Identification of neutrophils in the nonsensory epithelium of the vomeronasal organ in virus-antibody-free rats

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Abstract. Cells infiltrating the nonsensory epithelium of the vomeronasal organ of virus-antibody-free rats exhibited surface immunoreactivity for β_2-microglobulin and immunoglobulin (Ig) E. They were further characterized by using immunohistochemical techniques with antibodies to cell-specific markers or histochemical techniques for immunocytes with surface receptors for IgE. Localization of intracellular granules immunoreactive for lactoferrin and CD18, a leukocyte adhesion molecule, unequivocally identified these cells as neutrophils. The low number of IgA- and IgG-immunoreactive B lymphocytes, T lymphocytes, and accessory immunocytes in the vomeronasal organ as well as the rest of the nasal cavity confirmed the absence of infection. We hypothesize that the operation of the vomeronasal pump induces repeated episodes of transient focal ischemia followed by reperfusion, which results in release of neutrophil chemoattractants and modulation of adhesion factors that regulate the extravasation and migration of neutrophils into the nonsensory epithelium. The distribution of immunoreactivity for interleukin 8 suggests that it is not the primary neutrophil chemoattractant in this system while that of CD18 suggests its active involvement in neutrophil extravasation. In addition to their role in immune surveillance, neutrophils may stimulate ion/water secretion into the vomeronasal lumen, affecting the perireceptor processes regulating stimulus access and clearance from the sensory epithelium.

Key words: Vomeronasal organ – Neutrophils – Perireceptor events – Rat (Sprague Dawley)

Introduction

The nasal mucosae are continually exposed to airborne pathogens and other immunogens. To contend with this, they are equipped with a complex defense barrier that in-

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cludes immune components such as B lymphocytes that secrete primarily immunoglobulin (Ig) A or G; T lymphocytes that secrete a variety of cytokines; leukocytes, mast cells, macrophages, and non-specific defense factors such as lactoferrin and lysozyme that are secreted by certain immunocytes and glands (for review, see Kraehenbuhl and Neutra 1992).

Numerous reports have described the immune barrier function of the respiratory mucosa (see, e.g. Poliquin and Crepeau 1985; Brandtzaeg 1985), and several have focused on immune barrier function in the olfactory mucosa (Getchell and Getchell 1991; Mellert et al. 1992). However, little attention has been paid to the vomeronasal organ (VNO). Both the olfactory and the vomeronasal receptor neurons provide a direct access route for pathogens from the nasal cavity into the brain. Although previous studies have noted the occurrence of cells referred to as leukocytes or lymphocytes in the nonsensory epithelium of the VNO (see, e.g., Luckhaus 1969; Breipohl et al. 1979; Loo and Kanagasuntheram 1972; Adams 1986), no attempt was made to characterize the infiltrating cells, and often the immune status of the experimental animals was not reported.

During the course of our study on the distribution of immune barrier components in the olfactory mucosa of virus-antibody-free rats (Getchell and Getchell 1991), a large number of cells immunoreactive for β_2-microglobulin, a membrane-localized class I major histocompatibility complex marker, were observed in the VNO primarily in the nonsensory epithelium and its lamina propria, and at the junctions between the sensory epithelium and nonsensory epithelium. Histologically, the nonsensory epithelium appeared intact, and no obvious signs of tissue damage, such as epithelial sloughing, resulting from inflammatory responses were observed. The purpose of this study was therefore to 1) identify the β_2-microglobulin-immunoreactive cells in the nonsensory epithelium, and 2) investigate chemotactic, adhesion, and protective factors that might be associated with the accumulation of the β_2-microglobulin-immunoreactive cells in the VNO of these virus-antibody-free rats.

a   β₂-m

b   IgG

c   IgE

d   IgE

e   Lactoferrin

f   H-E
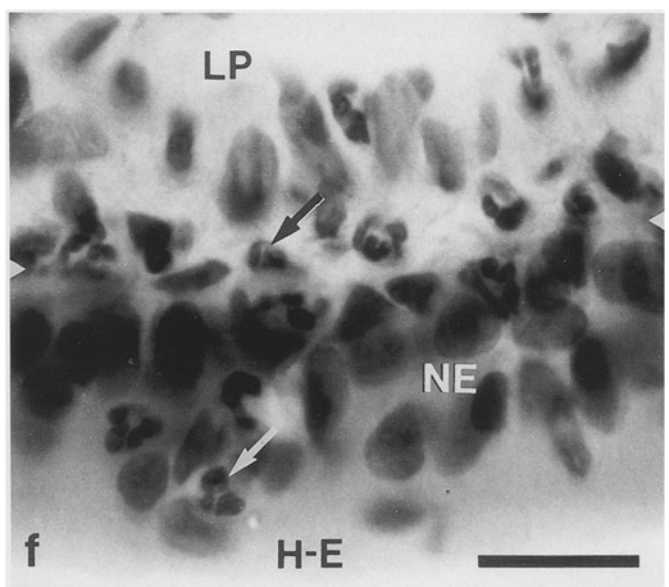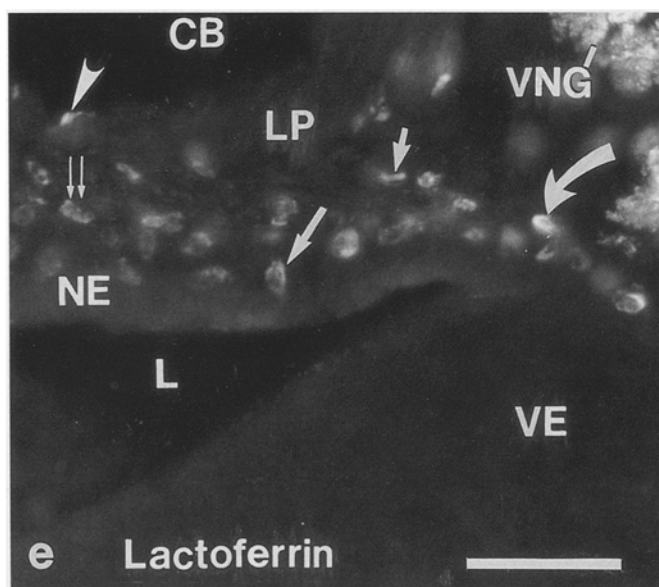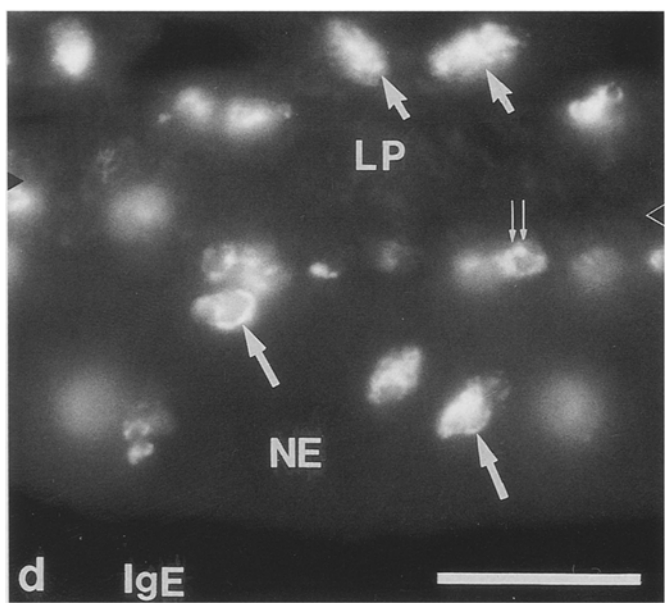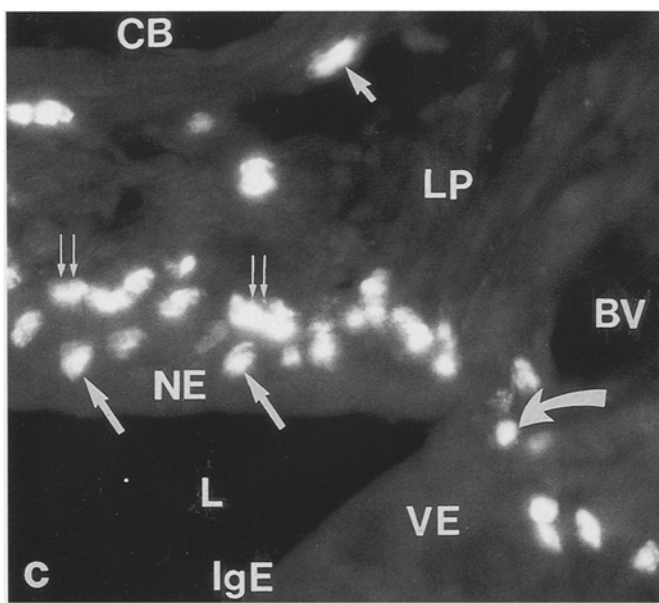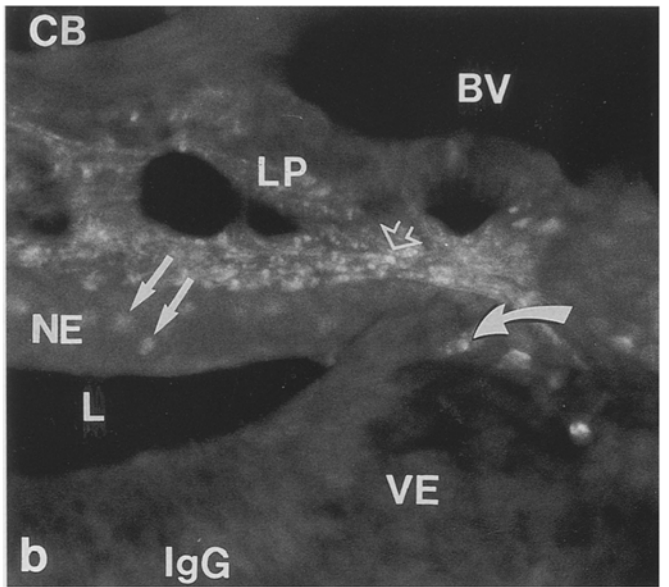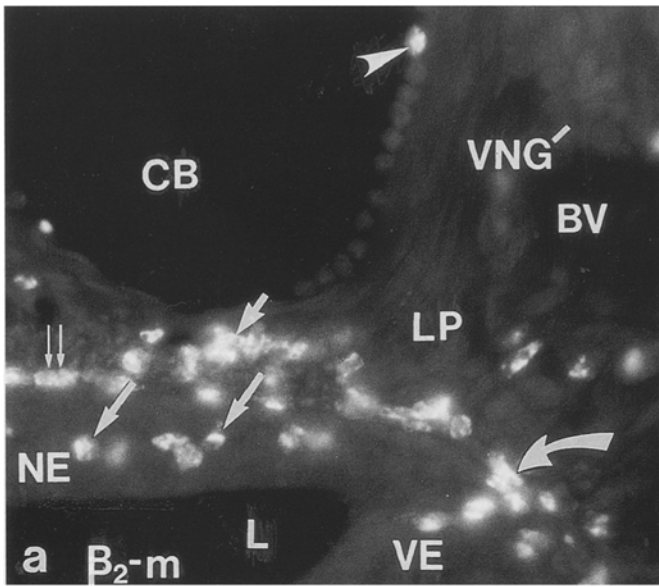
Materials and methods

Eight 3-5-week-old (60–140 g) virus-antibody-free male Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, Ind., USA or Charles River Laboratories, Wilmington, Mass., USA) and 2 pregnant virus-antibody-free Sprague Dawley rats (Harlan Sprague Dawley, Inc.) were shipped in double-screened filtered sterile boxes in environmentally controlled trucks and housed in autoclaved cages in a Bioclean unit with HEPA-filtered sterile, particle-free air in a facility maintained by the Department of Laboratory Animal Research (DLAR), University of Kentucky. Sentinel rats routinely monitored for common rat pathogens were housed in the same unit as the experimental animals and remained pathogen-free. All bedding, food, and acidified water provided for these rats were also autoclaved. The animals were maintained on a 12 h light:dark cycle. All rats were perfused within 2 weeks of their receipt. The rats had no detectable ecto- or endoparasites as determined by the suppliers. Serology performed by the suppliers and repeated by the DLAR on blood samples collected by cardiac puncture immediately before sacrifice revealed no detectable serum titers of antibodies against common rodent virus or bacterial pathogens. In addition, in order to localize and compare the occurrence and distribution of immunocytes in the nasal cavities of animals of different ages, rats at embryonic day (E) 19, and postnatal days (P) 2 and 11 were obtained from the pregnant rats as reported previously (Rama Krishna et al. 1994); the pups were kept in the same cage as their mother in the DLAR facility. The animals were brought to the laboratory in their cages immediately before use. For immersion-fixed tissues, the animals were anesthetized with Nembutal (80 mg/kg), the skull anterior to the posterior margin of the olfactory bulb (including the nasal cavity) was severed, cleaned of skin and muscles, and immersed in Zamboni's fixative for 2 h. Alternatively, anesthetized animals were perfused transcardially with physiological saline followed by Zamboni's fixative or 4% paraformaldehyde for 15 min; the anterior part of the skull was removed and postfixed in the appropriate fixative as described above. Tissues were cryoprotected sequentially in 10%, 20%, and 30% sucrose in phosphate-buffered saline (PBS, pH 7.4), embedded in OCT compound (Miles Inc., Elkhart, Ind., USA), and frozen. Fifteen-μm thick coronal sections of the nasal cavity were thaw-mounted onto gelatin-coated or Vectabond (Vector Labs, Burlingame, Calif., USA)-treated slides and stored at −20° C.

The antibodies used in this study are described in Table 1. Standard direct or two step immunofluorescence techniques as previously described (Getchell and Getchell 1991) were used for the antibodies to immunoglobulins, β_2-microglobulin and lactoferrin. Immunoperoxidase localization for the remaining antibodies was performed with Vectastain ABC kits (Vector Labs) and Histostain-SP kits (Zymed Labs, Inc., South San Francisco, Calif., USA) following the protocols provided with the kits. Briefly, sections were rehydrated in PBS, which was also used for rinses between incubations. Following application of PBS containing 0.4% Triton X-100 (Sigma Chemical Co., St. Louis, Mo., USA) and 1% globulin-free bovine serum albumin (BSA, Sigma Chemical Co.) for 30 min, sections were incubated with primary antibody in PBS containing 0.4% Triton X-100 and 1% BSA for 1 h; 0.1% Triton-X and 1% BSA were used with the antibodies to manganese (Mn) and copper-zinc (CuZn) superoxide dismutases. Sections were then incubated in the appropriate secondary antibodies, followed by quenching of endogenous peroxidase with 5% hydrogen peroxide in methanol or with 0.1% phenylhydrazine HCl, and incubated in the peroxidase conjugate. AEC or DAB was used as chromogen to visualize the reaction. Coverslips were mounted with glycerol-gelatin (Sigma Chemical Co.) for AEC or Permount (Fisher Scientific, Fair Lawn, N.J., USA) for DAB.

Biebrich scarlet histochemistry was used to identify eosinophils; briefly, sections were rehydrated in PBS, equilibrated in glycine buffer (pH 10.5), and stained for 1 h in 0.04% Biebrich scarlet (Sigma Chemical Co.) in glycine buffer (pH 10.5), rinsed in the same buffer and then in PBS (Spicer and Lillie 1961). Coverslips were mounted with glycerol-gelatin. Alcian blue at pH 1.0, which stains sulfated mucosubstances, was used to localize mast cells (Enerback et al. 1986); briefly, sections were rehydrated in PBS, stained for 1 h in Alcian blue G8X (Sigma Chemical Co.) in 0.1 N HCl (pH 1.0), rinsed in 0.1 N HCl, blotted with filter paper, and dehydrated. Coverslips were mounted with Permount. Hematoxylin and eosin staining was performed in the laboratory of the DLAR.

Negative controls consisted of omission of the primary antisera from the staining protocol and preadsorption controls. A 10–20-fold excess of β_2-microglobulin, lactoferrin, interleukin 8 (IL-8), and Mn and CuZn superoxide dismutase were incubated with their respective antibodies at the dilutions used in the staining protocols, incubated overnight at 4° C, and centrifuged; the supernatant was used in place of the primary antibody in the staining protocol. Staining of spleen and respiratory mucosa sections served as positive controls.

Fig. 1a–f. Identification and localization of immunocytes in the rat vomeronasal organ. **a** β_2-Microglobulin-immunoreactive cells (*long arrows*) lie close to the basement membrane (*double arrow*) in the nonsensory epithelium (*NE*), in the lamina propria (*LP*; *short arrow*), and at the junction between the nonsensory epithelium and the vomeronasal sensory epithelium (*VE*; *curved arrow*). A β_2-Microglobulin-immunoreactive cell (*arrowhead*) adhering to the endothelium of the cavernous body (*CB*) is observed. *BV* Blood vessel; *L* lumen; *VNG* vomeronasal glands. **b** Cell-surface staining for IgG is exhibited by cells distributed similarly to the β_2-microglobulin-immunoreactive cells in **a**. Weakly IgG-immunoreactive cells are located within the nonsensory epithelium (*small arrows*), and moderately IgG immunoreactive cells (*curved arrow*) are located at the junction between the nonsensory epithelium and the vomeronasal sensory epithelium. The connective tissue in the lamina propria is moderately immunoreactive for IgG (*open arrow*). **c** Cells intensely immunoreactive for IgE are localized in the nonsensory epithelium (*long arrows*) close to the basement membrane (*double arrows*) and in the lamina propria (*short arrow*) as well as at the junction between the nonsensory epithelium and vomeronasal sensory epithelium (*curved arrow*). **d** High magnification demonstrates that the IgE-immunoreactive cells in the nonsensory epithelium (*long arrows*) as well as those localized close to the basement membrane (*double arrows*) and in the lamina propria (*short arrow*) exhibit intense cell-surface staining. **e** Lactoferrin immunoreactivity is observed in the cells localized in the nonsensory epithelium (*small arrow*) and lamina propria (*short arrow*), close to the basement membrane (*double arrows*), and at the junction between the nonsensory epithelium and vomeronasal sensory epithelium (*curved arrow*) that are distributed similarly to those immunoreactive for IgE (**c**, **d**). A flattened neutrophil (*arrowhead*) is adhering to the wall of the CB. Secretory granules intensely immunoreactive for lactoferrin are observed in the VNG. **f** Hematoxylin and eosin staining demonstrates multilobed neutrophil nuclei (*arrows*) in the nonsensory epithelium and lamina propria. *Triangles* indicate the position of the basement membrane. *Bar:* a–c and e 50 μm, d 20 μm, f 15 μm

Results

Cellular organization of the vomeronasal organ

The VNO in rats is located at the base of the nasal septum in the anterior part of the nasal cavity. It consists of sensory epithelium and nonsensory epithelium separated by a fluid filled, crescent-shaped lumen (Fig. 1a) that communicates only with the nasal cavity through the vomeronasal duct. The sensory epithelium consists of vo-

Table 1. Antibodies used in this study. FITC, Fluorescein iso-thiocyanate-conjugated; ICAM, intercellular adhesion molecule; Ig, immunoglobulin; IL-8, inter-leukin 8; LFA, lymphocyte function associated antigen; m, monoclonal; p, polyclonal; rec, recombinant; SOD, superoxide dismutase; TRITC, tetramethyl rhodamine isothiocyanate-con-jugated

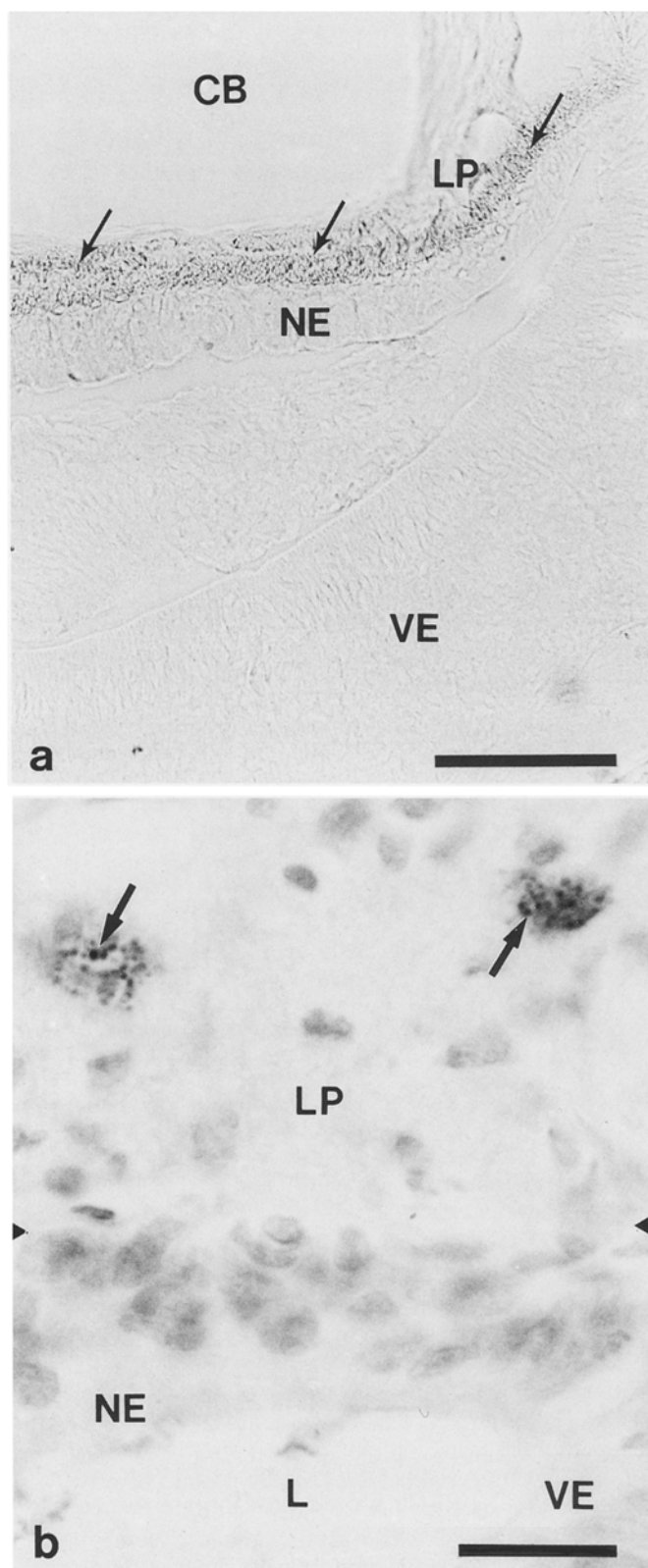| Primary antibody	Commercial source	Antigen source	Optimal dilutions
β_2-Microglobulin-FITC (p)	ICN	Human	1:30
ICAM 1 (CD54; m)	Serotec	Rat	1:30
IgA (p)	Sigma	Mouse	1:16
IgE-FITC (p)	Sigma	Human	1:30
IgG-FITC (p)	Jackson	Rat	1:30
IgM-TRITC (p)	Fisher Biotech	Rat	1:60
IL-8 (p)	Endogen	rec Human	1:50
Lactoferrin (p)	Accurate	Human	1:50
CD11a (LFA-1α chain; m)	Serotec	Rat	1:30
CD18 (LFA-1 β chain; m)	Serotec	Rat	1:30
Macrophage (ED2; m)	Serotec	Rat	1:200
MnSOD (p)	Biodesign	Human	1:250
CuZnSOD (p)	The Binding Site	Human	1:600
CD4 (helper T lymphocytes; m)	Serotec	Rat	1:50
CD8 (cytotoxic T lymphocytes; m)	Serotec	Rat	1:50

meronasal receptor neurons with axons in the lamina propria, sustentacular cells and basal cells. The nonsensory epithelium contains ciliated and nonciliated columnar cells. Vomeronasal glands are distributed along the rostrocaudal axis of the VNO at the boundary of the sensory epithelium and nonsensory epithelium and open into the lumen through ducts at the junction of sensory epithelium and nonsensory epithelium. A large blood vessel (BV), the cavernous body, and small arterioles, venules, and capillaries are situated lateral to the nonsensory epithelium in the lamina propria.

Localization of immunocytes in the vomeronasal mucosa

In the 3-5-week-old rats, cells intensely immunoreactive for β_2-microglobulin were clustered in the nonsensory epithelium (Fig. 1a) primarily near the basement membrane and at the junctions between the nonsensory and sensory epithelia. A few immunoreactive cells were also present in the lamina propria and adhering to the endothelial wall of the cavernous body (Fig. 1a). Rarely, a β_2-microglobulin-positive cell was observed in or just beneath the sensory epithelium. The localization of a band of immunoreactivity at the edges of many cells and a discontinuous punctate pattern of immunoreactivity over some cells indicated cell-surface staining. As a first step in identifying the β_2-microglobulin-immunoreactive immunocytes, we considered the possibility that the cells were either B or T lymphocytes, cell types commonly found in mucosae. To test these possibilities, sections were stained with antibodies to the α, γ, μ, and ε chains of IgA, IgG, IgM, and IgE respectively, whose cytoplasmic localization would identify the cells as Ig-producing B lymphocytes, and with antibodies to CD4 and CD8, which are membrane markers for helper and cytotoxic T lymphocytes, respectively (Table 1). Only one or two cells per section in the nonsensory epithelium were weakly immunoreactive for IgA in a pattern suggesting cell surface localization (data not shown). Additionally, a few cells exhibiting cytoplasmic IgA immunoreactivity were localized near the vomeronasal glands. Numerous

cells in the nonsensory epithelium and at the junctions between the sensory and nonsensory epithelium stained weakly in a pattern suggesting cell surface localization for IgG (Fig. 1b) and IgM (data not shown). The connective tissue in the lamina propria also exhibited IgG immunoreactivity. In contrast, numerous intensely stained IgE-immunoreactive cells (Fig. 1c, d) distributed similarly to the β_2-microglobulin-immunoreactive cells were observed in the nonsensory epithelium. Many were located near the basement membrane with a few close to the junctions of the sensory epithelium and nonsensory epithelium. In addition, several were present in the lamina propria in the connective tissue around the BVs (Fig. 1c), and adhering to the endothelial wall of the cavernous body. IgE-immunoreactive cells were also observed infrequently in the connective tissue ventral to the sensory epithelium and rarely in the sensory epithelium. The pattern of IgE immunoreactivity indicated cell-surface staining (e.g., Fig. 1d). This suggested that the cells were not IgE-synthesizing B-lymphocytes but rather cells with surface receptors for IgE. Few T lymphocytes were present. In the lamina propria of the nonsensory epithelium, 4–6 CD4+ and 1–2 CD8+ positive cells per section, most adhering to the endothelium of the cavernous body, were observed. Rarely, a single CD4+ and CD8+ positive cell was present in the lamina propria, and an occasional small cluster of 3–4 CD4+ cells but not CD8+ cells was observed in the nonsensory epithelium. No CD4+ or CD8+ positive cells were observed in or around the sensory epithelium or vomeronasal glands (data not shown). These results indicated that the infiltrating cells were not B or T lymphocytes but rather immunocytes with cell-surface receptor for Igs, particularly IgE.

Cell-surface receptors for IgE have been characterized on several types of immunocytes: eosinophils, mast cells, macrophages, neutrophils, basophils and platelets. Of these, eosinophils, mast cells, and macrophages normally occur in certain tissues in the absence of infection and were considered first. Biebrich scarlet at pH 10.5 stains both eosinophils and elastic fibres. No eosinophils were observed in the nonsensory epithelium. However,
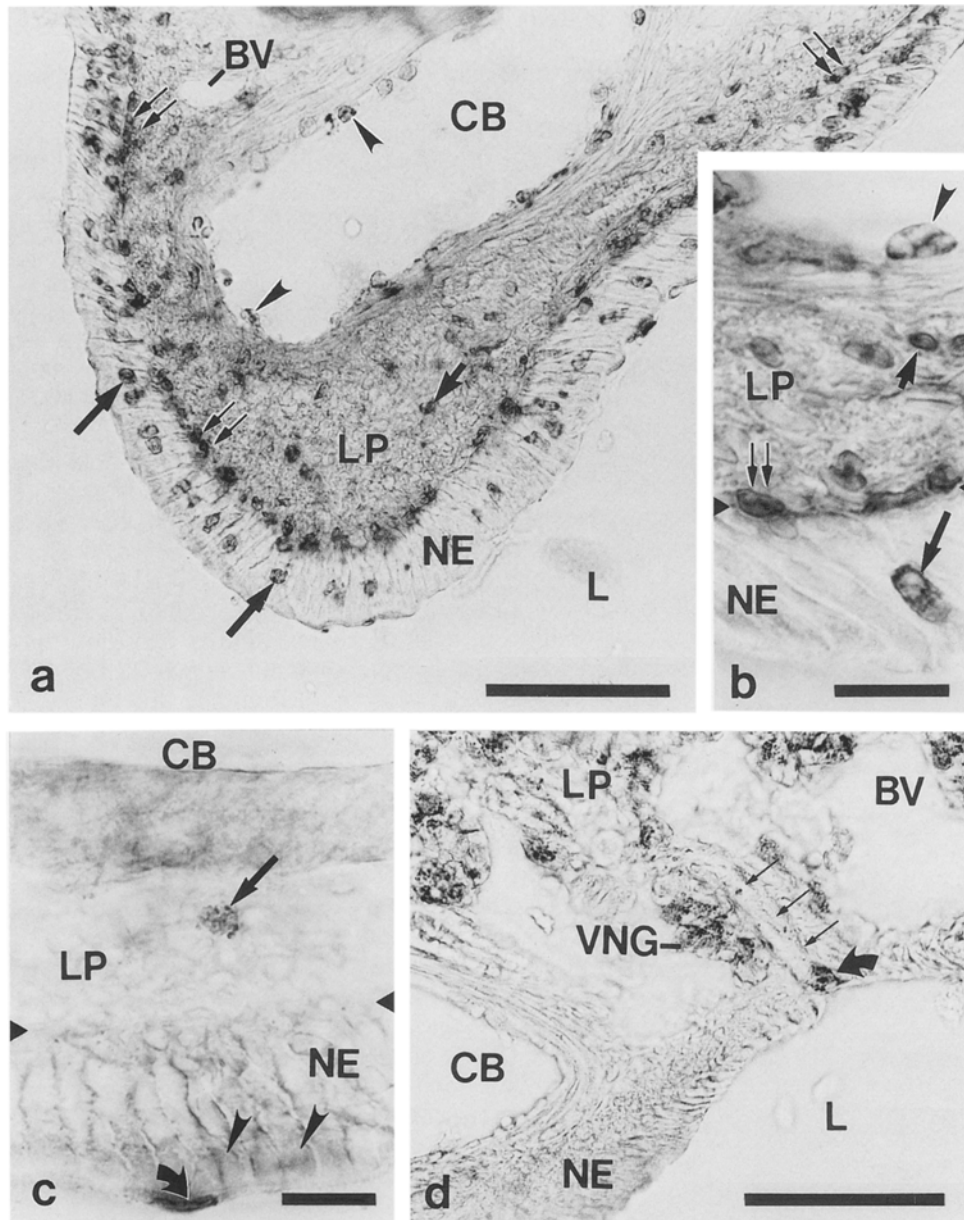
short elastic fibers that were evenly distributed throughout the lamina propria of the nonsensory epithelium surrounding the cavernous body were intensely stained (Fig. 2a). Alcian blue stained a few mast cells that were situated deep in the lamina propria of the nonsensory epithelium (Fig. 2b). Their distribution pattern was totally different from that of the β_2-microglobulin- and IgE-immunoreactive cells. No ED2-immunoreactive macrophages were observed in the nonsensory epithelium of the VNO, but 4 to 6 macrophages per section were localized near the vomeronasal glands. These results indicated that the infiltrating immunocytes were not eosinophils, mast cells, or macrophages.

Hematoxylin and eosin staining of the nonsensory epithelium revealed that the infiltrating immunocytes possessed distinctive multilobed nuclei (Fig. 1f), which most closely resembled the nuclei of neutrophils rather than basophils or platelets. To determine if these cells were neutrophils, immunoreactivity for lactoferrin, which among immunocytes is synthesized only by neutrophils, was investigated. Lactoferrin immunoreactivity was localized in cells distributed similarly to those immunoreactive for β_2-microglobulin and IgE (Fig. 1e), that is, in the nonsensory epithelium near the basement membrane and at the junction of sensory and nonsensory epithelia as well as in the lamina propria. The number of lactoferrin-immunoreactive cells in the nonsensory epithelium was comparable to that of the IgE-immunoreactive cells. These results, in conjunction with those described above for the other cell-specific antibody markers and histological stains, strongly suggested that the cells infiltrating the VNO of these virus-antibody-free rats were neutrophils.

In order to determine if the occurrence of the infiltrating immunocytes varied with the age of the animal, sections from E19, P2, and P11 rats were stained for β_2-microglobulin, IgE, and lactoferrin. No positive immunocytes were present in the VNO at E19. However, 2–3 immunoreactive cells/section were observed in the lamina propria near the cavernous body in the VNO at P2, and 3–5 immunoreactive cells/section were observed in the nonsensory epithelium by P11.

Localization of a potential chemotactic factor, adhesion molecules, and protective enzymes in the vomeronasal organ

In an attempt to determine what was causing infiltration of the uninfected nonsensory epithelium in these virus-antibody free rats by presumed neutrophils, the presence of a potent and specific neutrophil chemoattractant, IL-8, was investigated. Immunoreactivity for IL-8 was localized in the columnar cells of the nonsensory epithelium (Fig. 3c), in the granules and cytoplasm of the vomeronasal gland acini (Fig. 3d), and in the apical regions of their ducts (Fig. 3d). The surface of the nonsensory epithelium exhibited patchy but intense IL-8 immunoreactivity (Fig. 3c), whereas none was observed at the surface of the sensory epithelium. A few immunocytes adhering to the wall of the cavernous body ex-


Fig. 2a, b. Distribution of elastic fibers and mast cells in the nonsensory epithelium (*NE*) of the vomeronasal organ. **a** Biebrich scarlet-stained elastic fibers (*arrows*) are located in the lamina propria (*LP*) between the cavernous body (*CB*) and the nonsensory epithelium (*NE*). Note that no eosinophils are present in the nonsensory epithelium. *L* Lumen; *VE* vomeronasal sensory epithelium. **b** Alcian blue-stained granules (*arrows*) are seen in mast cells that are situated deep in the lamina propria. Note that no mast cells are present in the nonsensory epithelium. *Triangles* indicate the position of the basement membrane. *Bars:* **a** 100 μm, **b** 15 μm

Fig. 3a–d. Localization of CD18 and interleukin 8 (IL-8) immunoreactivity in the vomeronasal organ. **a** Immunocytes with intense CD18-immunoreactive membranes are observed in the nonsensory epithelium (*NE; long arrows*) and close to the basement membrane (*double arrows*). Numerous CD18-immunoreactive immunocytes (*short arrow*) are also present in the lamina propria (*LP*); they (*arrowheads*) are also observed adhering to the endothelium of the cavernous body (*CB*). **b** At higher magnification, intracellular granules exhibiting immunoreactivity for CD18 are observed in immunocytes in the nonsensory epithelium (*long arrow*) and lamina propria (*short arrow*) as well as those close to the basement membrane (*double arrows*) and adhering to the endothelium of the CB (*arrowhead*). **c** Weak IL-8 immunoreactivity is localized in the apical regions of columnar cells (*arrowheads*) in the nonsensory epithelium. Intense immunoreactivity is observed at the surface of the nonsensory epithelium (*curved arrow*). IL-8-immunoreactive granules (*arrow*) are present in a mast cell in the lamina propria of the nonsensory epithelium. **d** Secretory granules in the acini of the vomeronasal glands (*VNG*) as well as those (*curved arrow*) in the duct (*thin arrows*) are intensely immunoreactive for IL-8. *Triangles* indicate the approximate position of the basement membrane. *Bars:* **a, d** 100 µm, **b, c** 15 µm

pressed intense IL-8 immunoreactivity as did the granules in mast cells (Fig. 3c) that were localized near the cavernous body and small BVs. The infiltrating cells within the nonsensory epithelium were not immunoreactive for IL-8.

In an attempt to identify cellular adhesion mechanisms that might be involved in the infiltration of the nonsensory epithelium, immunoreactivity for CD18, a component of integrin adhesion complexes that are found on leukocytes, was investigated. Intense CD18 immunoreactivity was observed on cells distributed in the nonsensory epithelium like those that were immunoreactive for IgE and lactoferrin; these cells exhibited both surface staining and granular cytoplasmic staining (Fig. 3a, b). In adjacent sections, these cells exhibited no immunoreactivity for CD11a, a component of the LFA-1 adhesion factor. No specific immunoreactivity for the

endothelial cell-specific intercellular adhesion molecule (ICAM 1) was observed in the VNO.

Neutrophil infiltration is normally accompanied by histopathological changes in the tissue due to the release of neutrophil-derived oxygen free radicals. However, no histopathology was evident in the nonsensory epithelium. Oxygen free radical scavengers protect against tissue damage caused by neutrophil infiltration; among the oxygen free radical scavengers frequently found in tissues are the enzymes Mn and CuZn superoxide dismutase. Immunoreactivity for these enzymes was localized in the ciliated columnar cells of the nonsensory epithelium. Intense Mn superoxide dismutase immunoreactivity was localized at the base of these cells, with less intense but more granular immunoreactivity at their apices (Fig. 4a). A few cells demonstrated uniform immunoreactivity. Weaker staining was observed for CuZn superoxide
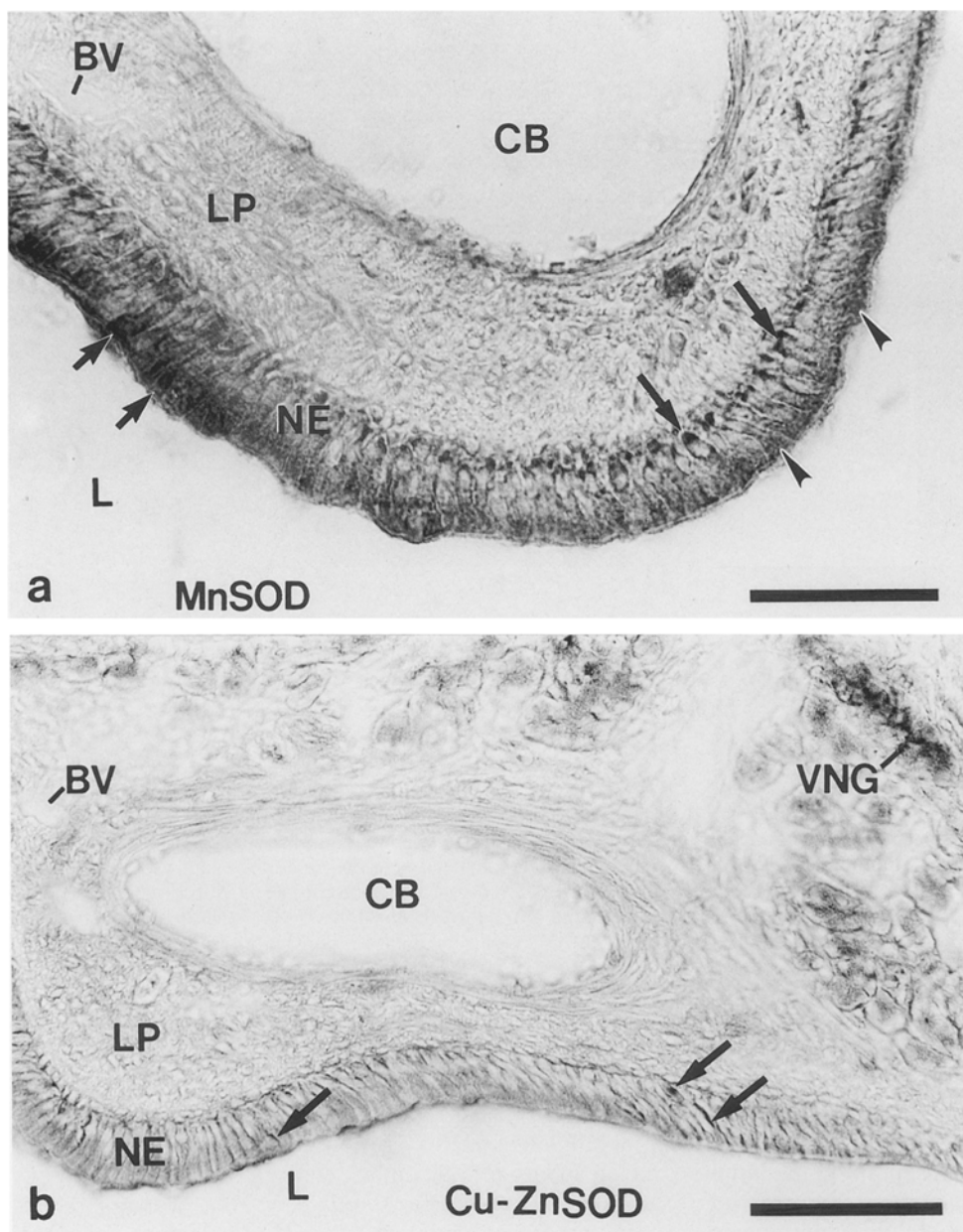
Fig. 4a, b. Localization of manganese (Mn) and copper-zinc (CuZn) superoxide dismutase immunoreactivity in the nonsensory epithelium (*NE*) of the vomeronasal organ. **a** Intense Mn superoxide dismutase immunoreactivity is localized in the foot processes (*long arrows*) with moderate immunoreactivity in apical regions (*arrowheads*) of the columnar cells. Some cells (*short arrows*) exhibited intense immunoreactivity throughout their cytoplasm. *BV* Blood vessel; *CB* cavernous body; *L* lumen; *LP* lamina propria. **b** Moderate CuZn superoxide dismutase immunoreactivity is observed in columnar cells (*arrows*) in the nonsensory epithelium. Intense CuZn superoxide dismutase immunoreactivity is localized in the acini of the vomeronasal glands (*VNG*). *Bars:* **a** 60 μm, **b** 100 μm

dismutase (Fig. 4b), with a few cells exhibiting moderate immunoreactivity in their basal regions. In addition, the vomeronasal glands exhibited moderate to intense Mn superoxide dismutase immunoreactivity (data not shown) and very intense CuZn superoxide dismutase immunoreactivity (Fig. 4b).

Comparative distribution of immunocytes in the olfactory and respiratory mucosae

The presence of infiltrating immunocytes in a tissue suggests the presence of infection. Because the rat's VNO communicates only with the nasal cavity, we examined the distribution of immunocytes in the nasal cavity to determine if there were any indications of infection. The olfactory mucosa contained very few immunocytes. On-

ly one β_2-microglobulin-immunoreactive cell was observed within the olfactory epithelium in one of the rats. A few β_2-microglobulin- and IgE-immunoreactive cells per section were observed in the lamina propria in proximity to BVs and Bowman's glands, or occasionally adhering to the endothelium of a BV (Fig. 5a, b). Lactoferrin-immunoreactive immunocytes were observed at locations similar to those of IgE-immunoreactive cells. A few Bowman's glands expressed weak immunoreactivity for lactoferrin. About 2–4 eosinophils per section were observed in the lamina propria (Fig. 5c), usually near the roof of the nasal cavity. A few mast cells but no ED2-immunoreactive macrophages were also observed in the lamina propria.

Compared to the olfactory mucosa, the respiratory mucosa contained a larger number but still relatively few immunocytes. In the septal respiratory mucosa, there
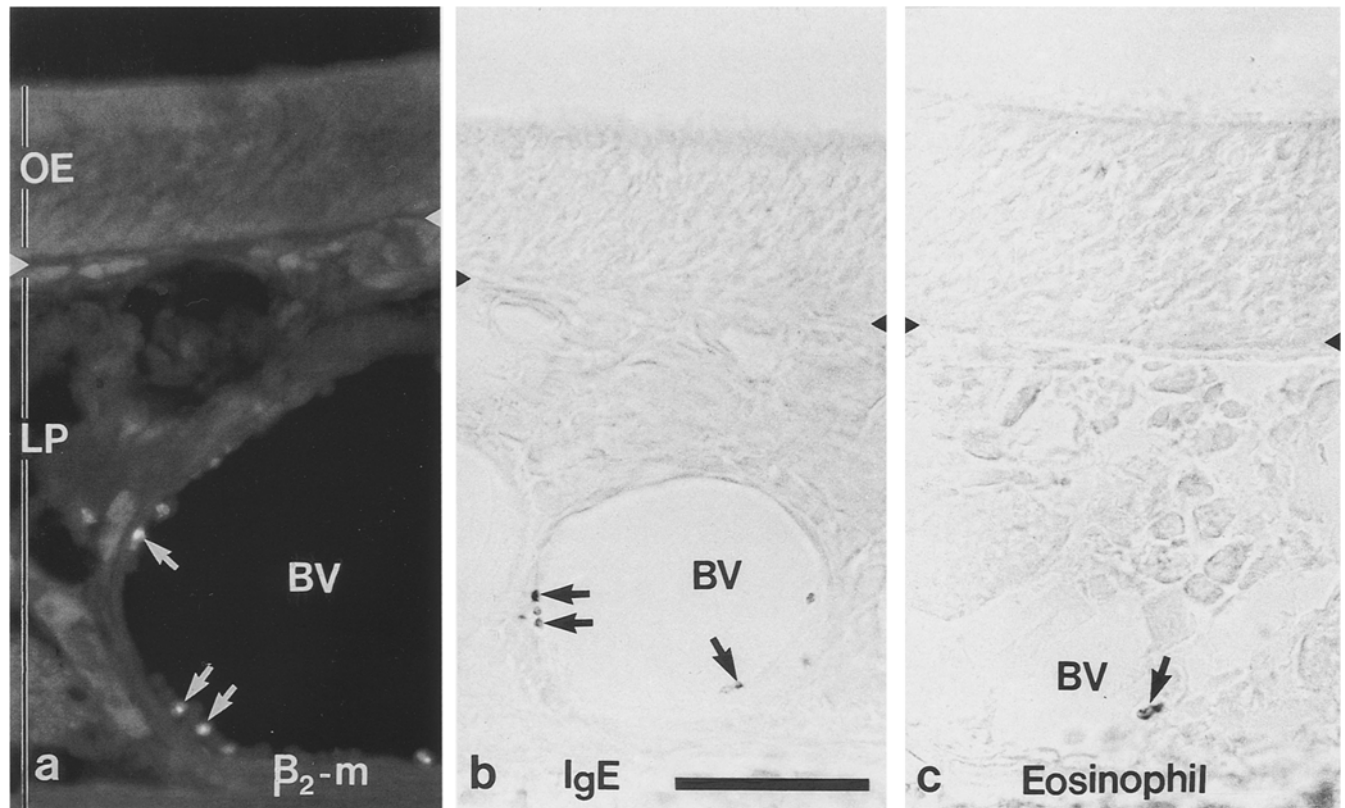
Fig. 5a–c. Localization of immunocytes in the olfactory mucosa of the nasal septum. **a** β_2-Microglobulin-immunoreactive cells (*arrows*) and **b** IgE-immunoreactive cells (*arrows*) adhere to the wall of a large blood vessel (*BV*) near t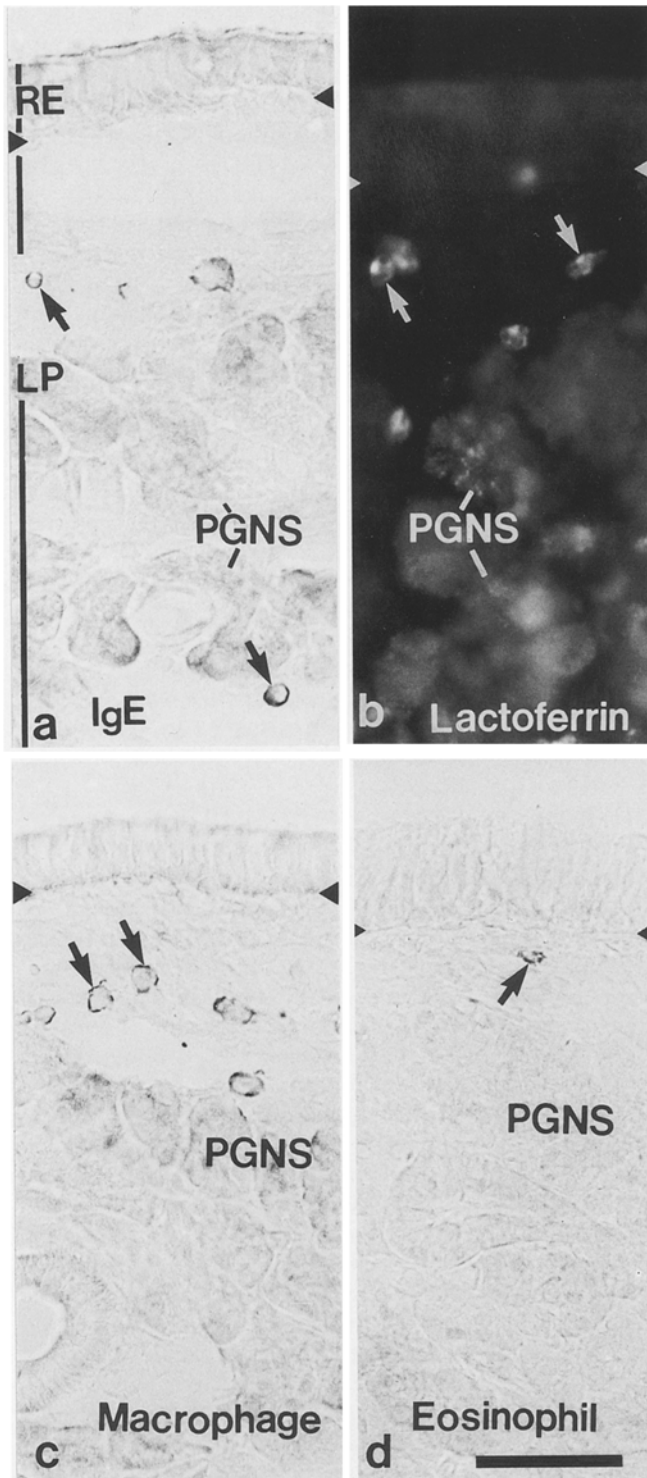he base of the lamina propria (*LP*). *OE* Olfactory epithelium. **c** A single, flattened eosinophil (*arrow*) is localized near the base of the lamina propria near a BV. *Triangles* indicate the approximate position of the basement membrane. *Bar:* **a–c** 50 μm

were several IgE-immunoreactive (Fig. 6a) and lactoferrin-immunoreactive (Fig. 6b) immunocytes. There were also a moderate number of macrophages (Fig. 6c), and very few eosinophils (Fig. 6d) and mast cells (data not shown). These immunocytes were primarily situated near the posterior glands of the nasal septum in the lamina propria with one or two located near the anterior glands. Rarely a single intraepithelial IgE-immunoreactive cell was observed. In the respiratory mucosa on the turbinates, several IgE- and lactoferrin-immunoreactive cells, macrophages, and eosinophils were localized in the lamina propria, often situated in and around BVs and respiratory glands. The secretory granules in the posterior glands of the nasal septum exhibited intense immunoreactivity for lactoferrin (Fig. 6b) and IL-8, whereas weak expression of β_2-microglobulin was observed on the gland cell membranes. A few IL-8-immunoreactive mast cells were observed in the lamina propria of the respiratory mucosa located ventral to the VNO capsule and on the lateral walls of the nasal cavity. Weak IL-8 immunoreactivity was observed in the supranuclear region of the columnar cells of the respiratory epithelium. The low numbers of immunocytes in the olfactory and respiratory mucosae and in particular the extremely low frequency of intraepithelial lymphocytes is strong confirmatory evidence for the lack of antigenic stimulation in the nasal cavities of these animals.

Controls

Omission of the primary antibody from the staining protocol and preadsorption of the antibodies to β_2-microglobulin, lactoferrin, Mn superoxide dismutase, and CuZn superoxide dismutase with the respective antigens resulted in no specific staining, whereas a major reduction in the staining intensity for IL-8 was observed when the antibody was preadsorbed with antigen. Weak nonspecific staining of the respiratory glands and the mucociliary and mucomicrovillar complexes in the VNO, probably due to basal levels of synthesis and secretion of peroxidase by the glands (e.g., Watanabe and Harada, 1990), persisted even when the primary antibody was omitted from the staining protocol or was preadsorbed with its antigen. Macrophages exhibiting intense peroxidase staining in all peroxidase-based protocols were observed in moderate numbers in myeloid tissue, in low numbers at the periphery of the nasal-associated tissue, and infrequently within the connective tissue of the laminae propriae of the nasal mucosae as described above. Spleen sections, which served as positive controls, exhibited the characteristic distribution of immunocytes (see e.g., Sprent, 1993).

ed in this study by Biebrich scarlet staining. The pump functions to draw stimulus-bearing air or mucus from the nasal cavity into the fluid-filled lumen of the VNO and expel its contents back into the nasal cavity, ensuring access of stimuli to and their clearance from the sensory epithelium (Meredith and O'Connell 1979; Eccles 1982; Meredith 1982). To activate the pump, adrenergic neuroeffector(s) are released from the sympathetic innervation of the BVs in the VNO (Meredith and O'Connell 1979), causing vasoconstriction. Hypothetically, the resultant reduced blood supply due to vasoconstriction of BVs in the lamina propria of the nonsensory epithelium would cause mild ischemia. This could stimulate the local release of several substances that activate perivascular C fibers, as demonstrated in cerebral ischemia (e.g., Moskowitz et al. 1988). The BVs and nonsensory epithelium in the VNO are densely innervated by C fibers derived from the nasopalatine branch of the trigeminal nerve as well as by autonomic fibers. The fibers innervating the BVs are immunoreactive for substance P (Finger et al. 1989), calcitonin gene-related peptide (CGRP, Silverman and Kruger 1989), and nitric oxide synthase (Kulkarni et al. 1994). When activated, these fibers would release the vasodilators substance P and CGRP, as has been demonstrated in cerebral ischemia (e.g., Macfarlane et al. 1991a, b), and presumably, nitric oxide. The ensuing vasodilation would result in reperfusion. Substance P additionally might act as a chemoattractant, while both substance P and CGRP might also stimulate neutrophil adherence to the endothelium (see below), resulting in neutrophil infiltration of the lamina propria and nonsensory epithelium.

Immunocyte infiltration of the VNO appears to be a general phenomenon among mammals in which the VNO is enclosed in a bony capsule and is organized in such a way that cavernous tissue can act as a pump. The infiltrating immunocytes, never previously characterized by the expression of cell-specific markers, in the nonsensory epithelium of the VNO has been previously observed in several mammalian species: rats (Breipohl et al. 1979), guinea pigs (Fig. 2, Ciges et al. 1977), dogs (Luckhaus 1969; Adams and Wiekamp 1984), gnotobiotic calves (Adams 1986), and tree shrews and slow lorises (Loo and Kanagasuntheram 1972). However, no infiltrating cells were observed in the human VNO (Getchell et al. 1993), which lacks a bony capsule and is located within the lamina propria of the respiratory mucosa with no special relationship to large BVs (Johnson et al. 1985; Stensaas et al. 1991). Additionally, the number of neutrophils in the nonsensory epithelium might be expected to increase as the animal ages, with continuous or repeated activation of the pump over time. Our results demonstrate such a relationship in virus-antibody-free rats, with no neutrophils present before birth, a gradual accumulation in the first few days of life, and large numbers in young adults.

Neutrophils infiltrate tissue due to the presence of chemoattractants. A specific attractant for neutrophils is IL-8 (Baggiolini and Clark-Lewis 1992), which has been implicated in reperfusion injury in respiratory tract ischemia (Sekido et al. 1993). IL-8 was localized in vomero-

nasal and posterior septal glands, in columnar cells in the nonsensory epithelium, and at the surface of the nonsensory epithelium, consistent with its previously reported localization in glands (Basolo et al. 1993; Anttila et al. 1992) and in human nasal epithelial cells (Bradding et al. 1993; Becker et al. 1993). However, this distribution of IL-8 suggests that it does not act as the primary neutrophil chemoattractant in the postulated ischemia/reperfusion-induced infiltration. Alternatively, substance P is also a chemoattractant for neutrophils, in some cases promoting neutrophil infiltration but not activation (e.g., Haines et al. 1993). As discussed above, substance P has been localized in fibers innervating the cavernous body, BV, and nonsensory epithelium; its release during ischemia would result in a distribution consistent with a possible role as a chemoattractant.

Neutrophils infiltrating a tissue must first adhere to the endothelium of a BV in the tissue and then migrate through the endothelium and perivascular tissue. Specific molecules expressed by both the endothelium and neutrophils in a precise temporal sequence regulate these processes (for review, see Butcher, 1992). The initial "capture" and rolling of neutrophils is mediated by selectins, such as ELAM-1, on endothelial cells and their ligands on the neutrophils. Subsequent firm adhesion is mediated by adhesion factors, such as ICAM-1, on endothelial cells and its ligands, such as LFA-1 (CD11a/CD18) and MAC-1 (CD11b/CD18), on neutrophils. The level of expression of ICAM-1 is not affected by conditions simulating ischemia (Arnould et al. 1993). However, the level of CD18 on circulating rat neutrophils is significantly upregulated following hepatic ischemia and reperfusion (Jaeschke et al. 1993), and antibodies that block CD18 interaction with its receptor prevent ischemia/reperfusion-induced neutrophil infiltration (Jaeschke et al. 1993). The intense CD18 immunoreactivity observed on the surface of the neutrophils in the VNO is consistent with its role in their adhesion and migration into the nonsensory epithelium. The localization of CD18 but not CD11a on these neutrophils suggests that the MAC-1 complex may be involved in adhesion and extravasation of these neutrophils as has been demonstrated in intestinal epithelial monolayers (Parkos et al. 1991). CGRP, which may be released from trigeminal innervation in response to ischemia as discussed above, has also been demonstrated to increase the adherence of neutrophils to endothelium (e.g., Sung et al. 1992). The timing of neutrophil adhesion, migration, and accumulation relative to ischemia/reperfusion and the identification of other adhesion factors that play a role in infiltration of the VNO are questions of interest for future studies.

Activated neutrophils release proteolytic enzymes and generate superoxide free radicals, resulting in tissue damage (e.g., Gallin, 1984). The absence of apparent tissue histopathology in the nonsensory epithelium, with no signs of cell sloughing or epithelial damage, strongly suggests that the neutrophils infiltrating the tissue were not activated. One possible explanation for the apparent lack of activation is that the neutrophils may migrate through the nonsensory epithelium and into the lumen

before activation occurs. It has been demonstrated that neutrophils isolated from ischemic liver after 1 hour of reperfusion do not secrete superoxide radicals or cause observable tissue necrosis but those isolated after 5 hours of reperfusion do (Jaeschke et al. 1990; Jaeschke and Farhood 1991). However, because we did not measure indicators of neutrophil activation and mediators of tissue damage, the possibility exists that a low level of activation might occur. At least 2 superoxide scavenging systems that might protect the BVs and the nonsensory epithelium from damage have been localized in the VNO. We have previously demonstrated the dense innervation of the cavernous body and small BVs in the lamina propria of the nonsensory epithelium by nitric oxide synthase-immunoreactive fibers (Kulkarni et al. 1994). The endothelial and possibly neuronal release of nitric oxide, which scavenges superoxide free radicals (Kubes et al. 1993), may reduce the occurrence of tissue damage. Also implicated in inactivating the highly reactive and destructive oxygen free radicals are the superoxide dismutases, whose expression is upregulated in hippocampal glia (Liu et al. 1993) during ischemia/reperfusion and which prevent or lessen superoxide damage when added to ischemic tissue (e.g., Uyama et al. 1992). The epithelial cells in the nonsensory epithelium exhibited strong immunoreactivity for both Mn and CuZn superoxide dismutase, suggesting that even if a low level of neutrophil activation were occurring, tissue damage due to superoxide anions might be minimal due to the high levels of these enzymes.

Could the neutrophils in the nonsensory epithelium have an effect on its function? Migration of neutrophils across intestinal epithelial monolayers reduces the transepithelial resistance through effects on transcellular and paracellular pathways. The transcellular resistance decrease is due to the early stimulation of chloride and obligatory water secretion (Parkos et al. 1992) by 5'-AMP, a neutrophil-derived secretagogue (Madara et al. 1992, 1993). Subsequently, a paracellular resistance decrease results from a transient increase in the permeability of tight junctions between the epithelial cells (Nash et al. 1987). The nonsensory epithelium is essentially an epithelial monolayer (e.g., Breipohl et al. 1979) whose apical surface consists of a mucociliary complex in contact with the fluid in the VNO lumen. A neutrophil-derived secretagogue may stimulate a transcellular movement of chloride ion and water into the VNO lumen that, along with secretions from the vomeronasal glands, contributes to the maintenance of fluid in the lumen, which is likely to be essential to the delivery of stimuli to and their clearance from the sensory epithelium. In addition, the neutrophils provide immune surveillance for the presence of pathogens in the material drawn from the nasal cavity into the VNO and can act as a first line of defense in preventing their invasion of the VNO.

In summary, we have characterized the cells that infiltrate the nonsensory epithelium of virus-antibody-free rats as neutrophils. We hypothesize that focal transient ischemia/reperfusion resulting from the activity of the vomeronasal pump may be responsible for this phenomenon and provide supporting evidence in the localization

of relevant adhesion molecules as well as enzymes that might protect against tissue damage caused by activated neutrophils. We further propose that neutrophils in the nonsensory epithelial monolayer may induce electrolyte/water secretion and play a role in the perireceptor processes regulating stimulus access and clearance from the lumen of the VNO.

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