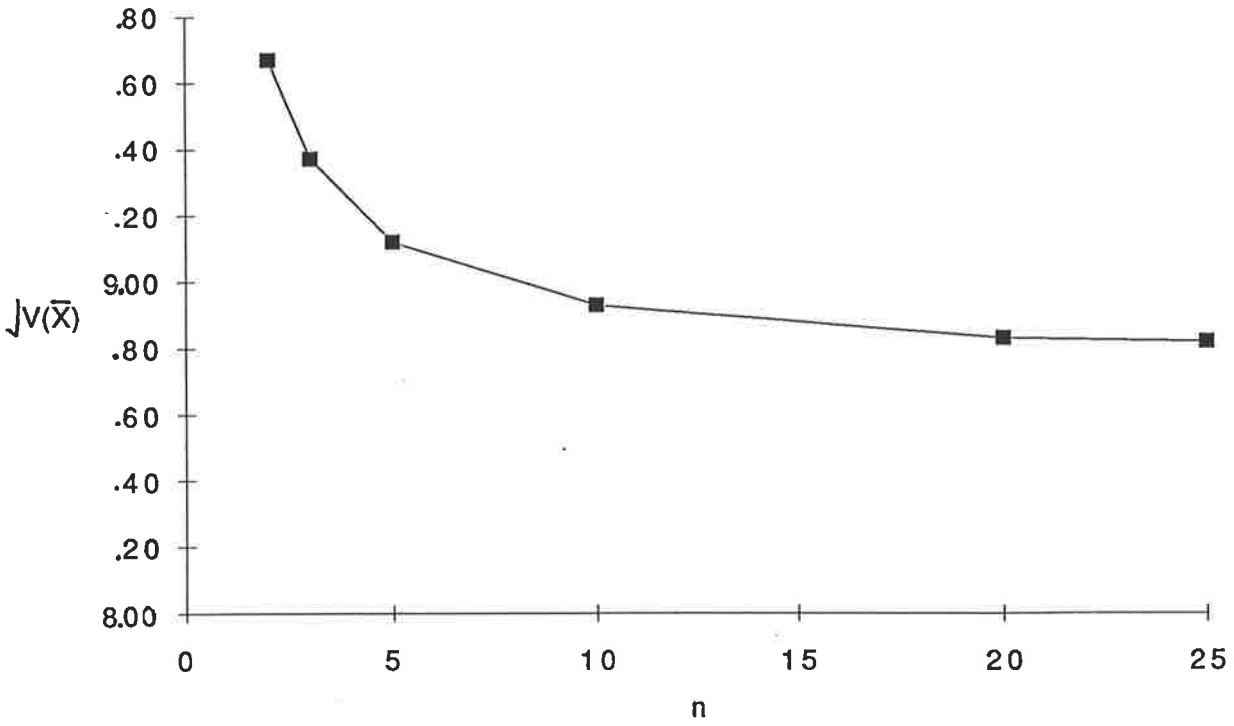


Table 20 & Figure 37. The relationship between the variance ($V(\bar{X})$) of the mean value of label density of type IV collagen and the number (n) of selected electronmicrographs/per animal when 10 animals/per group were used.

Table 20

n	$V(\bar{X})$	$\sqrt{V(\bar{X})}$
2	12.69	3.56
3	10.38	3.22
5	8.53	2.92
10	7.14	2.67
20	6.45	2.54

Figure 37

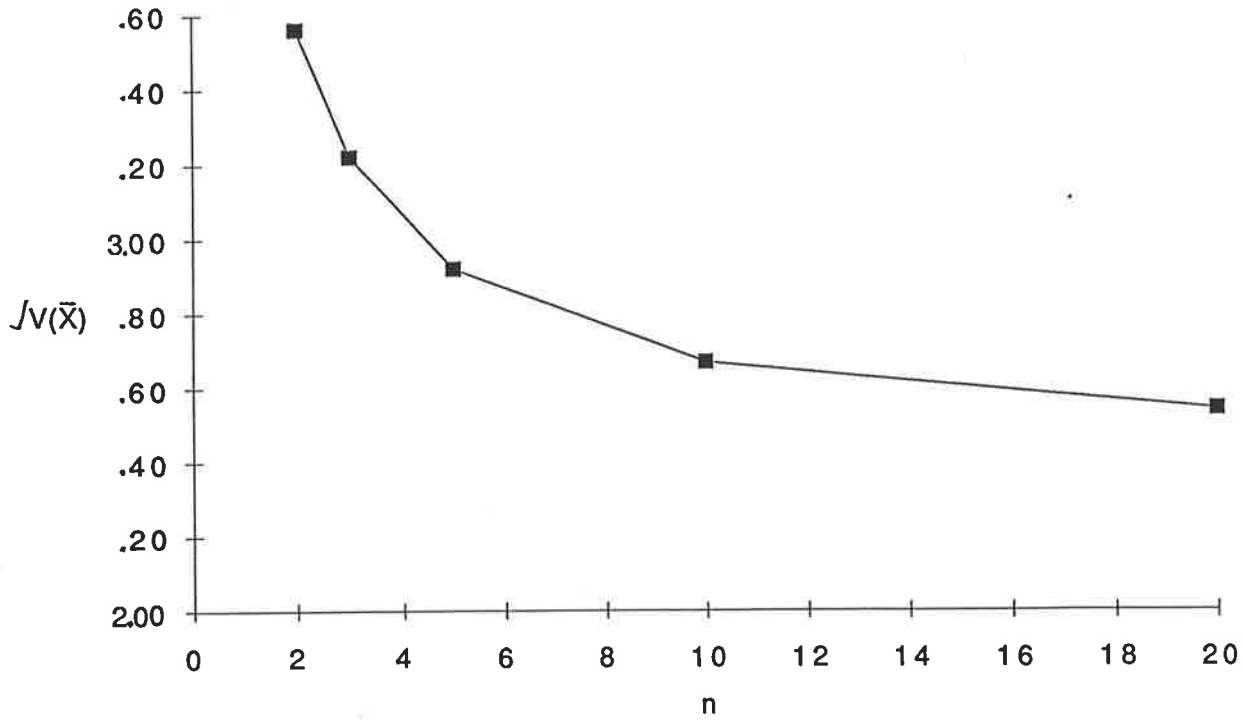
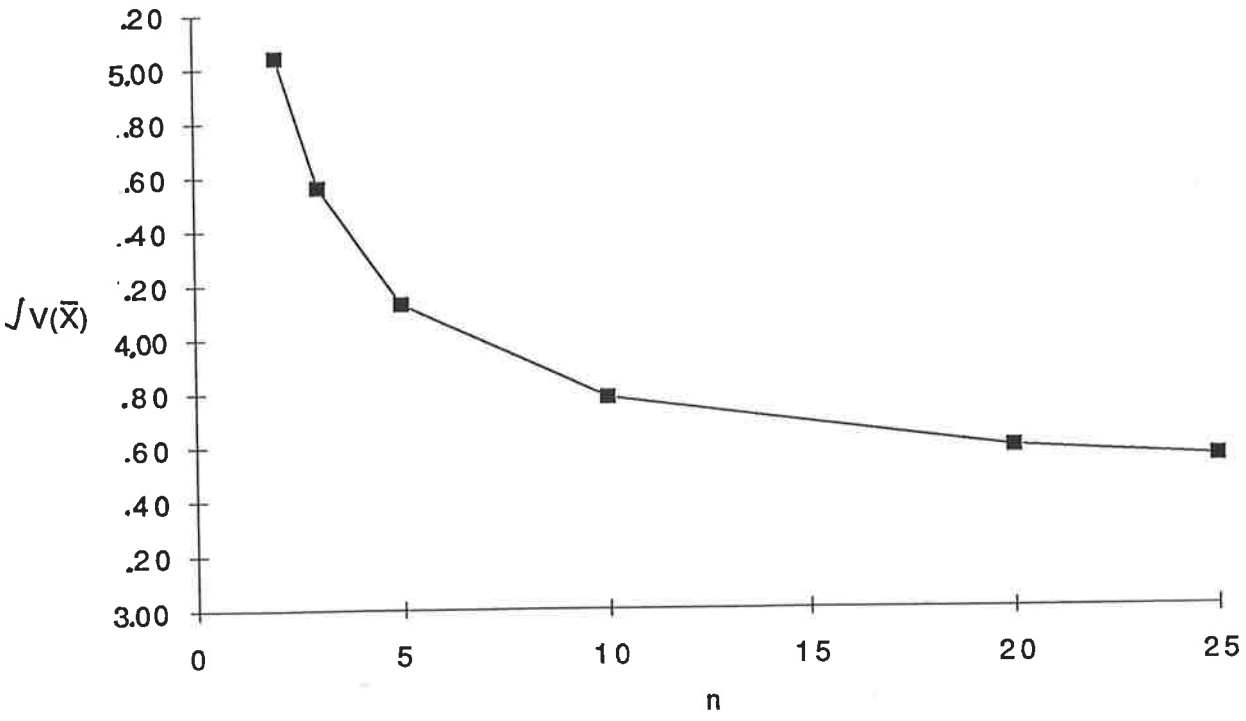


Table 21 & Figure 38. The relationship between the variance ($V(\bar{X})$) of the mean value of label density of type IV collagen and the number (n) of selected electronmicrographs/per animal when 5 animals/per group were used.

Table 21

n	$V(\bar{X})$	$\sqrt{V(\bar{X})}$
2	25.38	5.04
3	20.76	4.56
5	17.06	4.13
10	14.29	3.78
20	12.90	3.59
25	12.63	3.55

Figure 38



5.9. Quantitative analysis of the expression of basal lamina laminin and type IV collagen in normal rat tongue mucosa and induced oral carcinomas.

Methods: A total of 150 electronmicrograph (field) (5 fields per animal) were taken respectively for each antigen (laminin or type IV collagen) from 30 animals comprising the three groups using the sampling pattern described in Section 5.3 at an original magnification of 15,000 times. All electronmicrographs, standardized by the calibration grating and restricted to the central 18 X 24 cm areas, were printed on 20.3 X 25.4 cm (8 X 10 inch) photographic paper at a final magnification of 45,000 times. The label density of each antigen (the number of gold particles/per unit area) in lamina densa or in lamina lucida for each electronmicrograph was obtained by estimating the surface area of basal lamina using the point-counting method with the 5mm lined spacing grid and by counting the number of gold particles within the corresponding basal lamina areas. In addition, the label density of each antigen (the number of gold particles/per unit length) in only the lamina densa for each electronmicrograph was obtained by measuring the length of lamina densa using a MOP image analysis system and by counting the number of gold particles within the corresponding basal lamina areas. All data were subjected to analysis of variance using the BMDP statistical software package (1991 release, UCLA ed W.J.Dixon).

Results: Six variables were obtained by quantitative measurement:

(1). The label density of lamina densa laminin expressed as the number of gold particles/μm^2.
(2). The label density of lamina densa laminin expressed as the number of gold particles/μm.
(3). The label density of lamina lucida laminin expressed as the number of gold particles/μm^2.
(4). The label density of lamina densa type IV collagen expressed as the number of gold particles/μm^2.
(5). The label density of lamina densa type IV collagen expressed as the number of gold particles/μm.
(6). The label density of lamina lucida type IV collagen expressed as the number of gold particles/μm^2.

The distribution of each variable (the label density of antigen) is shown in Figs.39-44. Statistical analysis of quantitative data indicated that the label density of lamina densa laminin (the number of gold particles/μm^2 or the number of gold particles/μm) in the experimental oral carcinoma sample was significantly higher than that in the untreated group ($P<0.01$) (Table 22). There was no significant difference in the label density of lamina densa laminin (the number of gold particles/μm^2 or the number of gold particles/μm) between the untreated group and the propylene glycol-treated group ($P>0.5$) (Table 23) (Figs.45,46). Moreover, no significant difference in lamina lucida laminin (the number of gold particles/μm^2) among the three groups was apparent ($P>0.5$)(Table 26) (Fig. 49).

In contrast, the label density of lamina densa type IV collagen (the number of gold particles/μm^2 or the number of gold

particles/μm) in the experimental oral carcinoma sample was significantly lower than that in the untreated group ($P<0.01$) (Table 24). However, there was no significant difference in the label density of lamina densa type IV collagen (the number of gold particles/μm^2 or the number of gold particles/μm) between the untreated group and the propylene glycol-treated group ($P>0.5$) (Table 25) (Figs.47,48). There was also no significant difference in lamina lucida type IV collagen (the number of gold particles/μm^2) among the three groups ($P>0.5$) (Table 26) (Fig.50). It was interesting to note that there was a " consistent " factor of 2 in the mean value of label density of each antigen (laminin or type IV collagen), when a comparison was made between the untreated group and the experimental oral carcinoma.

Conclusions: In the experimentally induced oral carcinomas, lamina densa laminin appears to be significantly increased in tumour basal lamina. In contrast, lamina densa type IV collagen appears to be significantly decreased in tumour basal lamina. It appears that there is no significant difference in lamina lucida laminin and type IV collagen when normal tissues and carcinomas are compared.

Figure 39. Histogram showing the distribution of label density of lamina densa laminin (the number of gold particles/ μm^2) in the three groups.

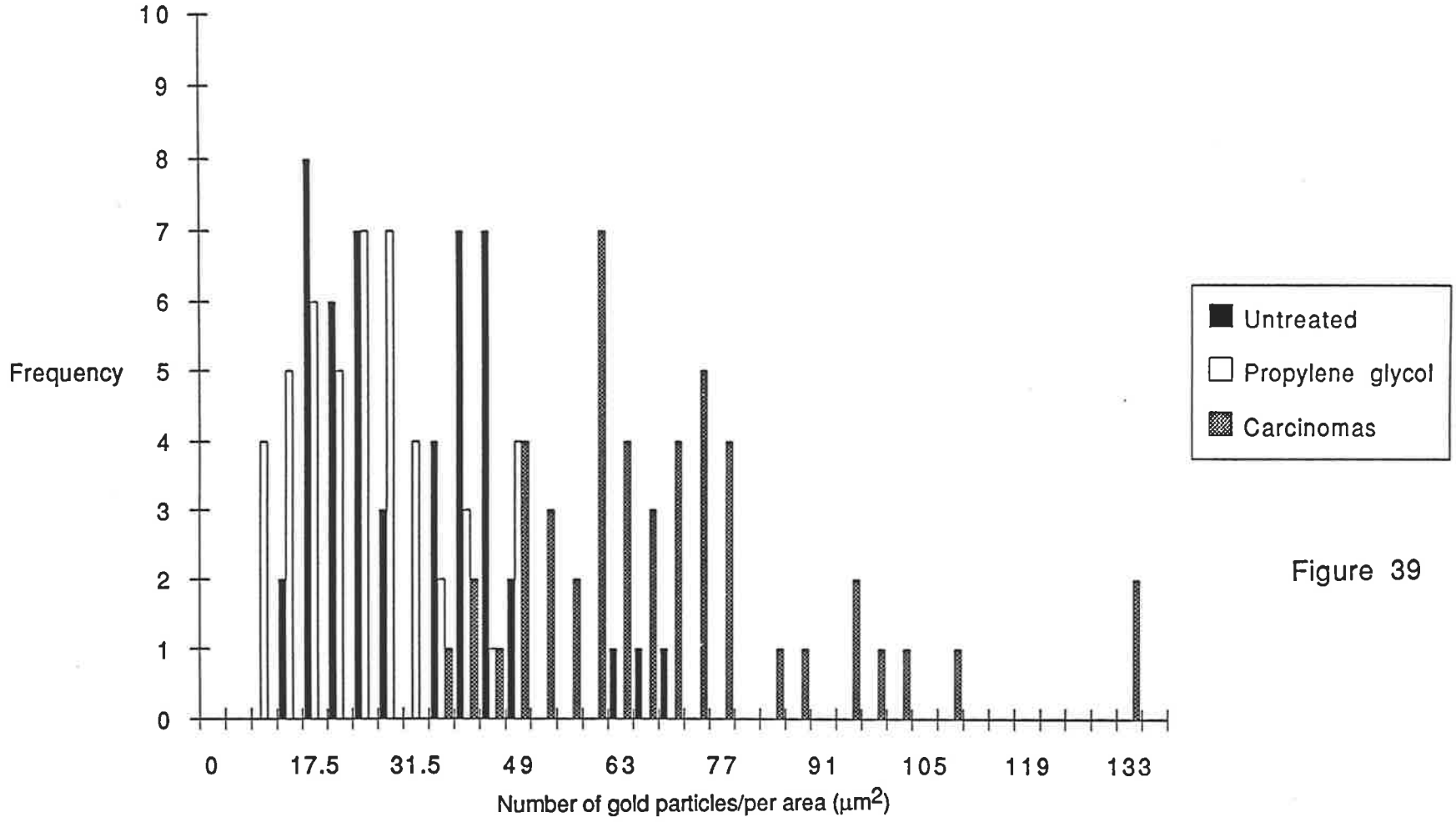

Frequency
10
9
8
7
6
5
4
3
2
1
0
0
17.5
31.5
49
63
77
91
105
119
133
Number of gold particles/per area (μm2)
Untreated
Propylene glycol
Carcinomas

Figure 39

Figure 40. Histogram showing the distribution of label density of lamina densa laminin (the number of gold particles/μm) in the three groups.

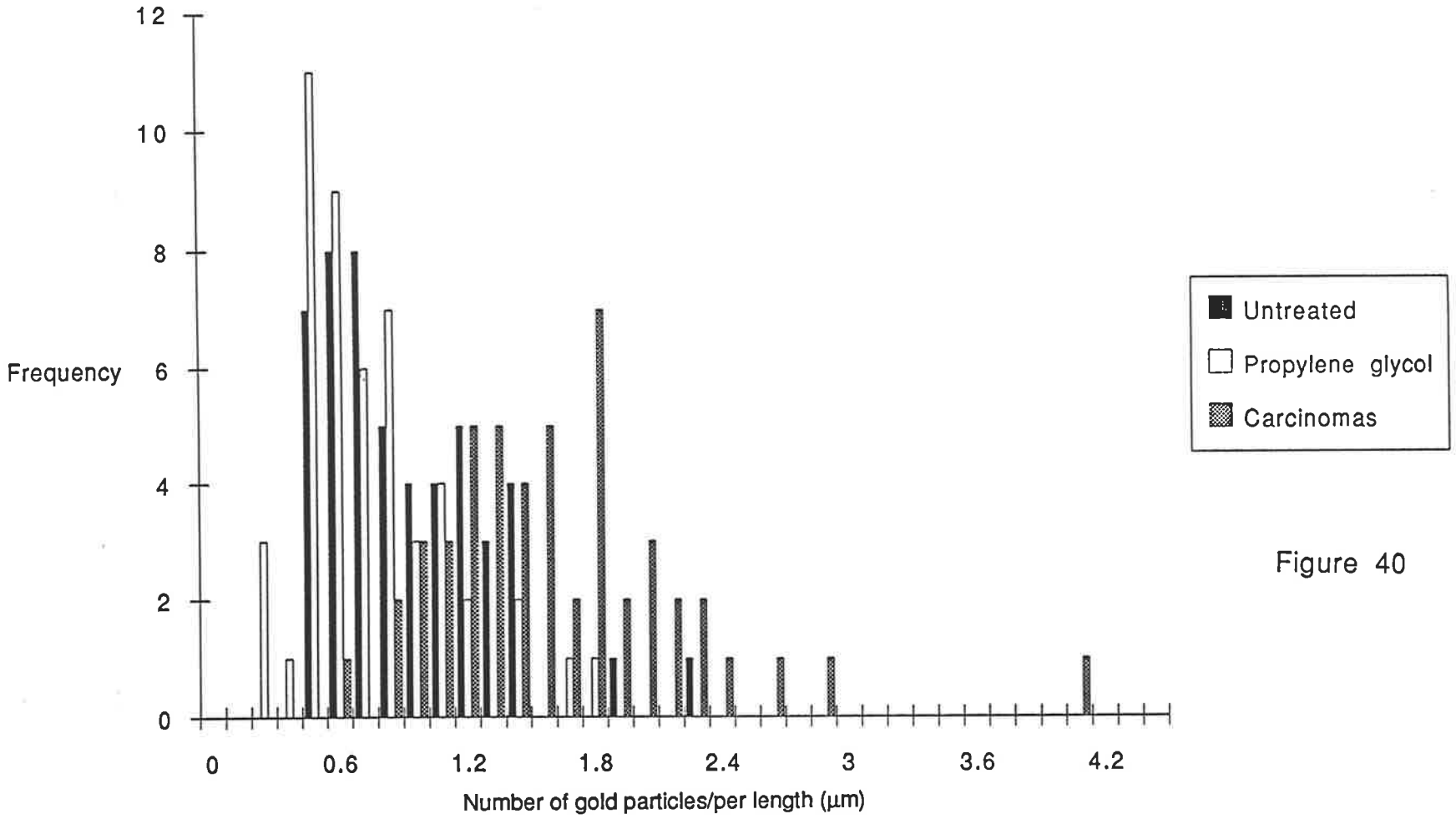

12
10
8
6
4
2
0
Frequency
0
0.6
1.2
1.8
2.4
3
3.6
4.2
Number of gold particles/per length (µm)
Untreated
Propylene glycol
Carcinomas

Figure 40

Figure 41. Histogram showing the distribution of label density of lamina lucida laminin (the number of gold particles/ μm^2) in the three groups.

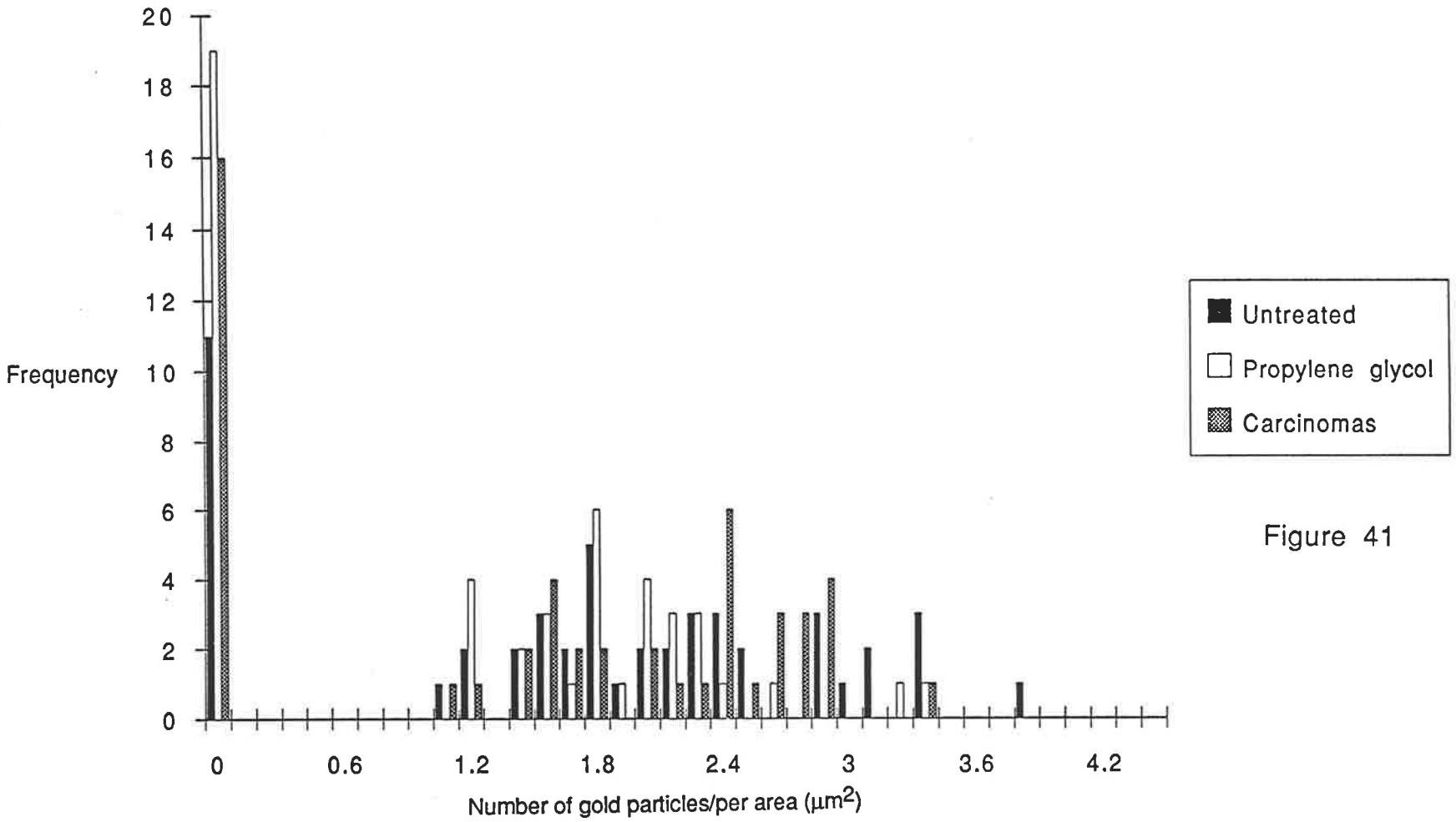

20
18
16
14
12
10
8
6
4
2
0
Frequency
0
0.6
1.2
1.8
2.4
3
3.6
4.2
Number of gold particles/per area (µm2)
Untreated
Propylene glycol
Carcinomas

Figure 41

Figure 42. Histogram showing the distribution of label density of lamina densa type IV collagen (the number of gold particles/μm^2) in the three groups.

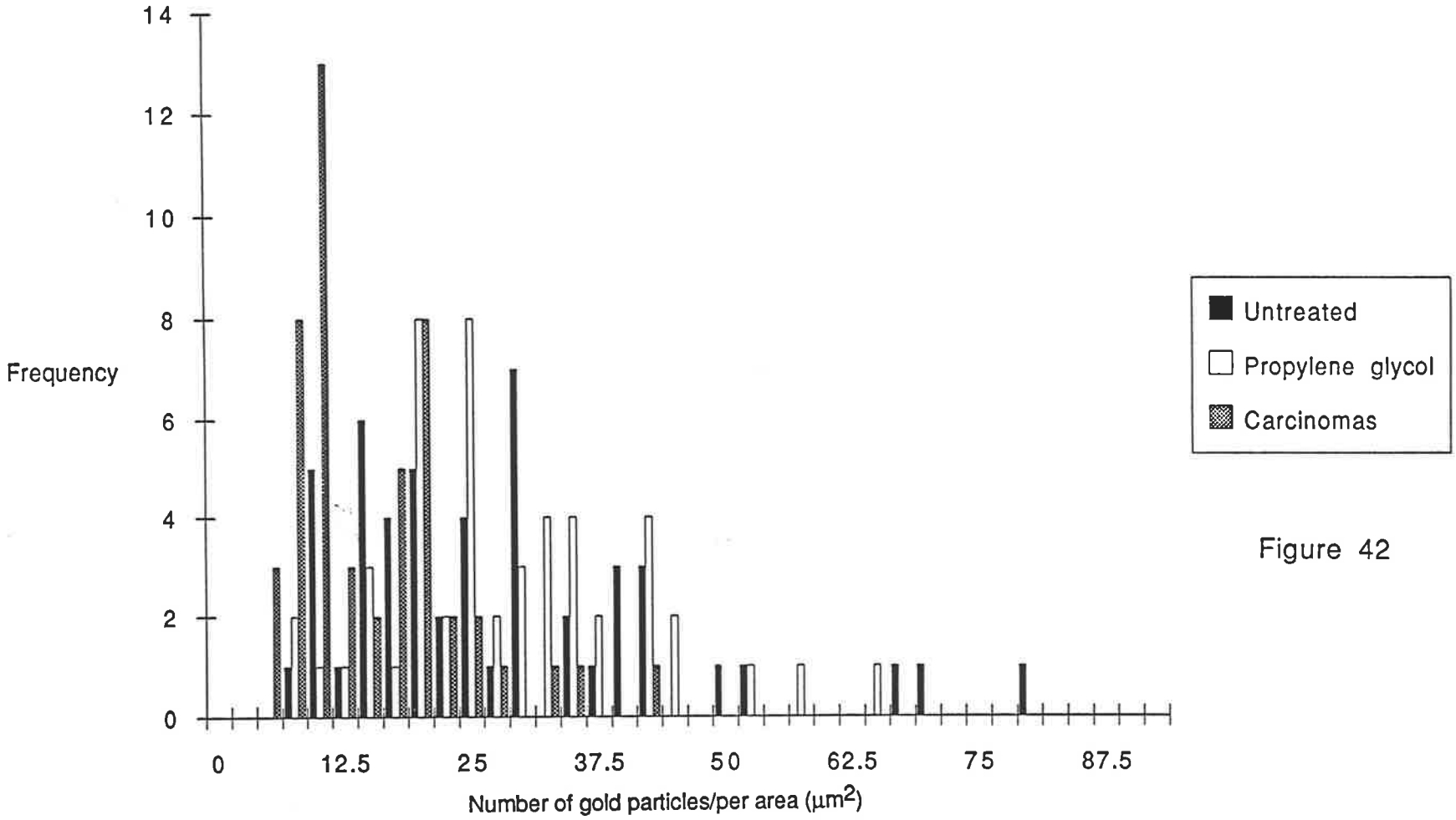

14
12
10
8
6
4
2
0
Frequency
0
12.5
25
37.5
50
62.5
75
87.5
Number of gold particles/per area (µm2)
Untreated
Propylene glycol
Carcinomas

Figure 42

Figure 43. Histogram showing the distribution of label density of lamina densa type IV collagen (the number of gold particles/μm) in the three groups.

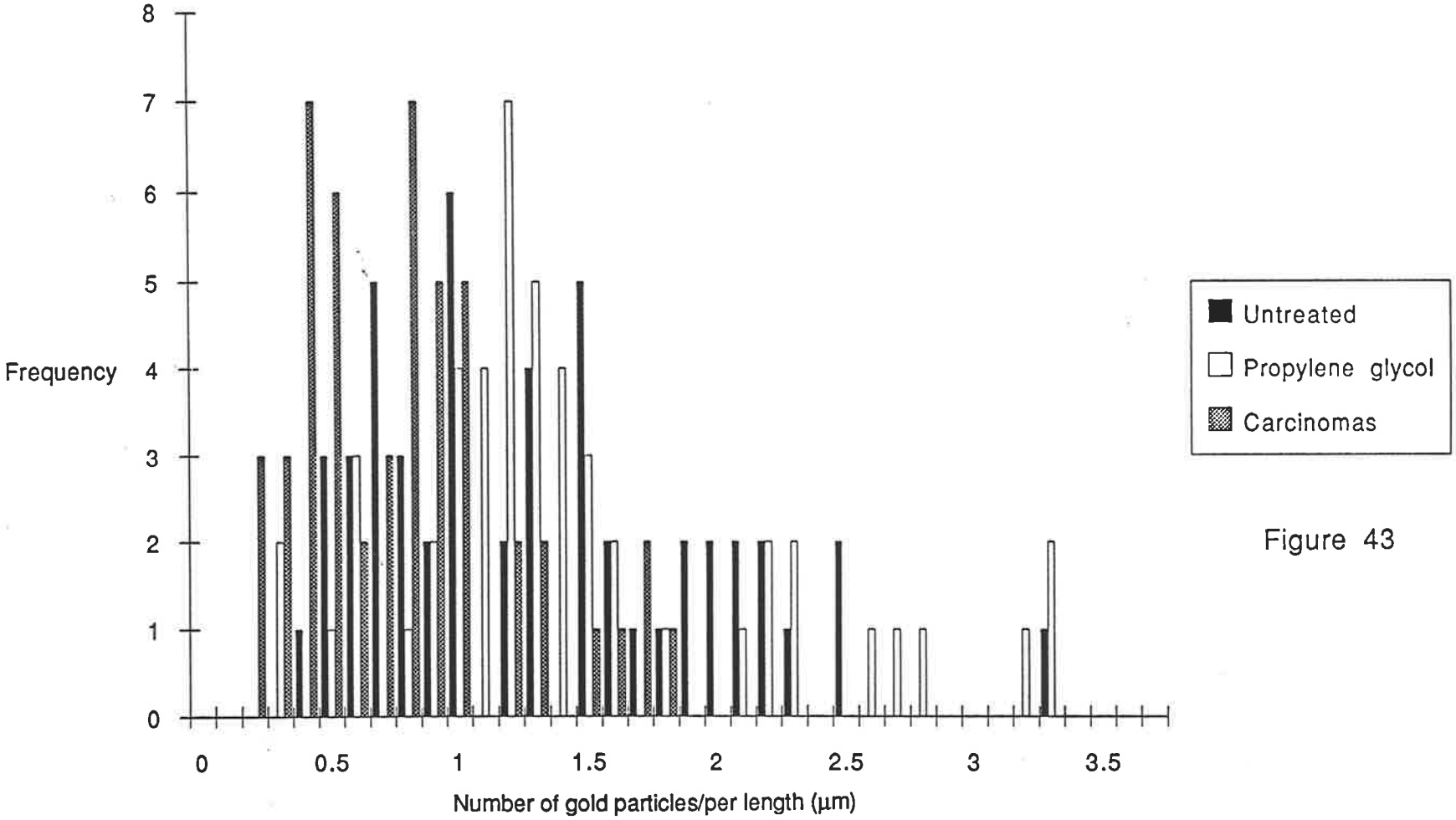

Frequency
8
7
6
5
4
3
2
1
0
0
0.5
1
1.5
2
2.5
3
3.5
Number of gold particles/per length (µm)
Untreated
Propylene glycol
Carcinomas

Figure 43

Figure 44. Histogram showing the distribution of label density of lamina lucida type IV collagen (the number of gold particles/μm^2) in the three groups.

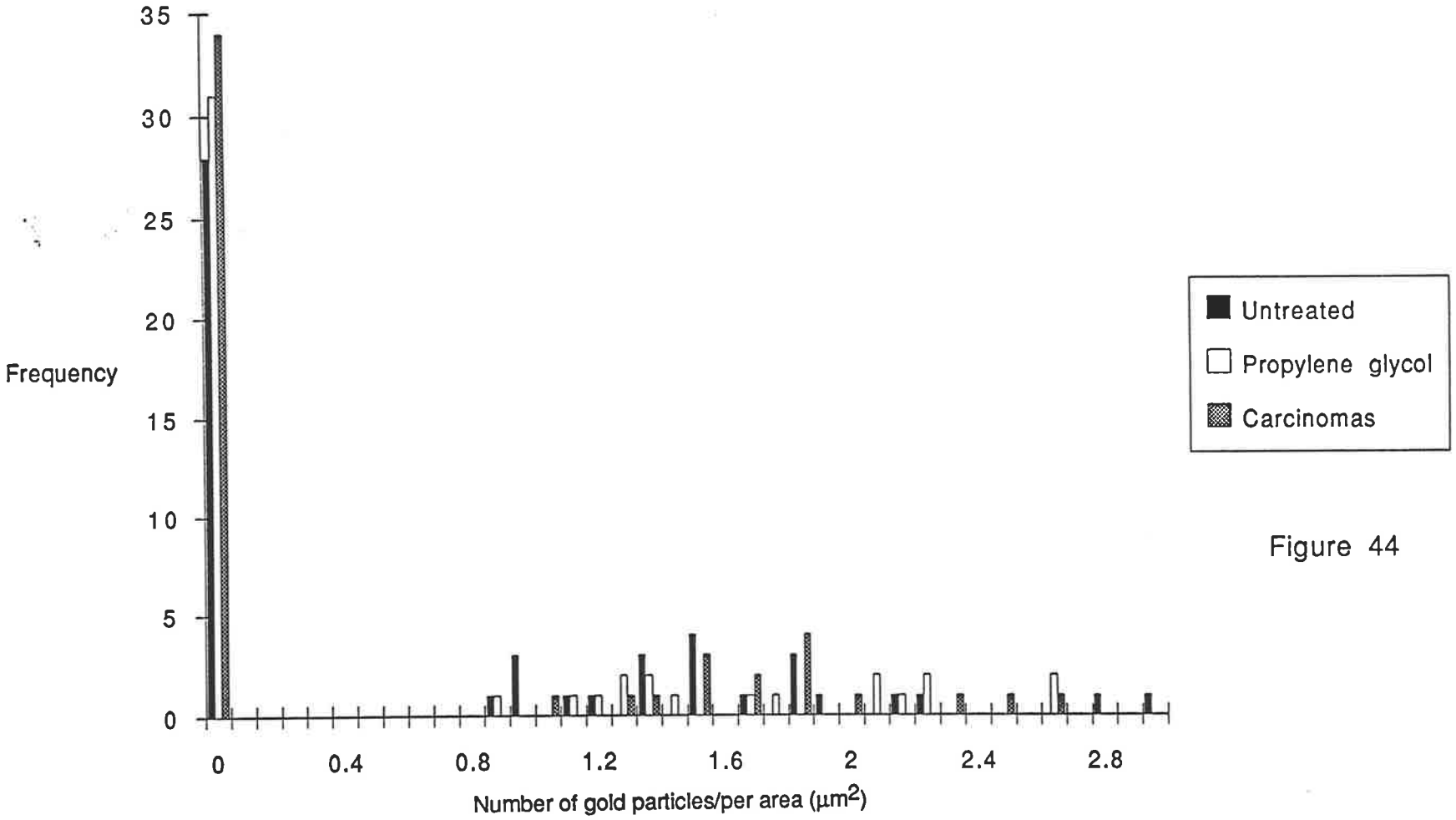

35
30
25
20
15
10
5
0
Frequency
0
0.4
0.8
1.2
1.6
2
2.4
2.8
Number of gold particles/per area (µm2)
Untreated
Propylene glycol
Carcinomas

Figure 44

Table 22. Label density of laminin on lamina densa of basal lamina in untreated normal rat tongue mucosa and experimental oral carcinomas expressed as the number of gold particles/μm^2 (1) and the number of gold particles/μm (2).

Table 23. Label density of laminin on lamina densa of basal lamina in untreated normal rat tongue mucosa and propylene glycol-treated rat tongue mucosa expressed as the number of gold particles/μm^2 (1) and the number of gold particles/μm (2).

Table 22

Variable	Group	
	Untreated	Carcinoma
	Mean±SD	Mean±SD
Laminin (1)	30.25±13.33	66.66±21.01*
Laminin (2)	0.86±0.39	1.56±0.59*

* P<0.01 there are significant differences between groups

Table 23

Variable	Group	
	Untreated	Propylene glycol
	Mean±SD	Mean±SD
Laminin (1)	30.25±13.33	24.647±11.08*
Laminin (2)	0.86±0.39	0.70±0.34*

*P>0.5 no significant difference between groups

Figure 45. Histogram showing the mean value and standard deviation of the label density (the number of gold particles/μm^2) of lamina densa laminin in the three groups.

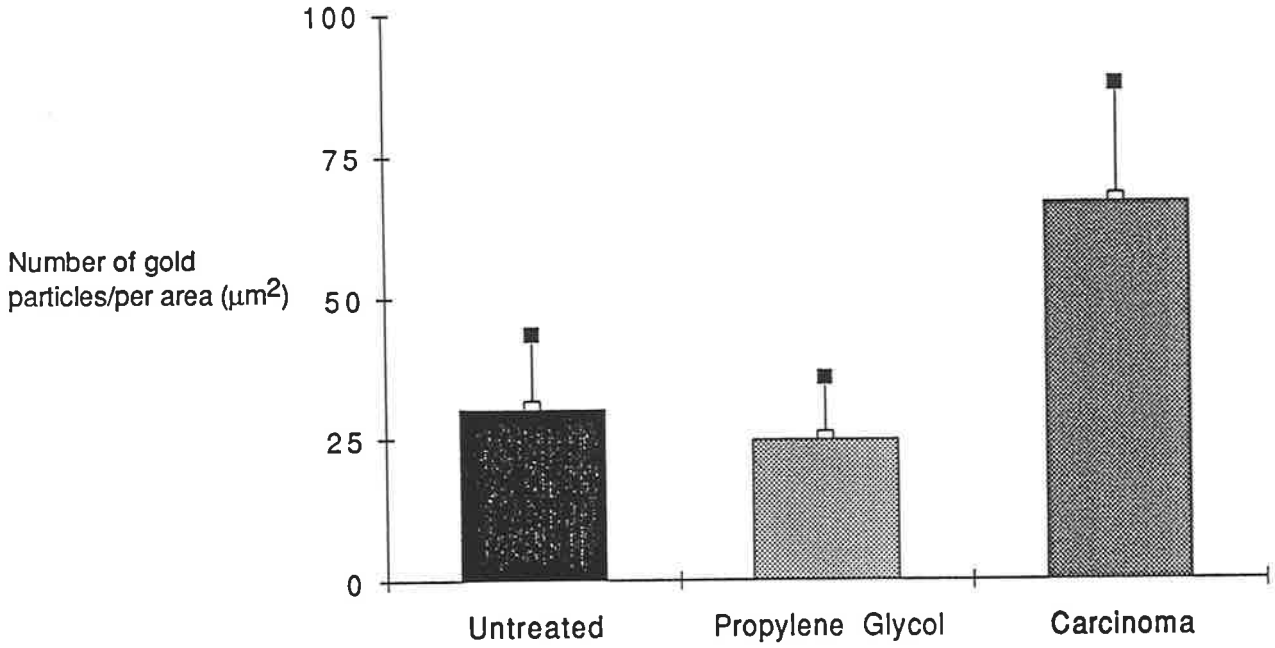

100
75
50
25
0
Number of gold particles/per area (μm2)
Untreated
Propylene Glycol
Carcinoma

Figure 45

Figure 46. Histogram showing the mean value and standard deviation of label density (the number of gold particles/μm) of lamina densa laminin in the three groups.

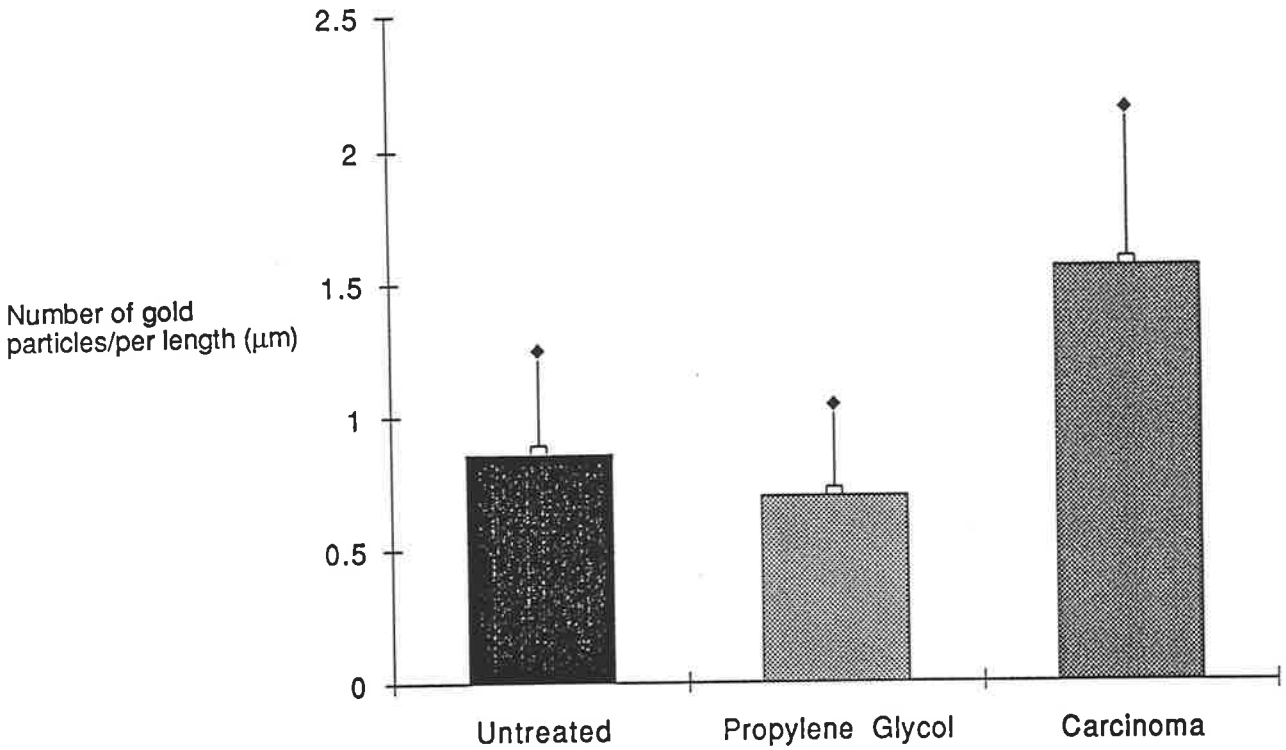

Number of gold particles/per length (μm)
2.5
2
1.5
1
0.5
0
Untreated
Propylene Glycol
Carcinoma

Figure 46

Table 24. Label density of type IV collagen on lamina densa of basal lamina in untreated normal rat tongue mucosa and experimental oral carcinomas expressed as the number of gold particles/μm^2 (1) and the number of gold particles/μm (2).

Table 25. Label density of type IV collagen on lamina densa of basal lamina in untreated normal rat tongue mucosa and propylene glycol-treated rat tongue mucosa expressed as the number of gold particles/μm^2 (1) and the number of gold particles/μm (2).

Table 24

Variable	Group	
	Untreated	Carcinoma
	Mean±SD	Mean±SD
Type IV collagen (1)	26.81±15.93	14.01±8.28*
Type IV collagen (2)	1.28±0.65	0.73±0.41*

*P<0.01 there are significant differences between groups

Table 25

Variable	Group	
	Untreated	Propylene glycol
	Mean±SD	Mean±SD
Type IV collagen (1)	26.81±15.93	27.60±12.31*
Type IV collagen (2)	1.28±0.65	1.42±0.73*

*P>0.5 no significant difference between groups

Figure 47. Histogram showing the mean value and standard deviation of the label density (the number of gold particles/μm^2) of lamina densa type IV collagen in the three groups.

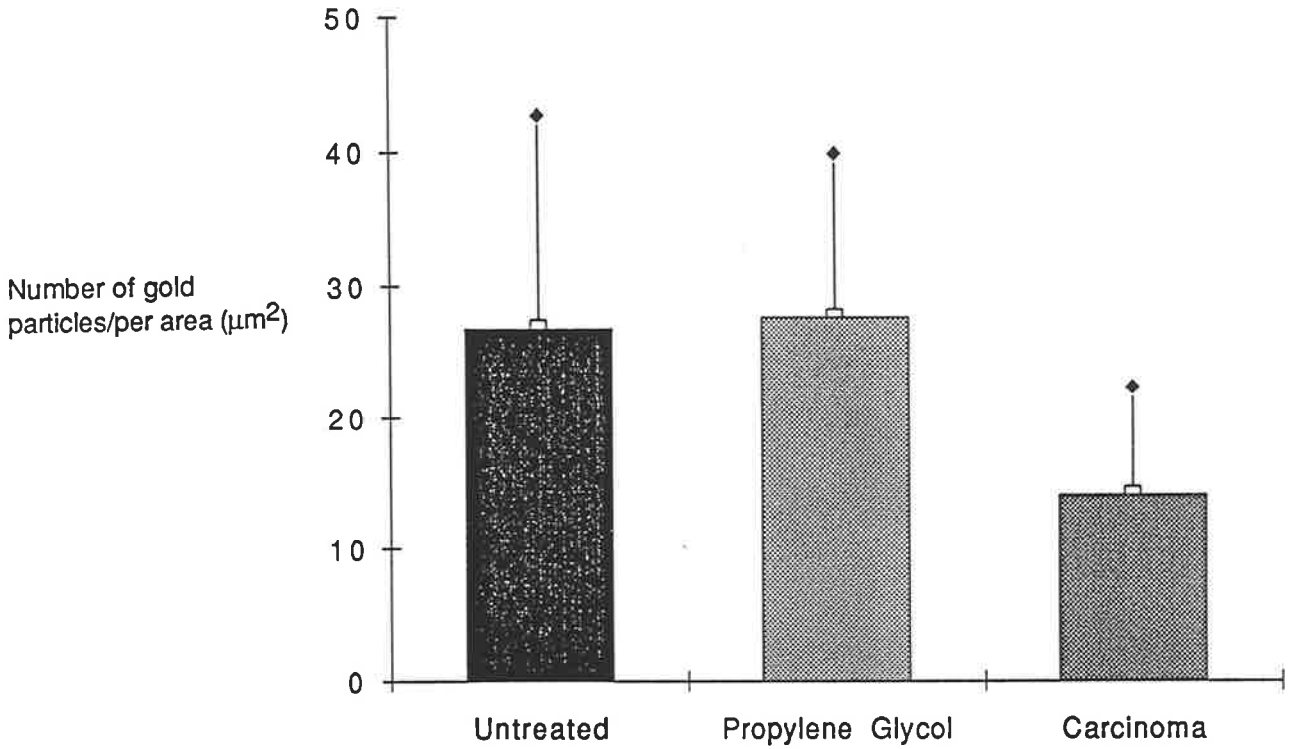

Number of gold particles/per area (μm2)
50
40
30
20
10
0
Untreated
Propylene Glycol
Carcinoma

Figure 47

Figure 48. Histogram showing the mean value and standard deviation of the label density (the number of gold particles/μm) of lamina densa type IV collagen in the three groups.

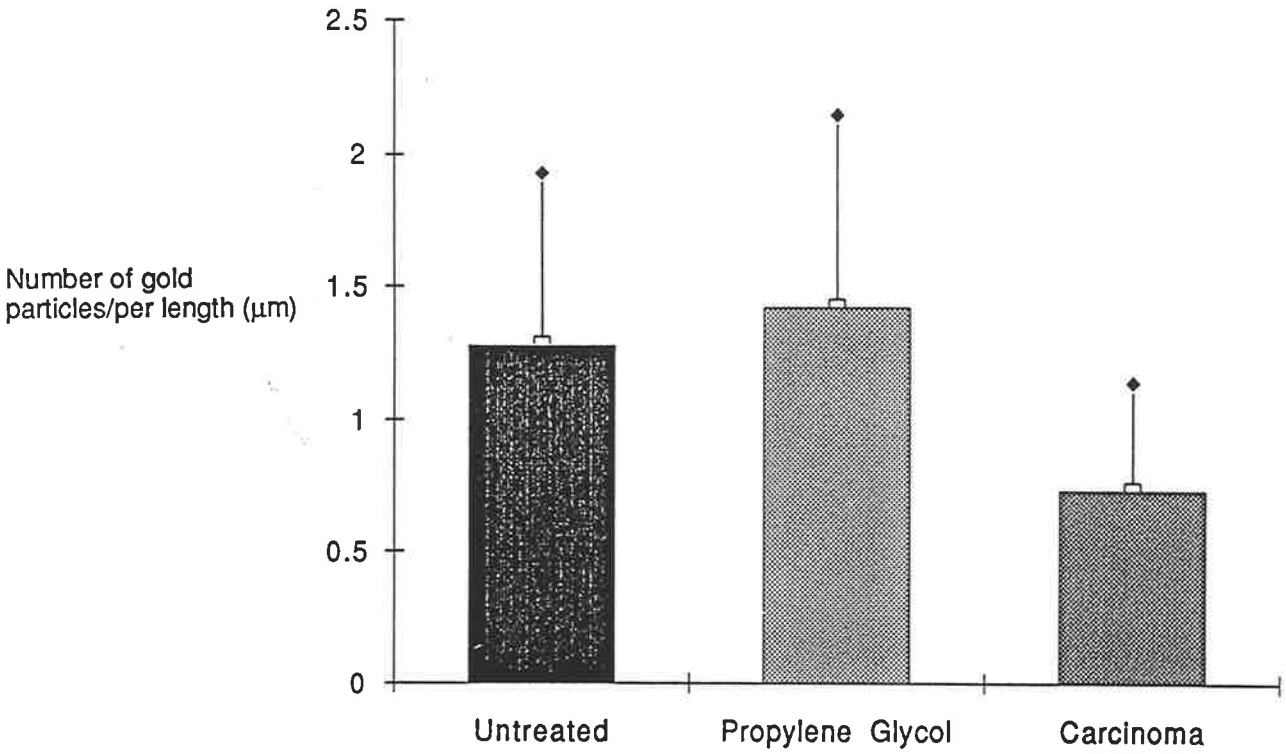

Number of gold particles/per length (µm)
2.5
2
1.5
1
0.5
0
Untreated
Propylene Glycol
Carcinoma

Figure 48

Table 26. Label density of laminin and type IV collagen on lamina lucida of basal lamina in the three groups expressed as the number of gold particles/μm^2.

Table 26

Variable	Group		
	Untreated	Propylene glycol	Carcinoma
	Mean±SD	Mean±SD	Mean±SD
laminin	1.70±1.09	1.17±1.00	1.46±1.11*
Type IV collagen	0.69±0.87	0.63±0.87	0.56±0.86*

*P>0.5 no significant difference between groups

Figure 49. Histogram showing the mean value and standard deviation of the label density of lamina lucida laminin in the three groups.

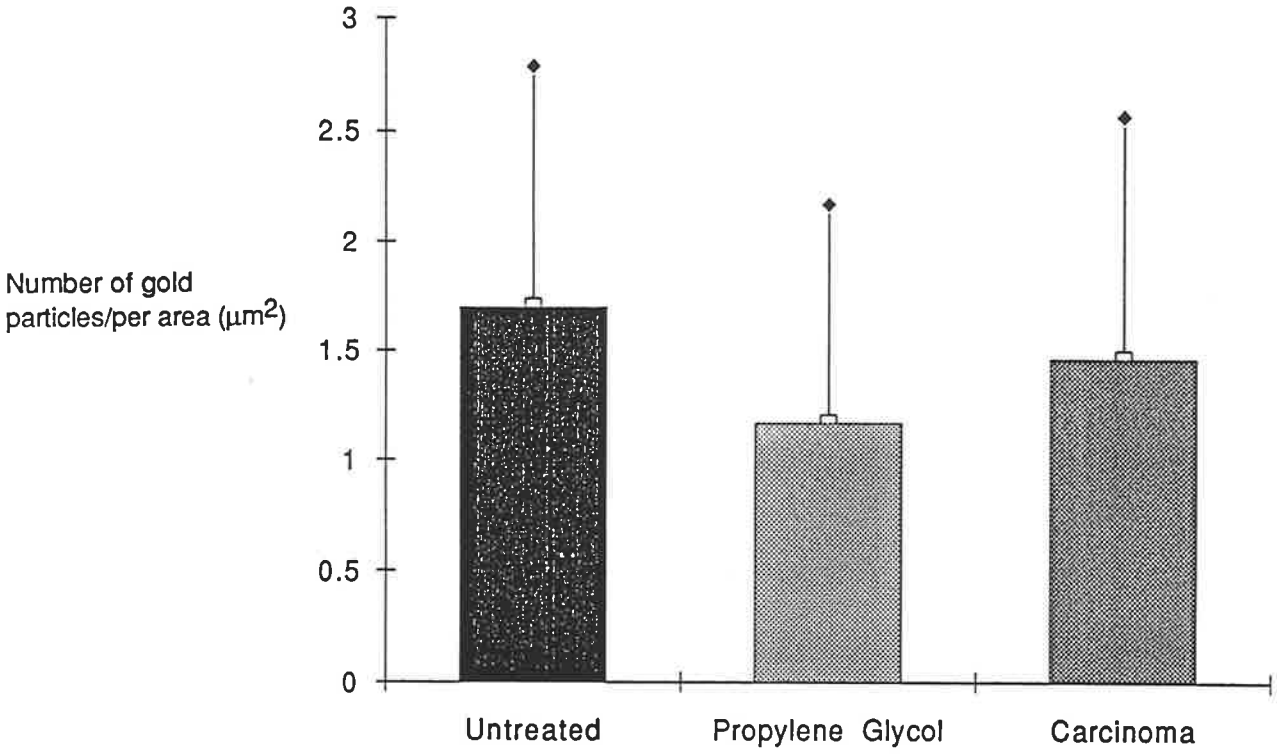

Number of gold
particles/per area (μm2)
3
2.5
2
1.5
1
0.5
0
Untreated
Propylene Glycol
Carcinoma

Figure 49

Figure 50. Histogram showing the mean value and standard deviation of the label density of lamina lucida type IV collagen in the three groups.

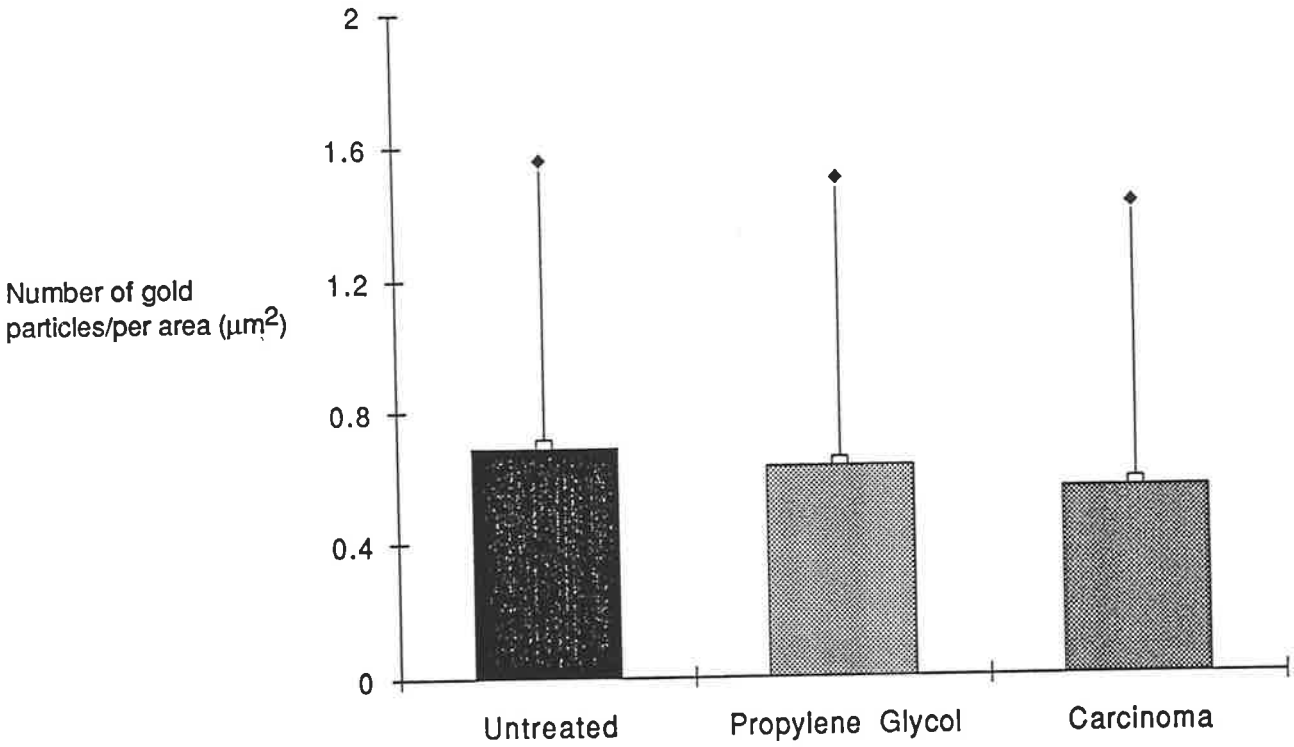

Number of gold particles/per area (μm2)
2
1.6
1.2
0.8
0.4
0
Untreated
Propylene Glycol
Carcinoma

Figure 50

5.10. Test of reproducibility of results in this study.

Methods: A total of 36 electronmicrograph (3 electronmicrographs per animal) for each antigen (laminin or type IV collagen) were randomly selected from 12 animals (4 per the three groups). The label density of each antigen in the lamina densa shown in each electronmicrograph used and marked for the original study was recalculated by remeasuring the surface areas of lamina densa using the point-counting method with the 5mm lined spacing grid and recounting without a knowledge of the original data the number of gold particles within the corresponding basal lamina areas. All new data were compared with original data.

Results: Essentially similar results were produced from both sets of calculations (See Appendix).

Conclusions: Methods for the quantitative evaluation of tissue antigen expression in the sections in this study are reproducible.

5.11. Discussion:

The Ultrastructural morphological changes (eg. thickened basal lamina) and optical microscopic immunostaining features (i.e. intensive staining) of basal lamina (Jiang, Wilson and Weibkin, 1993; Wilson et al, 1993) in 4NQO-induced oral carcinomas indicated that basal lamina changes might be related to qualitative and quantitative alterations in basal lamina type IV collagen and laminin. In the present study, the quantitative expression of basal lamina laminin and type IV collagen between rat normal tongue tissues and experimental oral carcinomas was investigated with the results obtained described in the previous Section (5.9).

A variety of methods including biochemical methods and morphometric methods have been used in the past to quantitate tissue antigens (Griffiths and Hoppeler, 1986). Using morphometric methods, it has been suggested that a "relative quantitation" of tissue components can be estimated from tissue sections (Bendayan, 1984). As described by Bendayan (1984), the differences in the label density of tissue antigen among cellular compartments or within subregions of a compartment might reflect relative differences in the concentration of antigen sites. Griffiths and Hoppeler (1986) also pointed out that only a postembedding immunostaining method with colloidal gold or a ferritin marker could give reliable results for the quantitative evaluation of tissue antigens on sections.

As described by Weibel (1969), accurate sampling is a critical for obtaining an unbiased estimate in quantitative studies. In the present study, sampling at all levels including the selection of tissue slices, tissue blocks, thin sections and basal lamina fields complied with the principles of random selection of samples described by Weibel (1979) and Gundersen et al (1988).

Although it has been reported that a variety of sampling patterns have been applied for tissues such as kidney and oral mucosa (Weibel, 1969; Gundersen and Østerby, 1981; Müller, 1980; Schroeder, 1981), it was found in pilot investigations that these sampling patterns were not suitable for the present study. For instance, Schroeder (1981) estimated the thickness of basal lamina in human oral epithelia using stereology. This investigator described that basal lamina regions at the base of epithelial ridges and above connective tissue papillae were randomly selected. However, in that study, the selected basal laminae were concentrated on only two regions of the whole basal lamina. In other words the sampling technique employed did not enable areas along the whole basal lamina to have the same chance of selection.

While observing basal lamina in tissue sections during the present investigation, it was found that basal lamina profile was characterized by marked irregularity due to epithelial cytoplamic projections. Moreover, neoplastic basal laminae, particularly neoplastic island basal laminae showed an irregular distribution. These features of basal lamina in normal tissues and neoplastic tissues caused difficulty in selection of representative basal

lamina fields from the sections using the sampling patterns described by previous investigators such as Gundersen and Østerby (1981) and Schroeder (1981).

Accordingly in order to obtain representative basal lamina fields in tissue sections, several sampling patterns had to be trialed in pilot studies. The method developed and described in Section 5.3 proved satisfactory as by using this sampling pattern, it enabled areas of basal laminae not only in normal tissues but also in neoplastic tissues including neoplastic islands to have the same opportunity for selection. Moreover, basal laminae in the three regions of normal tissue (i.e. epithelial ridges, above connective tissue papillae and between both regions) were able to be selected by adjusting an interval in the horizontal plane across the tissue sections.

In the preliminary study, a problem encountered in the application of the sampling pattern was that the sampling pattern did not properly incorporate with basal laminae on the tissue sections mounted on conventional 150 or 200 mesh grids because many basal lamina regions were often obstructed by the grid bars. To solve this problem, the conventional mesh grids were replaced with parallel lined grids. Although parallel lined grids provided a greater viewing area, it was found that parallel line structure was lacking of tissue section support. To enhance this grid support, formvar film was used to coat the grids. As a consequence, the sections mounted on these film-coated parallel lined grids provided sufficient basal lamina areas for selection.

As described by Gundersen and co-workers (1988), an isotropic feature of tissue is often required for most morphometric (sterological) studies. However, rat tongue mucosa tissues were anisotropic in the present study. Nevertheless a solution for anisotropic tissues in stereological studies was suggested by Gundersen and colleagues (1988) namely "vertical sections". These are as a plane of section perpendicular to a " given horizontal " (or reference) plane. In the present study, the epithelial surface was selected as a reference plane for obtaining the vertical sections. To ensure all sections were to cut perpendicular to this reference plane, thick sections were first cut and stained for histological examination and assessment of orientation. Blocks were then reorientated as necessary by reembedding.

In the application of morphometric methods to electron microscopic studies, it has been emphasized that several other variables should be considered in the methodology namely the choices of magnification, the size of measurement field and the appropriate measurement methods (Weibel, 1979; Haug, 1980). It is known that the magnification of electronmicrographs is inversely proportional to the size of the measurement field in that a high magnification will produce a distinct image of tissue structure, but a small measurement field. In practice, it is desirable to obtain a large measurement field on each electronmicrograph such that sample size can be reduced. However, a compromise is often reached between the choice of magnification and size of the measurement field in many studies (Haug, 1980). In the present study, electronmicrographs at the different magnifications were taken and then all

electronmicrographs were compared with each other in two aspects; the distinction of basal lamina structure and the size of basal lamina areas after enlargement. The principles for determining an optimum magnification were that electronmicrographs should contain the maximum basal lamina areas with a satisfactory resolution of basal lamina structure.

Previous studies have used a variety of measurement methods for quantitative studies (Weibel, 1979; Gundersen et al, 1988). In measuring surface areas of cellular compartments, it is evident that a point-counting method was often used in previous studies (Bendayan, 1984; Smith, 1990). As described by Weibel (1979), the choice of an appropriate measurement grid is required for the point-counting method. In the present study, a square measurement grid was employed. Owing to the features of basal lamina in tissue sections namely that it was long, thin and convoluted, two small spaced lined grids were trialed, superimposed on electronmicrographs in different positions in the preliminary study. By using trial exercises, an appropriate measurement grid was designed for the present study .

In morphometric studies, it has been suggested that distortion (eg. shrinkage and diffraction) of electronmicrographs may occur during the photographing and printing of images (Weibel, 1969; Smith, 1990). In the present study, this variables was investigated. It was found that the shrinkage of electronmicrographs during printing procedures (i.e. development, fixation and drying) could be detected, but it was very minor and consistent. As described by Smith (1990), the different

distortions might appear on electronmicrographs. In the present study, different degrees of distortion were detected in different areas of electronmicrographs. From the quantitative point of view, it was thought that this phenomenon would affect the accuracy of the estimates. To reduce substantial measurement errors caused by the distortion, all measurement fields were thus restricted consistently to the central areas of electronmicrographs (an area of less distortion).

Smith (1990) described that the plane of section would affect the estimate of the label density of basal lamina antigens in estimating the label density of basal lamina antigens in kidney. In the present study, it was found that several "appearances" of basal lamina labelled with gold particles occurred in tissue sections. For instance, some basal lamina showed distinct lamina densa and lamina lucida with almost equal width; however, in some areas, only a wide lamina densa areas, or, two layers of basal lamina of unequal width were seen in tissue sections.

In view of the fact that the plane of section could affect appearances of basal laminae in tissue sections, it was evident that substantial measurement errors could occur if the measured basal lamina areas were in an oblique sectioned plane. For instance, gold particles would possibly appear in the lamina densa region on the photographic plane, if they were located on the edge of the lamina lucida in a oblique sectioned plane; conversely, gold particles would possibly appear in the lamina lucida region or connective tissue on the photographic plane, if they were located on the edge of the lamina densa. For this reason, the structural

criteria for selecting basal lamina areas on electronmicrographs were established (Section 5.7) before estimating the surface areas and length of basal lamina in the present study .

In a morphometric study, Aherne and Dunill (1982) described that several phenomena may take place which affect the image namely tissue shrinkage, section compression, the Holmes effect and optically lost cap. Aherne and Dunill (1982) pointed out that it was essential to be aware of the substantial problem of tissue shrinkage due to fixation and dehydration and to avoid it, or, to correct it in quantitative studies. Haug (1980) described that the main cause of tissue shrinkage was due to interference in the water balance of tissue. In the present study, attempts to minimize tissue shrinkage during tissue preparation were made by adjusting solution osmolarity to a " physiological range " Despite this, it can not be excluded that some degree of tissue shrinkage still occurred in tissue specimens. In the present study, a correction for tissue shrinkage was not made, because it was assumed that the degree of tissue shrinkage in each group of specimens would be essentially similar and as such this problem would not affect the final results. To avoid tissue compression during sectioning, a " new " diamond knife for sectioning and chloroform to stretch sections were used.

The so-called Holmes effect and optically lost cap phenomena are explained as relating to section thickness (Weibel, 1969; Aherne and Dunill, 1982). Haug (1980) described that the influence of section thickness on surface or length estimate was small and might be ignored. Moreover, it has also been pointed out that the

Holmes effect and optically lost cap often compensate each other (Weibel, 1969; Aherne and Dunill, 1982). Based on these facts, the Holmes effect and optically lost cap phenomenon were ignored in the present study.

Bendayan (1984) described that two morphometric methods could be used in immunogold quantitation studies namely the point-counting method and image analysis. In the present study, both the point-counting method and an image analysis system were employed. It was found that the point-counting method was more convenient and reproducible for estimating the surface areas of basal lamina on tissue sections; while the image analysis system was more practical for measuring the length of basal lamina on tissue sections. Therefore, the label density of basal lamina laminin and type IV collagen was represented as the number of gold particle per unit area and per unit length using the point-counting method and the image analysis system respectively in the present study.

In morphometric studies, it has been emphasized that the selection of an adequate sample size is very important (Weibel, 1979; Gundersen and Østerby, 1981). A variety of methods determining sample size have been described in previous studies (Snedecor and Cohan, 1967; Weibel, 1969; Abadía-Fenoll, Navascues and Rios, 1980; Gundersen and Østerby, 1981). Notwithstanding these, there is little information available describing the best sample size for quantitating oral mucosa basal lamina components. Schroeder (1981) described that 6-10 basal lamina fields were selected from tissue sections in his

selected blocks (2-4 blocks/per group of biopsies) from the different regions of normal oral mucosa and pathological lesions for estimating the thickness of basal lamina in human oral epithelium. However, in that study, the method of determining sample size was not described.

As for the determination of sample size, Gundersen and Østerby, (1981) described that an optimum sample size should meet the demand that maximal representative information can be obtained with minimum variation and cost from available materials. In the present study, it was thought that variances at several levels might exist; among animals, blocks, sections and fields. In consultation with statistical expertise (Dr. Leppard, Dept. of Statistics, Uni. of Adelaide), a statistical " variance of component " model (Snedecor and Cohan, 1967) was recommended for use to determine sample size in the present study. By these exercises, the variations of the label density of basal lamina antigens at the different levels were obtained in pilot samples from each animal group. By analysing variation and cost, an optimum sample size was determined for the present study.

Having taken into account these variables in the experimental design, the label density of these basal lamina type IV collagen and laminin in the lamina densa and lamina lucida was subsequently and quantitatively analysed. From statistical analysis of quantitative data obtained, it was demonstrated that a very small amount of laminin and type IV collagen was detectable in the lamina lucida. Moreover, there was no significant change of these antigens in lamina lucida when normal tissues and

carcinomas were compared. Conversely, both basal lamina antigens were identified in the lamina densa and there were significant differences in the label density when normal tissues and carcinomas were compared.

In the present study, it was noted that immunogold labelling for laminin was distributed not only in the basal lamina regions of normal tissues and neoplastic tissues but also in the stroma of carcinoma. It was obvious that laminin in the stroma of carcinomas was more prominent compared with laminin in normal stroma as evidenced by negative labelling in the latter. Therefore, the quantitative comparison of the label density of laminin was limited to basal lamina regions of normal tissues and carcinomas only.

Quantitative analysis of basal lamina laminin in normal tissues and experimental oral carcinomas showed increased expression of lamina densa laminin in the experimental oral carcinomas. This finding confirmed the subjective observation made while locating the antigens. A number of studies have reported that " over-expression " of laminin is seen in relation to a variety of neoplasms using optical immunohistochemistry (Ormerod et al, 1985; Kallioinen, 1985; Fisseler-Eckhoff et al, 1990 Wilson et al, 1993). Further *In vitro and In vivo* studies on laminin have shown roles for laminin in tumour invasion and metastasis. For instance, Liotta and colleagues (1982) investigated the effect of laminin on isolated tumour cells in tissue culture. It was reported that laminin enhanced the activity of tumour cell attachment and metastasis. Sweeney and colleagues (1991) intravenously

injected the isolated tumour cells with laminin or without laminin into experimental animals. It was reported that laminin increased tumour incidence and growth. The finding in this study of increased basal lamina laminin thus appears to accord with the findings of other investigators.

The mechanism for the increased expression of laminin in experimental oral carcinomas is not clear. Kopf-Maier and Merker (1991) observed the development of basement membrane in xenografted human carcinomas following transplantation to athymic mice. It was reported that tumour cells synthesized basement membrane components including laminin. Ormerod et al (1983) reported that epithelial cells isolated from a DMBA-induced rat mammary tumour produced basement membrane components including laminin and formed a basement membrane-like material on floating collagen gels. It was suggested that the production of basement membrane components was related to the proliferation and differentiation of the mammary epithelial cells. In an ultrastructural study on experimental oral carcinomas (Jiang, Wilson and Wiebkin, 1993), it was found that the basal lamina was characterized by focal thickness of lamina densa. Moreover, carcinoma cells showed evidence of synthetic activity as shown by an increased number of enlarged mitochondria and well-developed Golgi apparatus. Incorporating these observed ultrastructural features with results of quantitative immunostaining, it is possible that the increased expression of laminin in the basal lamina of experimental oral carcinomas might be due to a higher rate of synthesis by carcinoma cells and the deposition of laminin within original basal lamina.

In comparison with laminin expression, type IV collagen exhibited contrasting results. Conflicting reports on its staining features have been described in a variety of neoplasms at the optical microscopic level. From previous studies (Wilson et al, 1993), it was demonstrated that type IV collagen labelling in neoplastic tissues was more intensive compared with those in normal tissues at the optical microscopic level. In the present study, the quantitative evaluation of basal lamina type IV collagen showed significantly decreased levels in tumour basal lamina using immunogold electron microscopic techniques. This finding is inconsistent with previous observations on type IV collagen at the optical microscopic level (Wilson et al, 1993). The explanations for this difference may be several; firstly, because the resolution of the light microscope is too low to distinguish between these locations, the intensive staining of basement membrane type IV collagen at the optical microscopic level might represent labelling of type IV collagen in basal lamina as well as some labelling in the stroma close to the basal lamina; secondly, the apparently diffuse characteristics of immunoperoxidase immunostaining for type IV collagen at the optical microscopic level might overlay non-staining areas of basement membrane or non-labelled areas of basal lamina which are demonstrated clearly by electron microscopy.

Analysis of the quantitative data indicated that occasionally there was greater expression of basal lamina type IV collagen in some carcinomas compared with some normal tissue specimens. However, despite this, quantitative analysis indicated that the

mean value of type IV collagen in the experimental oral carcinomas was decreased compared with that in normal tissues.

It is known that type IV collagen is related to the maintenance of basal lamina integrity in a physiological situation (Kleinman et al, 1982). The changes of basal lamina type IV collagen in malignancies have been related to its destruction and /or low rate of production (Carter et al, 1985; Verhoeven et al, 1990). For instance, Verhoeven and colleagues (1990) investigated the expression of type IV collagen in breast carcinoma. It was suggested that the production of type IV collagen in mammary glands was related to hormones and epithelial proliferation. Some investigators have reported that a high activity of enzymes and enzyme gene expression (eg. type IV collagenases, cathepsin B) are detected in malignant neoplasms and are related to the degradation of type IV collagen (Barsky et al, 1983; Pauli et al, 1983; Buck et al, 1992; Kusukawa et al, 1992; Pyke et al, 1993). Considering the aforementioned facts, it is suggested that the changes observed in basal lamina type IV collagen in the experimental carcinomas may be related to the inherent properties of the tumour cells (eg. differentiation) and /or microenviromental factors (eg. other extracelluar matrix components, enzymes) and that the decreased expression of basal lamina type IV collagen in experimental oral carcinomas may be due to inbalance between type IV collagen synthesis and degradation.

In the context of type IV collagen expression in neoplasms, many previous studies have also correlated changes in type IV collagen

with tumour invasion (Hirota, Yoneda and Osaki, 1990; Pitt, Hale and Buckley, 1992; Yoneda et al, 1992). For instance, Pitt and co-workers (1992) investigated the expression of type IV collagen in cervical carcinoma using optical immunohistochemistry. These authors reported a significant correlation between a decrease in type IV collagen and carcinoma invasion and metastasis. Despite this, it can not be concluded that the decrease in type IV collagen expression in the experimental oral carcinomas investigated in the present study is related to tumour invasion and metastasis, because the experimental oral carcinomas are characterized by low metastatic propensity.

It was interesting to note that the ratio of type IV collagen decrease appeared to be approximately equal to that of increased laminin, when a comparison of the label density of laminin and type IV collagen in lamina densa was made between normal tissues and carcinomas in the present study. Whether or not this phenomenon is associated with some biological properties of basal lamina laminin and type IV collagen in neoplasms remains unknown. Martinez-Hernandez and Amenta (1983) studied the ratio of basal lamina components in various tissues. It was suggested that the ratio of basal lamina components was associated with cell growth and differentiation and that a slight change of basal lamina components might affect its properties and functions. Bissell, Hall and Parry (1982) described that there was an interaction among extracellular matrix components and that the change of one component may selectively affect the properties of some extracellular matrix components. For instance, it was described that laminin in malignancies could stimulate the

release of type IV collagenases which would degrade type IV collagen (Campbell and Terranova, 1988; Sweeney et al, 1991).

The demonstration of quantitative alterations does not rule out possibility that the observed differences might also be related to qualitative changes (i.e. molecular structural changes in these antigens). For instance, Leu and Damjanov (1988) investigated the susceptibility of basement membrane laminin and type IV collagen to proteolytic digestion in normal tissues (i.e. skin, nerves, kidney, breast, lung) and neoplastic tissues (i.e. kidney adenocarcinomas, breast adenocarcinomas, gastric carcinomas). It was demonstrated that laminin could be removed more easily from all tissues in contrast to type IV collagen and also much more easily from malignant tumours than benign tumours or normal tissues. These phenomena were thought likely to be related to quantitative and qualitative differences in basement membrane compositions between normal tissues and neoplastic tissues.

CHAPTER SIX: SUMMARY AND CONCLUSIONS

In the present study, the effect of tissue preparation and immunostaining variables on the preservation of oral mucosal tissue morphology and the antigen retention of basal lamina laminin and type IV collagen was investigated. Experiments aimed at localizing and quantitating these antigens in the basal lamina of normal rat tongue mucosa and induced tongue carcinomas were carried out.

1. Effect of fixation, processing and immunostaining variables (Chapter three).

It was found that the ultrastructural identification of basal lamina laminin and type IV collagen depended on a number of variables related to tissue preparation (tissue fixation, dehydration and embedding conditions) and immunolabelling procedures (antibodies and gold-complex types). In general the observations deriving from these experiments confirmed findings described by previous investigators (eg. Bullock, 1984; Roth, 1986; Stirling, 1990). However, while previous investigators have shown that formaldehyde or formaldehyde-glutaraldehyde mixture fixatives are popular in immunocytochemistry (Tokuyasu, 1984), these fixatives did not appear to be suitable for the purposes of the present study. In contrast while it is generally thought that glutaraldehyde is not an optimum fixative for the ultrastructural localization of tissue antigens due to damage to tissue antigens (Hayat, 1989), tissues fixed in mild glutaraldehyde fixative exhibited an acceptable level of immunolabelling in the present study. Furthermore, by regulating glutaraldehyde fixative conditions (eg. the adjustment of fixation osmolarity, the addition

of picric acid and PVP), marked improvement in tissue morphology and immunolabelling were seen. These findings indicated that picric acid and PVP additives affected the morphological preservation of oral mucosal epithelium and the immunolabelling of basal lamina laminin and type IV collagen and that use of additives to a mild concentration of glutaraldehyde fixative could improve antigen retention in basal lamina, and at same time, compensate for loss of morphological preservation due to the reduction of glutaraldehyde concentration.

In the use of L.R.White resin for embedding, two different dehydration procedures have been reported in a number of investigations (Bendayan, Nanci and Kan, 1987; Newman and Hobot, 1987; Lin and Essner, 1990; Herken and Miosge, 1991). Partial dehydration (up to 70% ethanol) has been strongly recommended for L.R.White resin (Newman and Hobot, 1987). However, no obvious differences in immunolabelling of basal lamina laminin and type IV collagen were observed when complete dehydration (up to 100% ethanol) and partial dehydration were compared in the present study.

Two different polymerization conditions for L.R.White resin have been used by a number of investigators (Newman et al, 1983; White et al, 1988; Fanning et al, 1991). Newman and Hobot (1987) reported that the two different polymerization conditions did not significantly affect immunolabelling when immunogold techniques were used. However, the results of the present study indicated that the temperature for the polymerization of L.R.White resin

affected immunolabelling of basal lamina laminin and type IV collagen.

A number of authors have emphasized that immunostaining procedures are critical for the demonstration of tissue antigens (Roth, Taatjes and Warhol, 1989; Stirling, 1990). The present study indicated that inappropriate antibodies could result in a low level of immunolabelling and/or a high level of background staining and that the use of gold complex types also affected immunolabelling of basal lamina laminin and type IV collagen.

To assess the specificity of immunolabelling observed in the present study, several control experiments were carried out. It was interesting to note that the immunoreactivity of type IV collagen in rat tissue sections could be blocked by combined human type IV collagen with bovine type IV collagen, but not by each of them individually.

Conclusions from the present study:

1). A mild glutaraldehyde fixative appears to preserve the antigens of basal lamina laminin and type IV collagen in rat oral mucosa.

2). Some additives (picric acid and PVP) added to fixatives appear to improve morphological preservation of epithelium and antigen retention of basal lamina.

3). Fixation osmolarity is related to buffer systems and some additives (sucrose and PVP) and appears to affect the preservation of tissue morphology.

4). Phosphate buffer (PB) and phosphate-buffered saline (PBS) appear to contribute to fixation osmolarity which is associated with morphological preservation of epithelium, but not antigen retention of basal lamina laminin and type IV collagen.

5). A hydrophilic resin (L.R.White resin) as an embedding medium appears to be satisfactory for retention of basal lamina antigens.

6). Using L.R.White resin, the dehydration procedure employed appears to be associated with morphological preservation, but not the immunolabelling of basal lamina laminin and type IV collagen.

7). The polymerization temperature used for L.R.White resin appears to affect the degree of antigen retention of basal lamina laminin and type IV collagen.

8). The selection of an appropriate antibody and gold complex in immunostaining appears to be critical for the successful localization of tissue antigens.

9). The results of this study indicate that there is a heterogeneity in molecular structure of type IV collagen between rat tissue, human tissue and bovine tissue .

2. Localization and quantitation of basal lamina laminin and type IV collagen (Chapter four and Chapter five).

In the present study, tissue processing and immunogold post-embedded techniques developed in a series of experiments were employed to identify the sites of location of basal lamina laminin and type IV collagen in normal rat tongue mucosa and experimentally induced oral carcinomas. It was found that laminin and type IV collagen were located in the lamina densa of rat tongue mucosal basal lamina and induced oral carcinoma basal laminae. The reported site of laminin in the basal lamina of rat tongue mucosa in the present study is different from that described for other tissues (eg. kidney, colon, muscle) as described by other investigators (Bendayan, 1985; Grant and Leblond, 1988; Desjardins and Bendayan, 1989). However, the location of type IV collagen in basal lamina of rat oral mucosa is similar to that described for other tissues (Grant and Leblond, 1988; Desjardins and Bendayan, 1989). Laminin was detected in stromal fibroblasts of normal tissue and carcinoma tissue and in the stroma of carcinomas in the present study.

These results indicate that oral mucosa basal lamina may have structural and functional characteristics different from basal laminae in other tissues and that stromal fibroblasts may be involved in the production of laminin.

The results of quantitative analyses showed that the expression of laminin in carcinomas was significantly higher than that in normal tissues; in contrast, the expression of type IV collagen in

carcinomas was significantly lower than that in normal tissues. These observations offer an explanation for the ultrastructural morphological changes previously observed (thickening of basal lamina in induced oral carcinoma) (Jiang, Wilson and Weibkin, 1993) and confirm the subjective observations made earlier in this study (Chapter four).

CHAPTER SEVEN: SUGGESTIONS FOR PROSPECTIVE STUDIES

As a consequence of the results of this study it is felt that future studies of the type indicated as follows would be of benefit:

1). It has been described that formaldehyde and formaldehyde-glutaraldehyde mixture are good fixatives for immunocytochemistry. However, poor preservation of tissue morphology and tissue antigens was observed in the present study. The reasons for this are poorly understood. It would be worthwhile to further investigate these fixatives relative to tissue types and fixation time.

2). Although tissue processing conditions used in the present study produced good antigen retention, morphological preservation was still not very satisfactory. Further improvement of morphological preservation might be expected by more experimentation of tissue preparation conditions (eg. use of other additives and microwave treatment techniques).

3). In the present study, antibodies used were polyclonal anti-human and/or bovine antigens, because specific anti-rat antibodies were not available at the time the experiments were carried out. Experiments using more specific antibodies (anti-rat antibody and monoclonal antibodies) now available would be of value.

4). It is known that basal lamina comprises a number of components. Aside from laminin and type IV collagen, type VI collagen, heparan sulfate, feotal antigen 2, entactin, tenascin and thrombospondin have been reported in the basal laminae of various

tissues. The immunocytochemical demonstration of other basal lamina constituents may further help our understanding of the structure of basal lamina in normal oral mucosa and in pathological processes such as neoplasia.

5). In the present study, investigation of the distribution of basal lamina laminin and type IV collagen was carried out in relation to normal tissues and induced oral carcinomas. Similar investigations on premalignant lesions might be very of value in relation to the early diagnosis of carcinoma.

6). It is indicated that the methods developed in the present study will be extended to human oral tissues and human oral carcinomas to compare the characteristics of human oral carcinoma basal lamina components with the results described for experimental rat oral carcinomas, thus testing the relevance of the experimental rat carcinoma model to the human situation as well as providing further data on oral carcinomas not presently available.

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Test of reproducibility of results							
Animal	Counting time	Laminin*			Type IV collagen*		
		Electronmicrographic number			Electronmicrographic number		
		1	3	5	1	3	5
KN2	1	34.15	22.86	22.58	16.67	27.78	9.3
	2	32.14	22.22	24.14	17.24	27.63	9.76
KN4	1	60.98	46.67	14.71	13.51	39.13	16.67
	2	58.14	44.83	10.34	11.9	36	18.75
KN6	1	40.43	13.56	19.35	18.75	25	27.78
	2	39.58	14.81	16.67	18.75	22.22	31.25
KN8	1	26.83	35.9	28	25	28.57	32.821
	2	26.83	38.36	26.53	23.26	32	31.82
KP2	1	32.35	7.5	10.91	40.38	24	23.33
	2	28.57	8.11	13.98	37.04	24.49	22.58
KP4	1	47.37	28.13	25	44.83	11.11	35
	2	44.19	27.78	25.58	46.43	10.81	35
KP6	1	20	17.91	9.38	18.52	27.78	29.17
	2	21.05	20	11.94	18.52	30.61	32.56
KP8	1	15.25	48	36.11	34.48	26.67	28.57
	2	15.52	48	38.89	36.67	23.53	25.81
KT2	1	72.41	58.17	72	16.67	3.45	9.09
	2	70	45.83	72	12.5	3.33	9.09
KT4	1	35.71	58.33	130	25	13.64	20
	2	34.48	66.67	131.58	28.57	17.65	23.53
KT6	1	59.38	70	95	8.33	40.91	4.55
	2	62.07	63.64	105.56	6.45	34.42	4.35
KT8	1	91.67	92.31	50	16.67	15.25	12.9
	2	75	95.83	50	18.33	16.07	12.5

* All data represent the number of gold particles/per unit area (μm^2)