Histol Histopath (1991) 6: 175-181

**Histology and Histopathology**

# Effects of alcohol on laminin in rat gastric mucosa

**Kathryn S. Rightor and Karmen L. Schmidt**
Department of Pathology and Laboratory Medicine, The University of Texas Medical School at Houston, Houston, Texas, USA

**Summary.** Following ethanol exposure, the gastric surface epithelium often exfoliates, leaving a denuded basal lamina. Viable cells from the gland migrate along the basal lamina to repair the defect, a process known as restitution. Laminin, the major non-collagenous glycoprotein of basal laminae, functions in cellular adhesion and migration and, therefore, any alteration of this molecule by ethanol may influence subsequent restitution. After a 5 or 60 minutes treatment with saline, 50% or 100% ethanol, gastric tissues were removed from fasted female Sprague-Dawley rats, fixed in 1% paraformaldehyde and processed in Lowicryl. Once embedded and sectioned, specimens were incubated in anti-laminin followed by protein A-gold. The area of lamina densa from interfoveolar, pit and gland regions was measured and gold particles counted. Absolute ethanol caused diminished immunogold binding in all regions at all time periods, except the gland at 60 minutes. Exposure to 50% ethanol for 5 minutes did not alter laminin binding, although 60 minutes after 50% alcohol, laminin immunolabelling was increased. Alcohol concentration alters laminin immunogold binding, and therefore may influence restitituion.

**Key words:** Basal lamina, Basement membrane, Immunocytochemistry, Restitution, Alcohol, Gastric mucosa, Rat

## Introduction

The gastric mucosal basal lamina serves as the interface between the gastric epithelium and the lamina propria. Alcohol is known to alter the ultrastructure of the basal lamina. After the gastric mucosa is subjected to an acute dose of alcohol, it displays necrotic, hemorrhagic lesions (Lacy, 1987). Under these conditions, the gastric epithelium exfoliates, exposing its underlying basal lamina to luminal alcohol, particularly in the interfoveolar region. The basal lamina appears flocculent and thickened in tissues severely damaged by alcohol exposure, and the epithelial and endothelial basal laminae often rupture, permitting cellular and vascular components to extravasate into the adjoining connective tissue and gastric lumen (Rutten and Ito, 1983; Lacy and Ito, 1984; Lacy, 1987). Despite these effects on the basal lamina, the epithelium and vasculature have remained the major focus of attention regarding alcohol injury (Lacy, 1985). Little attention has been directed toward the effects of alcohol upon the basal lamina or its individual components although these may be important targets of alcohol-mediated injury. Such a contention is supported by our recent findings that alcohol elicits a dose-dependent decrease in immunolabelling of type IV collagen, the major protein of basal laminae (Rightor et al., 1991). In addition, Black and colleagues reported that acid decreased the number of anionic sites corresponding to glycosaminoglycans in basal laminae of rat gastric mucosa (Black et al., 1985).

The basal lamina is composed of several proteins and glycoproteins. Laminin is the major non-collagenous glycoprotein of the basal lamina and it mediates growth, differentiation, migration, agglutination, and assembly of the extracellular matrix (Kleinman et al., 1985). More pertinent to the present study, laminin is a high molecular weight attachment protein which links the epithelium and endothelium to the basal lamina, apparently by mediating the attachment of epithelial cells to type IV collagen (Terranova et al., 1980; Timpl et al., 1984). Also, laminin has been reported to promote growth and motility of Schwann cell-derived tumor cells and mouse melanoma cells *in vitro* (Baron-Van Evercooren et al., 1982; McCarthy et al., 1983; McCarthy and Furcht, 1984). These haptotactic responses show cell movement which is directed by adhesion gradients in response to substratum-bound constituents.

*Offprint requests to:* Dr. Karmen L. Schmidt, Ph. D., Department of Pathology and Laboratory Medicine, The University of Texas Medical School at Houston, 6431 Fannin Street, Houston, Texas 77030, USA

Since restitution is an important repair process in gastric mucosa which depends upon rapid migration of viable cells and laminin mediates cell adhesion and migration, alcohol alteration of molecular laminin may impede repair by restitution. The purpose of this study was to determine the effect of ethanol upon laminin in gastric mucosal basal lamina during periods of restitution. The intensity of immunogold binding to laminin was used to assess the antigenic and therefore, the molecular integrity of this molecule (Novotny et al., 1986).

## Materials and methods

Female Sprague-Dawley rats (TIMCO, Houston, TX) weighing approximately 200 g were fasted overnight in wire-bottom cages to prevent coprophagia. The next day they received 1 ml of either saline, 50% or 100% ethanol via orogastric tube. Absolute alcohol was used to induce gastric injury since it has been used frequently in previously published work and produces a consistent and predictable pattern of injury (Robert et al., 1979; Lacy and Ito, 1982; Guth et al., 1984; Schmidt et al., 1985, 1986). A 50% concentration of alcohol was also used because it is less toxic than 100% ethanol and provides reproducible damage (Schmidt and Miller, 1988). In addition, our previous work has demonstrated that 50% and 100% alcohol alter the basal lamina and yet still permit the restitution process to be studied (Smith and Schmidt, 1989). Gastric tissues were retrieved after 5 and 60 min because the restitution process actively engages and proceeds during this time period (Lacy and Ito, 1984; Schmidt et al., 1985, 1986; Ito and Lacy, 1988). Animals were administered ether anesthesia and sacrificed using aortic perfusion with 1% paraformaldehyde. To ensure adequate preservation, fixative was also injected into the gastric lumen through the forestomach. Paraformaldehyde fixative was selected because it optimally preserved antigenicity of laminin molecule although ultrastructuere was not optimal (Stephens et al., 1982; Smith and Schmidt, 1987). Gastric tissues were obtained from identical regions of the gastric corpus by a method previously published (Schmidt et al., 1985) and prepared for immunogold (IG) electron microscopy. All animals procedures were approved by the animal welfare committee.

For IG, tissues were dehydrated in increasing concentrations of alcohol while concomitantly decreasing the temperature (Bendayan, 1984), and then embedded in Lowicryl K4M (Carlemalm et al., 1982). Gastric specimens were polymerized under UV light at -30° C for 60 hours and then at room temperature for 24 hours. Subsequently, tissue blocks were thin-sectioned and placed on Butvar-coated nickel grids (Handley and Olsen, 1979). Sections were then incubated in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA), placed on a drop of a 1:200 solution of anti-laminin (Collaborative Research, Bedford, MA) for 1 hour, washed in PBS several times and then in PBS containing BSA. Sections were incubated for 30 minutes in a 1:4 solution of 20 nm colloidal protein A-gold (EY Laboratories, San Mateo, CA), followed by final washes in PBS and distilled water. Staining controls included omission of anti-laminin and substitution of pre-immune serum for the anti-laminin.

Tissue sections were stained in uranyl acetate and viewed on a JEOL 1200-EX transmission electron microscope at 60kV. A minimum of 5 photographs/animal were taken from each of the interfoveolar, mid-pit, and gland isthmus regions. Since the epithelium and basal lamina often separated under the duress of alcohol, making the lamina rara no longer discernable, the area of lamina densa was measured. The area of lamina densa was measured using a trapezoidal algorithm program and a digitizing tablet, and the number of gold particles counted within that area. A minimum of 400 counts/region/animal were obtained to reduce error (Loud, 1987). The density of immunogold labelling of the laminin molecule was used as an indication of the antigenic and molecular integrity of laminin (Novotny et al., 1986) and compared among the groups. Since 5 and 60 minute groups were gold-reacted on separate days, control values were normalized and the remaining values adjusted accordingly. Gold particles/area lamina densa were evaluated using analysis of variance and Student-Newman-Keuls' test for multiple comparisons ($\alpha = 0.05$) (Newman, 1939; Keuls, 1952).

## Results

The presence of electron-dense gold particles overlying the laminae rara and densa indicated specific laminin binding. Background counts were not significantly different between groups ($\alpha = 0.05$). Tissues incubated with either no primary antibody or with pre-immune serum revealed minimal, non-specific immunogold binding. In specimens treated with anti-laminin, however, immunogold binding was specific to the basal lamina, primarily the lamina densa (Figs. 1a-2a). Binding to the laminae rara did occur in control tissues, but since the laminae rara could not be identified in alcohol-treated tissues where damaged epithelial cells had detached from the basal lamina, only the lamina densa was used as a measure of basal lamina area.

Quantitative data for 5 and 60 min are shown in Figure 3. IG binding in tissue sites after 5 min exposure to 50% alcohol (Fig. 1b) was equivalent to controls. However, after acute gastric insult for 5 min using 100% ethanol, laminin binding was significantly diminished in all three mucosal regions compared with control levels (Fig. 1c). At 60 min, tissues subjected to 50% ethanol, showed an enhanced degree of binding in all regions when compared with controls (Fig. 2b). Laminin binding was decreased in the interfoveolar and pit regions after exposure to 100% ethanol (Fig. 2c), while laminin binding in the gland isthmus had increased to control levels (Fig. 2d).

Comparing 5 and 60 min time points (Fig. 4), 50% ethanol resulted in increased binding in all regions after 60 min, although binding was not significantly different

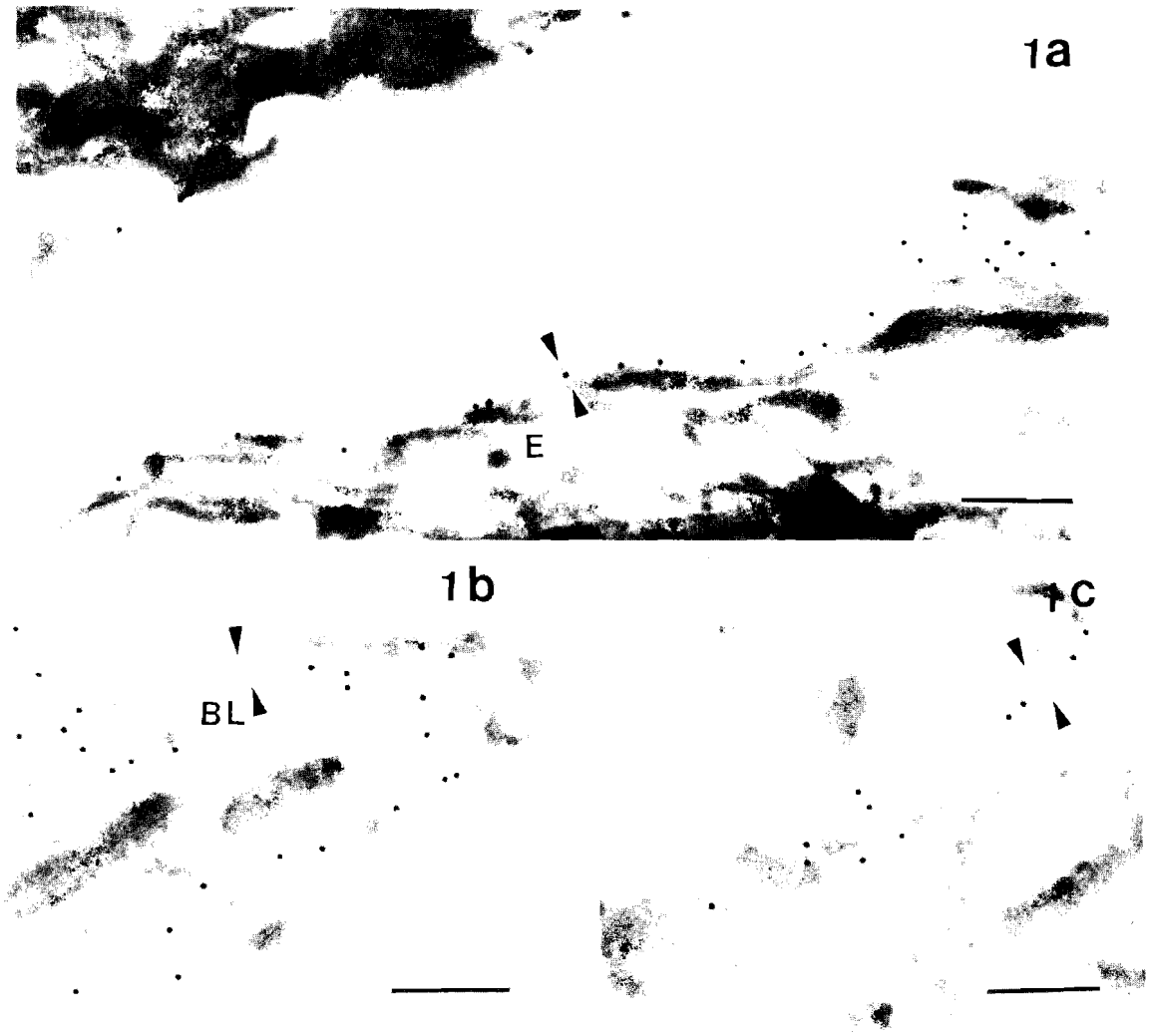


**Fig. 1.** 5 min tissue. **a.** Gastric epithelium (E) showing laminin immunobinding to the basal lamina (arrows) in the interfoveolar region of 5 min control tissue. Bar = 0.05 μm. **b.** Laminin immunobinding in the interfoveolar region of gastric mucosa after 5 min exposure to 50% ethanol. Lamina densa of basal lamina (BL) is thickened and plicated. Bar = 0.05 μm. **c.** Gastric tissue treated with 100% ethanol for 5 min, showing laminin immunolabelling. Note flocculent appearance of basal lamina (arrows) separated from epithelium. Bar = 0.05 μm.

from controls at 5 or 60 min. After either 5 or 60 minutes exposure to 100% alcohol, the interfoveolar and pit region lamina densa showed an intense decrease in IG binding. A similar decrease in gold binding occurred in the gland at 5 min after 100% alcohol. However, in the gland after 60 min exposure to 100% ethanol, binding was not significantly different from controls.

## Discussion

Superficial damage of the gastric mucosal epithelium is mended through restitution, a process which entails migration of viable epithelial cells from the gland isthmus to replenish the denuded surface (Lacy, 1985; Schmidt et al., 1986). In the absence of hemorrhagic necrosis, this process is completed in minutes to hours (Lacy and Ito, 1984; Schmidt et al., 1986). Extensive necrotic injury is repaired by healing, a slow process which involves inflammation and cell replication (Silen and Ito, 1984). Morris and Wallace (1981) were the first to propose that an unbroken basal lamina is vital for gastric mucosal restitution. They found that when gastric tissue was exposed to 40% ethanol and then 50mM hydrochloric acid, any basal lamina exposed following cell exfoliation was destroyed. These authors proposed that removal of the substratum provided by the basal lamina could interfere with re-establishment of the epithelium. In support of this contention, Lacy (1987) has shown that in regions where the basal lamina is discontinuous, cellular lamellipodia do not bridge such ruptures, underscoring the importance of this structure as a scaffold for cell attachment and migration. Similar

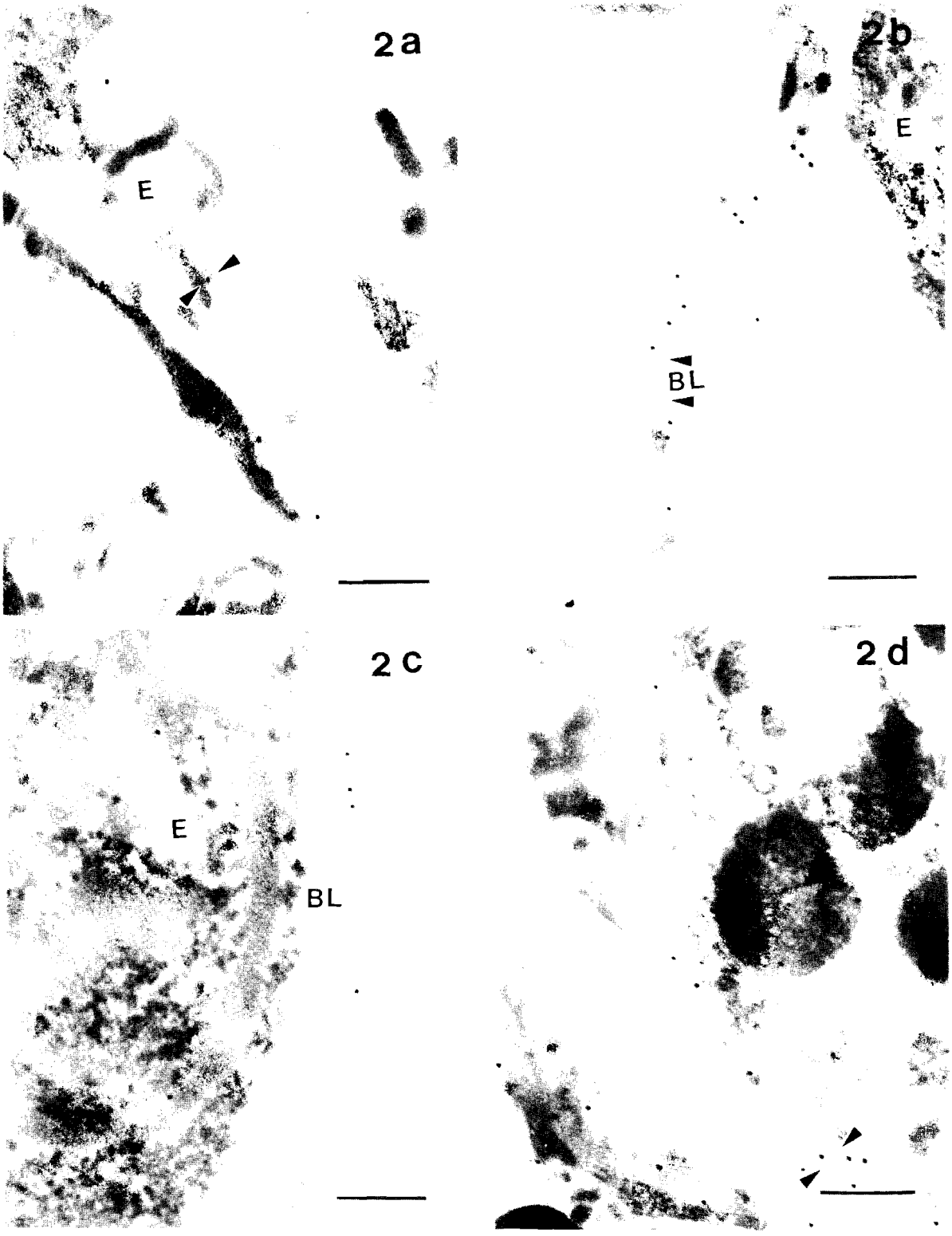


**Fig. 2.** 60 min tissue. **a.** Laminin immunobinding to control basal lamina (arrows) in the interfoveolar zone of 60 minute gastric epithelium (E). Bar = 0.05 μm. **b.** Interfoveolar region of rat gastric mucosa after 60 min exposure to 50% ethanol, showing enhanced binding when compared with controls (2a). Epithelium (E) has separated from the basal lamina (BL). Bar = 0.05 μm. **c.** Gastric pit epithelium (E) after 60 min exposure to 100% ethanol, demonstrating reduced laminin binding. The basal lamina (BL) is floccular, the epithelium distressed. Bar = 0.05 μm. **d.** Immunolabelling in gland of 100% ethanol-treated tissue after 60 min. Note the increased binding (arrows) when compared to gastric pit labelling after the same treatment for an equivalent time (Fig. 2a). Bar = 0.05 μm.

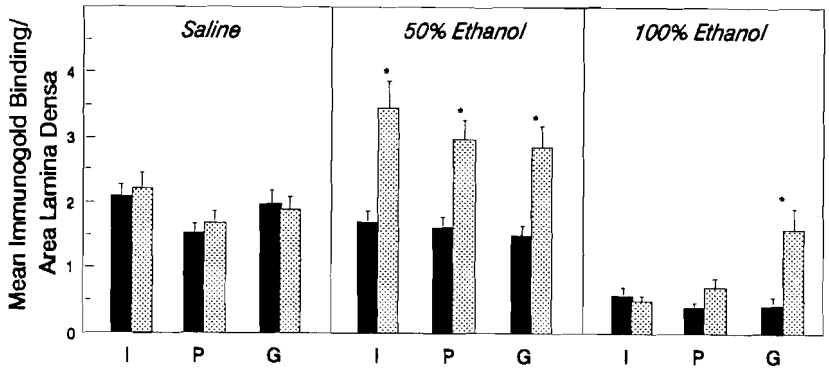


**Fig. 3.** Mean laminin binding in gastric lamina densa comparing treatments. * Treatments were significantly different from corresponding controls ($\alpha$ = 0.05). Sixty minute data (b) was normalized such that saline controls were equivalent to five minute controls. I = interfoveolar, P = pit, G = gland.

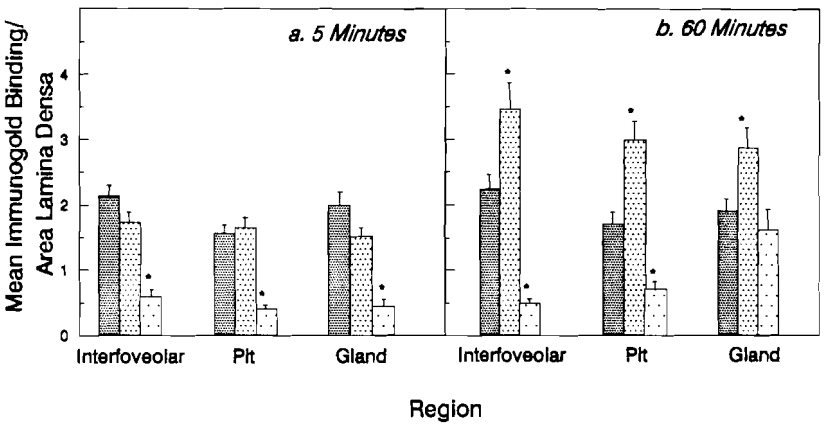


**Fig. 4.** Mean laminin binding in gastric lamina densa comparing 5 and 60 min. I = interfoveolar, P = pit, G = gland. * Laminin binding at 60 min was significantly different from 5 min binding ($\alpha$ = 0.05).

findings have been reported in other systems (Vracko, 1974). Repair of necrotic lesions, a long-term process taking weeks to months, requires cell proliferation and differentiation as well as migration. Lacy and colleagues have shown that viable epithelial cells fail to migrate and repair damaged gastric surface when the basal lamina, disfigured by alcohol, is accompanied by swollen collagen fibrils (Lacy et al., 1983). These studies clearly indicate that a damaged basal lamina may impede restitution and healing processes.

Alcohol-induced distortions and ruptures of the basal lamina which are not bridged by migrating epithelial cells suggest that alterations may have occurred in the molecular integrity of laminin, the major non-collagenous glycoprotein of basal lamina. Since laminin mediates cell adhesion and migration, any alteration in its structure may profoundly affect restitution and healing processes.

In the present study, we investigated the possibility that alcohol attacks molecular laminin. In order to test this hypothesis, we measured the immunogold binding characteristics of laminin following two concentrations of alcohol administered over two time periods. Actual changes in binding properties due to experimental manipulation were relative rather than absolute numbers of binding sites. Grant and Leblond (1988) set a precedent for this approach by using standard fixation and processing techniques and immunolabelling densities of laminin and type IV collagen to compare thick and thin basement membranes.

Our findings show that laminin binding in gastric mucosal basal lamina is not affected after a 5 min exposure to 50% ethanol, but is profoundly diminished following treatment with 100% ethanol for the same length of time. The intense drop in binding induced by absolute alcohol may be caused by decreased affinity for immunogold due to changes in laminin molecular conformation or, possibly, to alterations in surrounding molecules which may in turn affect laminin binding capacity. The laminin molecule of rat gastric mucosa appears to be relatively impervious to the effects of 50% ethanol.

Our findings also indicate that at 60 min after exposure to absolute ethanol, laminin binding in interfoveolar and pit regions remains at the same low level observed following 5 min exposure. However, after 60 min, binding levels in the gland zone have returned to near control levels. These findings suggest that absolute alcohol maximally damages laminin in all zones within the first minutes after exposure but increased gold binding in the gland region at 60 minutes after absolute ethanol may indicate synthesis of new laminin.

This study also showed that after a 5 min exposure to 50% ethanol, laminin binding appears unchanged. However, 60 min after exposure to 50% alcohol, the degree of immunogold binding to the laminin molecule has increased in all three regions to well above control levels. This interesting finding may be due to synthesis

of new laminin by viable epithelial cells or to the unmasking by 50% ethanol of previously unavailable binding sites on the laminin molecule. In attempt to determine if laminin was being synthesized under the above conditions, the cytoplasm of epithelial cells was examined for gold particles. No evidence of specific binding was found in the epithelial cells. This does not, however, rule out synthesis because unsecreted laminin found within the cell may not be capable of binding the antibody used in this study.

In summary, alcohol elicits several effects on the basal lamina and on laminin in particular. Laminin immunolabelling is severely reduced in all regions within 5 min after 100% ethanol exposure, and remains at this low level in the interfoveolar and pit regions after 60 min. Immunogold levels in the gland increased to control levels in this time frame, however, suggesting that the gland is in the process of healing. After exposure to 50% ethanol, no changes in binding were apparent after 5 min, but after 60 min, binding levels were increased. These findings indicate absolute alcohol decreases the binding capacity of laminin but lower concentrations of alcohol increase binding capacity of the laminin molecule. Since these alterations occur during periods of restitution, and laminin is the basal lamina component which mediates cell migration and adhesion, such changes may influence the capacity of gastric tissues to repair following alcohol insult. Studies to examine this contention are underway.

*Acknowledgements*: Grand support: NIAAA grant AA 06887 and NIH grant DK 37260 to Dr. Schmidt.

## References

Baron-Van Evercooren A., Kleinman H.K., Seppa H.E.J., Rentier B. and Dubois-Dalco M. (1982). Fibronectin promotes rat Schwann cell growth and motility. J. Cell Biol. 93, 211-216.

Bendayan M. (1984). Protein A-gold electron microscopic immunocytochemistry: methods applications and limitations. J. Electron Microsc. Tech. 1, 243-270.

Black B.A., Morris G.P. and Wallace J.L. (1985). Effects of acid on the basal lamina of the rat stomach and duodenum. Virchows Arch. (B) 50, 109-118.

Carlemalm E., Garavito R.M. and Villiger W. (1982). Resin development for electron microscopy and an analysis of embedding at low temperature. J. Microsc. 126, 123-143.

Grant D.S. and Leblond C.P. (1988). Immunogold quantitation of laminin, type IV collagen, and heparan sulfate proteoglycan in a variety of basement membranes. J. Histochem. Cytochem. 36, 271-283.

Guth P.H., Paulsen G. and Nagata H. (1984). Histologic and microcirculatory changes in alcohol-induced gastric lesions in the rat: Effect of prostaglandin cytoprotection. Gastroenterology 87, 1083.

Handley D.A. and Olsen B.R. (1979). Butvar B-98 as a thin support film. Ultramicroscopy 4, 479-480.

Ito S. and Lacy E.R. (1988). Morphology of rat gastric mucosal damage, defense and restitution in the presence of luminal ethanol. Gastroenterology 88, 250-260.

Keuls M. (1952). The use of the "studentized range" in connection with an analysis of variance. Euphytica 1, 112-122.

Kleinman H.K., Cannon F.B., Laurie G.W., Hassell J.R., Aumailley M., Terranova V.P., Martin G.R. and Dubois-Dalco M. (1985). Biological activities of laminin. J. Cell Biochem. 27, 317-325.

Lacy E.R. (1985). Prostaglandins and histological changes in the gastric mucosa. Dig. Dis. Sci. 30, 83S-94S.

Lacy E.R. (1987). Gastric mucosal defense after superficial injury. Clin. Inv. Med. 10, 189-200.

Lacy E.R. and Ito S. (1982). Microscopic analysis of ethanol damage to rat gastric mucosa after treatment with a prostaglandin. Gastroenterology 93, 619.

Lacy E.R. and Ito S. (1984). Rapid epithelial restitution of the rat gastric mucosa after ethanol injury. Lab. Invest. 51, 573-583.

Lacy E.R., Rutten M.J. and Ito S. (1983). The role of the basal lamina in gastric mucosa reepithelization after surface cell destruction. Anat. Rec. 205, 104-105a.

Loud A.V. (1987). Electron microscopic morphometry. Anal. Quant. Cytol. Histol. 9, 7-12.

McCarthy J.B. and Furcht L.T. (1984). Laminin and fibronectin promote the haptotactic migration of B16 mouse melanoma cells in vitro. J. Cell Biol. 98, 1474-1480.

McCarthy J.B., Palm S.L. and Furcht L.T. (1983). Migration by haptotaxis of a Schwann cell tumor line to the basement membrane glycoprotein laminin. J. Cell Biol. 97, 772-777.

Morris G.P. and Wallace J.L. (1981). The roles of ethanol and of acid in the production of gastric mucosal erosions in rats. Virchows Arch. B 38, 23-28.

Newman D. (1939). The distribution of range in samples from a normal population, expressed in terms of an independent estimate of standard deviation. Biometrika 31, 20-30.

Novotny J., Hanaschumacher M., Haber E., Bruccoleri R.E., Carlson W.B., Fanning D.W., Smith J.A. and Rose G.D. (1986). Antigenic determinants in proteins coincide with surface regions accessible to large probes (antibody domains). PNAS 83, 226-230.

Rightor K.S., Mitchell P.A., Miller T.A. and Schmidt K.S. (1991). Colloidal gold localization of type IV collagen in the extracellular matrix of rat gastric mucosa: Influence of alcohol and prostaglandin. Anat. Rec. (in press).

Robert A., Nezamis J.E., Lancaster C. and Hanchar A.J. (1979). Cytoprotection by prostaglandins in rats: Prevention of gastric necrosis produced by alcohol, HCl, NaOH, hypertonic NaCl and thermal injury. Gastroenterology 77, 433.

Rutten M.J. and Ito S. (1983). Morphology and electrophysiology of guinea pig gastric mucosal repair in vitro. Am. J. Physiol. 244, G171.

Schmidt K.L., Henagan J.M., Smith G.S., Hillburn P.J. and Miller T.A. (1985). Prostaglandin cytoprotection against ethanol-induced gastric injury in the rat: a histologic and cytologic study. Gastroenterology 88, 649-659.

Schmidt K.L., Bellard R.L., Smith G.S., Henagan J.M. and Miller T.A. (1986). Influence of prostaglandin on repair of rat stomach damaged by absolute ethanol. J. Surg. Res. 41, 367-377.

Schmidt K.L. and Miller T.A. (1988). Morphological characteristics of prostaglandin cytoprotection. Toxicol. Pathol. 16, 223-236.

Silen W. and Ito S. (1984). Mechanism for rapid re-epithelialization of the gastric mucosal surface. Am. Rev. Physiol. 47, 217-229.

Smith K.B. and Schmidt K.L. (1987). Protein A-gold localization of

type IV collagen in gastric mucosa. Tex. Soc. Electron Mircosc J. 18,2.

Smith K.B. and Schmidt K.L. (1989). Quantiative Immunogold localization of laminin and type IV collagen in gastric mucosa. Proc. Ann. EMSYA 47, 934-935.

Stephens H., Bendayan M., Silver M. (1982). Immunocytochemical localization of collagen types and laminin in skeletal muscle with the protein A-gold technique. Biol. Cell 44:81-84.

Terranova V.P., Rohrbach D.H. and Martin G.R. (1980). Role of laminin in the attachment of PAM 212 (epithelial) cells to basement membrane collagen. Cell 22, 719-726.

Timpl R., Fujiwara S., Dziadek M., Aumailley M., Weber S. and Engel J. (1984). Lamini, proteoglycan, nidogen and collagen IV: structural models and molecular interations. Ciba Found. Symp. 108, 25-43.

Vracko R. (1974). Basal lamina scaffold: anatomy and significance for maintenance of orderly tissue structure: a review. Amer. J. Patho. 77, 314-338.

Accepted October 12, 1990

Histol Histopath (1991) 6: 183-189

Histology and Histopathology

# Immunocytochemistry of epithelial markers in citral-induced prostate hyperplasia in rats

**Ruth Massas[1], Ciro Servadio[2], Uriel Sandbank[1] and Armand Abramovici[1]**

[1]Laboratory of Developmental Pathology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv and [2]Urology Department, Beilinson Medical Center, Petah-Tikva, Israel

**Summary.** Immunocytochemical characterization of several epithelial markers using the PAP technique was analyzed during different stages of induced prostatic hyperplasia in rats. Intact adolescent rats (42 days old) were treated with citral (3,7 dimethyl - 2,6 octadienal) for 10, 30 and 100 days and their ventral prostate compared to untreated, matched-age animals. Among the epithelial markers studied the prostatic specific acid phosphatase was present in hyperplastic prostates of rats. The immunoreaction showed a fair correlation with the severity of lesion and duration of treatment. The prostatic specific antigen showed equally immunoreactive in both control and treated rats. The hyperplastic and normal rat prostates did not show immunoreactivity towards the other epithelial cell markers such as epithelial membrane antigen, carcinoembrionic antingen and alpha-fetoprotein antisera.

It is concluded that prostatic specific acid phosphatase, and to a lesser extent prostatic specific antigen, might represent valuable markers for comparative studies of prostatic hyperplasia in rodents.

**Key words:** Epithelial markers, Hyperplasia, Citral, Immunocytochemistry, Prostate, Rat

## Introduction

During the last decades understanding of the pathogenesis and the evolution of prostate hyperplasia in man has been improved using animals models such as dogs and rats which develop analogical lesions either spontaneously (Franks, 1967; Brendler et al., 1983; Berry et al., 1985) or experimentally induced by hormonal (Walvoored et al., 1976; Cabanes et al., 1978; Jacoby et al., 1978; De Klerck et al., 1979; Habenicht et al., 1986) and nonhormonal treatments (Abramovici et al., 1985; Servadio et al., 1986). All these studies have revealed that the endocrine and biological behaviour of prostate hyperplasia is similar in both animals and men. However, some interspecies differences concerning the clinical expression of the disease were noted (Issacs, 1984; Purnell et al., 1984; Berry et al., 1985). The different symptomatology seems to originate mostly from the topographical localization of the prostatic gland in these species and its relationship with other intrapelvic organs, rather than endocrine.

The introduction of new immune histochemical technique in the prognosis of prostatic growth anomalies in man have also improved our knowledge in the evaluation of this disease (Miller, 1983; Ellis et al., 1984). The characterization of specific cell markers for prostatic gland is achieved by using antisera raised against some cytoplasmic constituents (Miller, 1983; Ellis et al., 1984). This technique became a useful tool in modern diagnostic pathology. Among the most useful markers for human prostate are prostatic-specific acid phosphatase, PSAP, (Jobsis et al., 1981; Nadji and Morales, 1982) prostatic-specific antigen, PSA, (Nadji et al., 1981) and epithelial membrane antigen, EMA, (Heyderman et al., 1979; Sloane and Ormerod, 1981; Ellis et al., 1984). Other epithelial markers such as carcinoembryonic antigen, CEA, (Heyderman and Neville, 1977; Heidl, 1982; Ellis et al., 1984; Purnell et al., 1984) and the controversial findings about alpha-fetoprotein, AFP, (Ellis et al., 1984) antisera were reported in neoplastic growth of the prostatic gland.

To the best of our knowledge there are no reports about the application of immunohistochemical technique in the study of experimental prostate hyperplasia in animal models. In our opinion such an approach might provide an additional and valuable parameter for a better comparative analysis of prostate

*Offprint requests to:* Prof. A. Abramovici, Laboratory of Developmental Pathology, Sackler School of Medicine, Tel-Aviv University, Ramat Aviv, 69978 Tel-Aviv, Israel

hyperplasia in laboratory animals and men.

The aim of this study is a first attempt to characterize and define the existence and evolution of such epithelial markers in normal and hyperplastic ventral prostate of rats induced by citral (3,7 dimethyl - 2,6 octadienal) (Abramovici et al., 1985; Servadio et al., 1986) at different stages of development.

## Materials and methods

Adolescent male Wistar rats (42 days old, 150 g weight) from Beilinson Hospital strain were housed in a climatized environment with 12-hour light/dark cycle. Standard Embar pellets (Emek Hefer, Israel) and water were supplied ad libitum. Citral (Fluka, Switzerland) was dissolved in 70% ethanol solution and the rats were smeared daily, on the shaved back skin. The animals were divided into 3 groups as follows: group A, 30 rats, were treated with a dose of citral 150 mg/kg body weight/day; group B, 30 rats, received the same amount of solvent only (70% ethanol); group C, 15 untreated control rats. Animals from all the 3 groups were sacrificed by cervical dislocation after 10, 30 and 100 days of treatment, corresponding to 52, 72 and 142 days of age, respectively.

Preliminary studies for fixative efficiencies for the demonstration prostatic markers revealed that the Stieve and Bouin solutions were more effective that the 4% formalin-saline and 4% paraformaldehyde fresh solutions. The latter fixatives showed a reduced reactivity and were also accompanied by a slight background staining. The ventral prostates were freshly dissected and fixed in Stieve solution for 16 hours at room temperature, then dehydrated, paraffin embedded and cut into 8 μm thick sections. Deparaffinized tissue sections were immunostained using the antibody peroxidase-antiperoxidase (PAP) method of Sternberger (1979). To abolish the endogenous peroxidase, tissue sections were previously treated with 3% $H_2O_2$ in methanol solution, thereafter dehydrated and preabsorbed with nonimmune goat serum (Bio-Yeda, Israel) and washed in PBS. Commercially polyclonal-specific antisera kits for PSAP, PSA and AFP (Biomeda, England) as well as CEA and EMA (Dako, USA) were used. The immunostaining procedure was applied according to the customer instructions. The sections were counterstained with Harris' hematoxylin then dehydrated and mounted in Merckoglas (Merck, W-Germany).

For each antisera characterization, the following controls were run accordingly:

1. Elimination of primary antibody (nonspecific binding of secondary IgG).
2. Elimination of both primary and secondary antisera (presence of endogenous peroxidase).
3. Positive primary antisera controls checked with known immunoreactive tissues of human benign prostatic hyperplasia (BPH) and prostatic carcinoma.

In order to facilitate a comparative analysis of the immunohistochemical findings a standard evaluation of

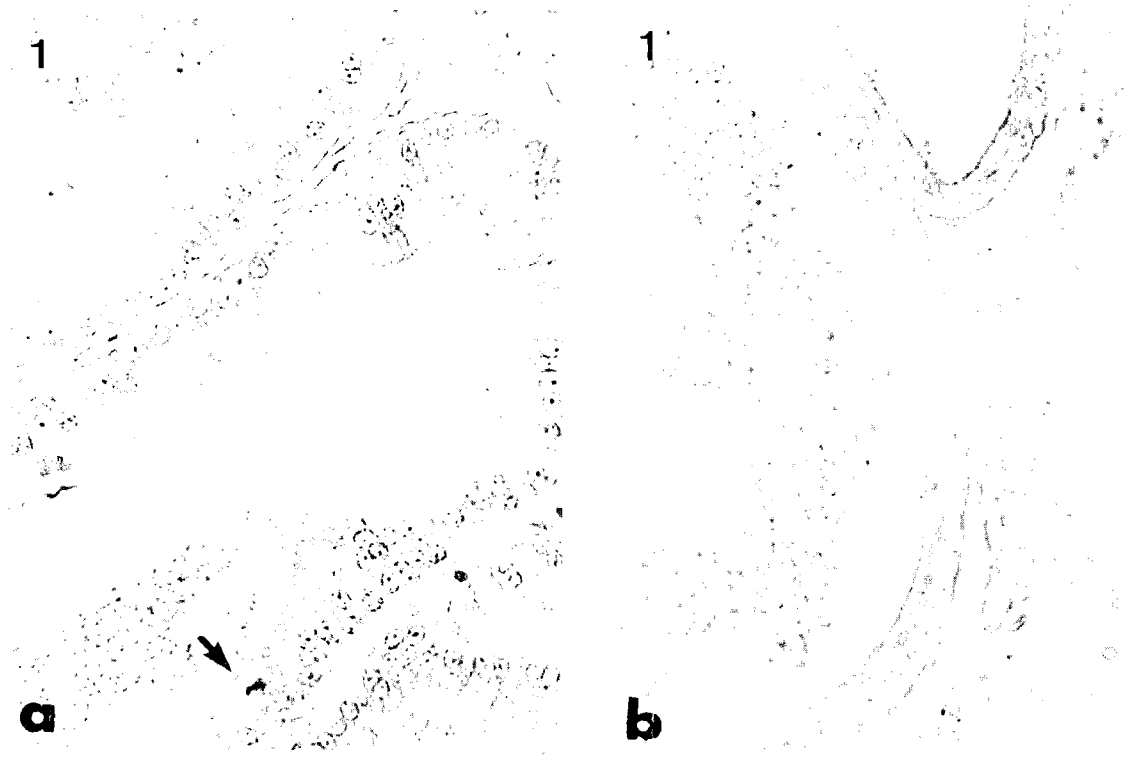


**Fig. 1.** PSAP-like immunoreactivity in prostatic acini after citral administration for 30 days (a), as compared to the normal untreated rats of 72 days old (b). Note the positive reactivity among the hyperplastic tall cells as compared to the normal cuboidal cells. The secretory material is seen in the luminal surface of the hyperplastic cells. Note the unreactivity of a mitotic cell (arrow). × 250

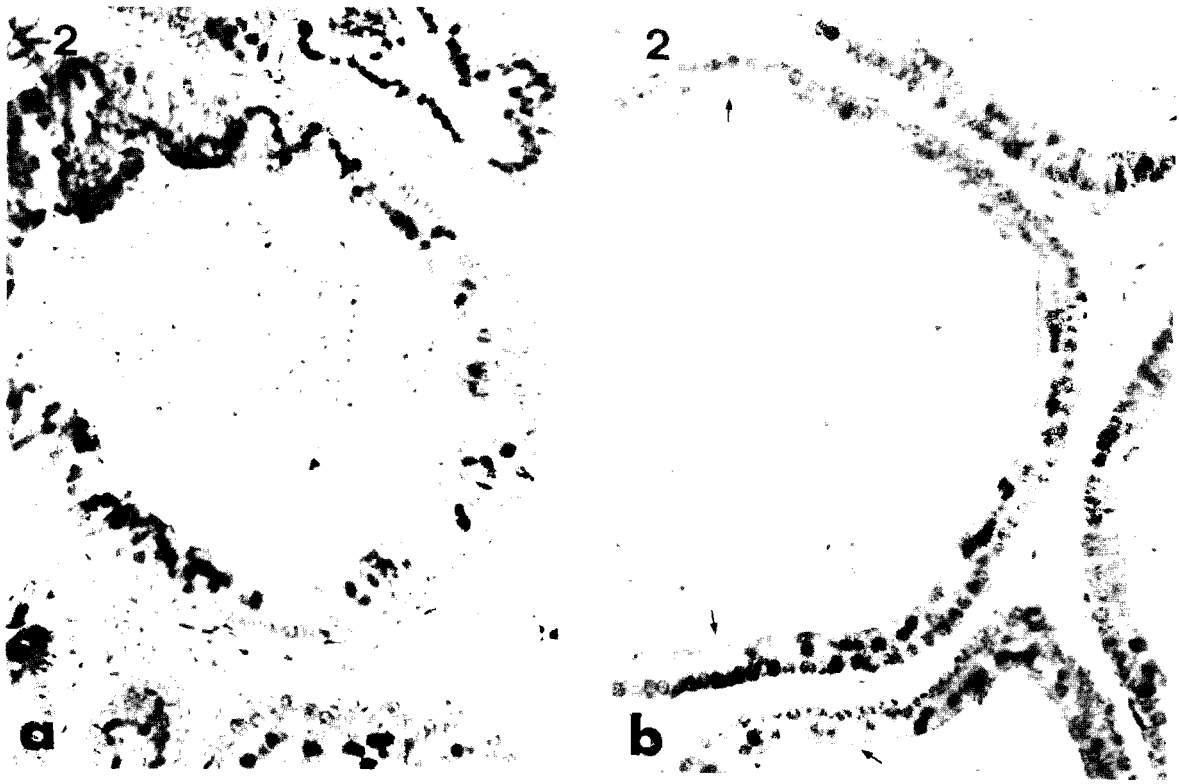


**Fig. 2.** PSAP-like immunoreactivity in prostatic acini after citral administration for 100 days (a), as compared to the normal untreated rats of 142 days old (b). A high increase in intensity and frequency of epithelial cells is seen in the treated rat prostates. The normal acini epithelia shows an unequal number of reactivity cells, some of them being completely empty (arrows). × 250.

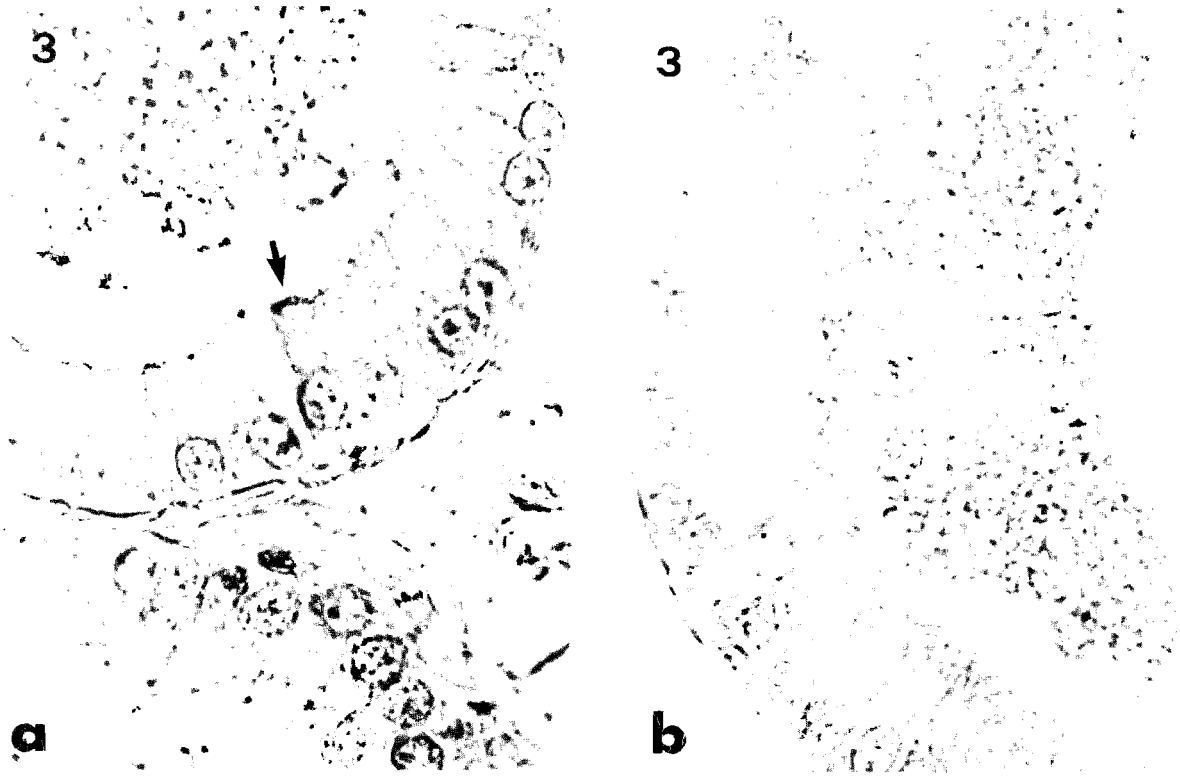


**Fig. 3.** PSA-like immunoreactivity in prostatic acini of citral treated rats for 30 days (a), and normal untreated rats (b). The diffuse intracytoplasmatic material is slightly more reactive among the treated rat prostates. Isolated cells might show conspicuous positive chromogen material. × 630

**Table 1.** Score evaluation of colour intensity and immunoreactive cell frequency in a field.

| Colour intensity | Frequency | |
|---|---|---|
| 0 negative | 0 negative | |
| 1 yellow | A isolated cells | ( 5-10%) |
| 2 brown | B scattered cells | (15-35%) |
| 3 dark brown | C clustered | (40-70%) |
| 4 black | D numerous | (75%) |

**Table 2.** The immunocytochemical characterization of PSAP and PSA in normal and hyperplastic ventral prostates of the rats.

| Teatment period (days) | Group | Antisera | | | | |
|---|---|---|---|---|---|---|
| | | PSAP | PSA | CEA | EMA | AFP |
| 10 | control* | 1/B | 1/A | 0 | 0 | 0 |
| | citral | 1/B | 1/A | 0 | 0 | 0 |
| 30 | control* | 1/B | 1/A | 0 | 0 | 0 |
| | citral | 1-2/C | 1/A | 0 | 0 | 0 |
| 100 | control* | 2/C | 1/A | 0 | 0 | 0 |
| | citral | 3/C-D | 1/A | 0 | 0 | 0 |

* The control group includes both ethanol-treated and untreated rats.

colour intensity and frequency is offered in Table 1. The intensity of PAP chromogenic reaction was graded from 0 to 4 where the negative immunoreactive staining received a zero scored while the high reactive cells received a 4 score. The frequency of reactive cells was scored on an A-D scale, according to the approximate percentage of immunoreactive cells in the medium power of microscope field. The assessment of background staining was performed in the same way.

## Results

The immunohistochemical characterization of different epithelial prostatic markers has shown a wide range of reactivity. Typical hyperplastic lesions of ventral prostate reacted positively with PSAP and to a lesser extent with PSA antibodies (Table 2).

A substantial increase in PSAP immunoreactivity both in colour intensity as well as cell frequency of the hyperplastic prostate acini was found after 30 days of citral treatment. The chromogen was localized mainly in the mid-apical region of the higher columnar epithelial cells both of normal and treated animals. The frequency of positive reactive cells was higher (approximately 40-70%) in the treated group than in the control one (approximately 10%) (Figs. 1a,b). A further increase in cell frequency and intensity was found among acini of the 100 days of citral treatment as compared to the normal rats of cross-matched age (Figs. 2a,b). Intraluminal secretion also showed a positive PSAP immunoreactivity. A shorter period of treatment (10 days) did not show substantial difference from the normal (52-day-old rats) acini reactivity which was identical to the normal, 72-day-old rats. PSAP reactivity was absent in mitotic cells (Fig. 1a) as well as the interface epithelial nuclei and fibrovascular stromal cells.

PSA immunoreactivity was found scattered in the cytoplasm of the acinar epithelium (Figs. 3a,b). The treated prostate seemed to possess a slightly higher immunoreactivity especially at the luminal margins of the cells compared to the normal untreated acini (Fig. 3 a,b). Variations of PSA immunoreactivity were found among different acini; some cells showed conspicuous intracytoplasmic positive chromogen material. The intraluminal fine granular secretion stained more intensely than the epithelial cells in both normal and hyperplastic acini. The fibrovascular stroma remained unreactive to PSA marker. Noteworthy is the absence of variation of PSA immunoreactivity at different stages of treatment.

Other tumor makers such as AFP, CEA and EMA-like immunoreactivity were absent in both hyperplastic and normal rat ventral prostate.

## Discussion

The present findings revealed positive immunoreactivity of both PSAP and PSA in rat hyperplastic prostate. The PSAP reactivity was found to be mainly localized in the mid-apical (Golgi) areas of normal and hyperplastic epithelial cells, but no reactivity was observed in the fibro-vascular stroma. This marker seems to be a permanent constituent of the differentiated epithelial cells of prostate as its reactivity was found to be age-related, increasing from adolescence towards adult period (Massas et al., 1987).

The hyperplastic epithelial response to citral treatment showed a definite more intense reactivity to PSAP than the normal age-matched rats. This difference tends to enhance with length of the treatment, reaching a maximum expression after 100 days of citral administration, and corraborates well with our previous histopathological findings (Abramovici et al., 1985; Servadio et al., 1986). However, the PSAP reactivity was not sensitive enough to define early hyperplastic lesions after 10 days of citral treatment (Servadio et al., 1986). Noteworthy is that intracellular variations in PSAP reactivity were found in the same acinar level, especially among the younger rats. The adult rats (142 days old) showed a more uniform staining, quite comparable to the human hyperplastic prostate. Reactivity variations were also noted between different grades of human prostatic carcinoma (Ellis et al., 1984), the differentiated tumors reacting more intensely with PSAP antisera than the undifferentiated cases. Admitting that the polyclonal antisera do cross-react with rat and human prostate, then it can be assumed that the apparent interspecies variations is a matter of differentiation or functional state of the cells (Potter, 1978) rather than a genetic one. According to this view, the well-differentiated cells will react more intensely to PSAP than the mitotic cells or young cells (Massas et al., 1987) or anaplastic prostate tissue (Ellis et al., 1984).

It is commonly accepted that prostatic growth and function is monitored by androgens which also seem to control, somehow, the acid phosphatase activity (Jobsis et al., 1981). Our observations on the age distribution of PSAP activity in rat prostate were found to be related to the plasma androgen levels during the sexual maturation (Massas et al., 1987).

The question arises whether the PSAP represented a single enzyme or a complex of many isoenzymes which express themselves in various conditions. The rat prostatic acid phosphatase consists of at least two isoenzymes, one a lysosomal form and the other a secretory one (Tenniswood et al., 1976, 1978). The isoenzymes differ in the electrophoretic migration, pH and sensitivity to tartrate and formaldehyde inhibition (Tenniswood et al., 1976). A different behaviour of these two isoenzymes was also noted after castration where the tartrate-sensitive phosphatase persists while the formaldehyde-sensitive isoenzyme disappears (Tenniswood et al., 1978). The existence of two distinct isoenzymes might well explain the decrease in reactivity of PSAP and increase in background staining after formalin-fixation of rat prostate already mentioned in our findings.

The demonstrations of positive immunoreactivity of PSA in rat ventral prostate showed a different pattern of localization and behaviour. The protein seemed to be dispersed across the entire epithelial cytoplasm at random, with some enhanced reactivity toward the luminal surface. The intensity and cell distribution was significantly reduced as compared to PSAP marker, and no differences between the control and experimental prostates could be noted. The length of treatment and the severity of lesions did not seem to affect the initial low reactivity of the acinar cells. The presence of positive PSA-like immunoreactivity has already been described in both hyperplastic (Nadji et al., 1981) and neoplastic (Ellis et al., 1984) human prostates. The fact that the authors mentioned that PSA reactivity was higher in human neoplastic prostate while remaining quite unchanged in normal and hyperplastic conditions is suggestive for an amplification of this antigen synthesis under the influence of the oncogenic process. New reports have revealed the presence of PSA reactivity in other tissues such as breats and sweat glands (Papotti et al., 1989) challenging the previous assumption that the PSA is a good marker for prostatic tumor diagnosis. Recently a new group of monoclonal antisera was even proposed originating from human prostatic carcinoma cells (Bazinet et al., 1988) which do offer a better identification for low-graded prostatic carcinoma, but which do not react with normal, hyperplastic or well-differentiated carcinoma of prostate.

The EMA is a glycoprotein which has only been partially purified (Ormerod et al., 1983) and cross reacts with antisera raised against milk fat globule membrane (Ceriani et al., 1977). Previous studies with this antigen in humans have shown EMA to be present in a wide variety of benign and malignant tissues (Heyderman et al., 1979; Sloane and Ormerod, 1981) as well as in prostate gland (Sloane and Ormerod, 1981; Ellis et al., 1984). Recently, some doubts on the efficiency of EMA immunoreactivity in the diagnosis of human prostatic carcinoma have been reported (Pinkus and Kurtin, 1985; Thomas and Battifora, 1987). According to these authors the anti-EMA poor sensitivity index for prostatic epithelium makes it a less specific marker for this tumor diagnosis than for other epithelial neoplasia.

The negative immunoreactivity of CEA and EMA in rat prostate hyperplasia might be explained by the fact that these antigens are merely regarded as a marker for malignant tumors. Originally the CEA was found in the coloreetal carcinoma (Gold and Fredman, 1965), and since it has been demonstrated in other tissues (Heyderman and Neville, 1977; Purnell et al., 1984) including prostate carcinoma (Heyderman and Neville, 1977; Ellis et al., 1984; Berry et al., 1985).

The absence of AFP-like material in rat ventral prostate hyperplasia was not surprising, since this marker is usually considered as a negative control for prostatic epithelial markers (Ellis et al., 1984).

*Conclusion.* Experimental prostate hyperplasia in rats shared some common epithelial markers with the human prostate hyperplasia. The immunoreactivity of PSAP was found to be the most reliable marker for the differentiation state of prostatic tissues, and can be confidently used in further comparative studies. The remaining epithelial markers EMA and CEA which are better characterized in human neoplastic conditions could not be identified in our experimental model which deals only with hyperplastic changes. Our findings concerning the induction of atypical hyperplastic lesions in rat ventral prostate (Abramovici et al., 1987) might offer a good opportunity for further studies dealing with the characterization of these markers in what is considered premalignant conditions.

*Acknowledgements.* We thank Mrs. H. Wassung for her skillful technical assistance and to H. Choen for microphotographs.

## References

Abramonoci A., Serradio C. and Sandbark U. (1985). Benign hyperplasia of ventral prostate in rats induced by a monterpene. Prostate 7, 389-394.

Abramovici A., Servadio C., Schmueli J. and Sandbank U. (1987). Experimental induction of atypical hyperplasia in rat ventral prostate. In: Prostate Cancer, Part A: Research, Endocrine Treatment and Histopathology. Murphy G.P., Khoury S., Kuss P., Chatelain C. and Denis L. (eds). Alan R. Liss, Inc. New York. pp 559-568.

Bazinet M., Cote R.J., Cordon-Cardo C., Myc A., Fair W.R. and Old J.J. (1988). Immunohistochemical characterisation of two monoclonal antibodies P25.48 and P25.91, which define a new prostate specific antigen. Cancer Res. 48, 6938-6942.

Berry S.J., Coffey D.S. and Ewing L.L. (1985). A comparison of human and canine benign prostatic hyperplasia. In: Regulation of androgen action. Bruchovski N., Chapdelaine A. and Neumann F. (eds). Congressdruch R. Bruckner. West Berlin. pp 59-68.

Brendler C.B., Berry S.J., Ewing L.L., McCullough R.A., Cochran R.C., Strandberg J.D., Zirkin B.R., Coffey D.S., Wheaton L.G., Hiler M.L., Bordy M.J., Niswender G.D., Scott W.W. and Walsh P.C. (1983). Spontaneous benign prostatic hyperplasia in the beagle. J. Clin. Invest. 71, 1114-1123.

Cabanes T.F., Secchi J., Robel P. and Baulieu E.E. (1978). Combined effects of testosterone and estradiol on rat ventral prostate in organ culture. Cancer Res. 38, 4126-4134.

Ceriani R.L., Thompson K., Peterson J.A. and Abraham S. (1977). Surface differentiation antigens of human mammary epithelial cells carried on the human milk fat globule. Proc. Natl. Acad. Sci. USA. 74, 582-586.

De Klerck D.P., Coffey D.S., Ewing L.L., McDermott I.R., Reiner W.G., Robinson C.H., Scott W.W., Strandberg J.D., Talaly P., Walsh P.C., Wheaton L.G. and Zirikin B.R. (1979). Comparison of spontaneous and experimentally induced canine prostatic hyperplasia. J. Clin. Invest. 64, 842-849.

Ellis D.W., Leffers S., Davies J.S. and Alan B.P. (1984). Multiple immunoperoxidase markers in benign hyperplasia and adenocarcinoma of the prostate. Am. J. Clin. Pathol. 81, 279-284.

Franks M.L. (1967). Normal and pathological anatomy and histology of the genital tract of rats and mice. In: Pathology of laboratory rats and mice. Cotchin E. and Roe F.J.C. (eds). Blackwell Scientific Public. Oxford. pp 469-499.

Gold P. and Fredman S.O. (1965). Demonstration of tumor-specific antigens in human colonic carcinoma by immunological tolerance and absorption techniques. J. Exp. Med. 121, 439-462.

Habenicht U.F., Schwartz K., Schweiker H.U., Neumann F. and Etrebg M.F.E. (1986). Development of model for induction of estrogen-related prostatic hyperplasia in the dog and its response to the aromatase inhibitor 4-hydroxy-4-androstene-3, 17 dione: preliminary results. Prostate 8, 181-194.

Heidl G. (1982). Demonstration of carcinoembryonic antigen (CEA) by means of the immunoperoxidase technique in paraffin embedded specimens of tumor tissues. Exp. Pathol. 21, 79-88.

Heyderman E. and Neville A.M. (1977). A shorter immunoperoxidase technique for the demonstration of carcinoembryonic antigen and other cell products. J. Clin. Pathol. 30, 138-140.

Heyderman E., Steele K. and Ormerod M.G. (1979). A new antigen of the epithelial membrane: its immunoperoxidase localisation in normal and neoplastic tissue. J. Clin. Pathol. 32, 35-39.

Isacs J.T. (1984). Common characteristics of human and canine benign prostatic hyperplasia. In: New approaches to the study of benign prostatic hyperplasia. Kinball F.A., Buihl A.E., Caster D.B. and Alan R. (eds). Alan R. Liss, Inc. New York. pp 217-234.

Jacoby G.H., Moore R.J. and Wilson J.D. (1978). Studies on the mechanism of 3 alpha-androstanediol induced growth of the dog prostate. Endocrinology 102, 1748-1755.

Jobsis A.C., DeVries G.P., Meifer A.E.F.H. and Ploem J.S. (1981). The immunohistochemical detection of prostatic acid phosphatase: Its possibilities and limitations in tumor histochemistry. Histochem. J. 13, 961-973.

Massas R., Servadio C., Bruhis S., Sandbank U. and Abramovici A. (1987). Ontogenetic evolution of acid phosphatase immunoreactivity in different lobes of rat prostate. (Abstr). Histochem. J. 19, 184.

Miller G.J. (1983). The use of histochemistry and immunohistochemistry in evaluating prostatic neoplasia. In: Progress in surgical pathology. Vol 5. Fenoglio C.M. and Wolff M. (eds). Masson Publishing. New York. pp 115-129.

Nadji M., Tabei S.Z., Castro A., Chu T.M., Murphy G.P., Wang M.C. and Moroles A.R. (1981). Prostatic specific antigen. An immunohistologic marker for prostatic neoplasms. Cancer 48, 1129-1232.

Nadji M. and Morales A.R. (1982). Immunohistochemistry of prostatic acid phosphatase. Ann. N.Y. Acad. Sci. 390, 133-141.

Ormerod M.G., Steel K., Westwood J.H. and Mazzini M.N. (1983). Epithelial membrane antigen: partial purification assay and properties. Br. J. Cancer 48, 533-541.

Papotti M., Paties C., Peveri J., Moscuzza L. and Bussolati G. (1989). Immunocytochemical detection of prostate specific antigen (PSA) in skin adnexal and breast tissues and tumors. Basic. Appl. Histochem. 33, 25-29.

Pinkus G.S. and Kurtin P.J. (1985). Epithelial membrane antigen - a diagnostic discriminant in surgical pathology: Immunohistochemical profile in epithelial mesenchymal and hematopoetic neoplasm using paraffin sections and monoclonal antibodies. Human Pathol. 16, 929-936.

Potter van R. (1978). Phenotypic diversity in experimental hepatomas: The concept of partially blocked ontogeny. Br. J. Cancer 38, 1-23.

Purnell D.M., Heatfield B.M. and Trump B.F. (1984). Immunocytochemical evaluation of human prostatic carcinomas for carcinoembryonic antigen nonspecific cross-reacting antigen, ß-chorionic gonadotrophin, and prostate specific antigen. Cancer Res. 44, 285-292.

Servadio C., Abramovici A., Sandbank U., Savion M. and Rosen M. (1986). Early stage of the pathogenesis of rat ventral prostate hyperplasia. Eur. Urol. 12, 195-200.

Sloane J.P. and Ormerod M.G. (1981). Distribution of epithelial membrane antigen in normal and neoplastic tissue and its value in diagnostic tumor pathology. Cancer 47, 1786-1795.

Sternberger L.A. (1979). Immunocytochemistry. John Wiley and Sons. New York.

Tenniswood M., Bird C.E. and Clark A.F. (1976). Acid phosphatase: androgen dependent markers of rat prostate. Can. J. Biochem. 54, 350-357.

Tenniswood M., Abrahams P.P., Bird C.E. and Clark A.F. (1978). Effects of castration and androgen replacement on acid phosphatase activity in the adult rat prostate gland. J. Endocrinol. 77, 301-308.

Thomas P. and Battifora H. (1987). Keratins versus epithelial membrane antigen in tumor diagnosis: An immunohistochemical comparison of five monoclonal antibodies. Human Pathol. 18, 728-734.

Walvoored D.J., Resnick M.I. and Grayhack J.T. (1976). Effect of testosterone, dihydrotestosterone, estradiol and prolactin on the weight and citric acid content of the lateral lobe of rat prostate. Invest. Urol. 14, 60-65.

Accepted November 2, 1990