Sensory Innervation of Conjunctival Lymph Follicles in Cynomolgus Monkeys

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Purpose. The importance of neuroregulation of immunoresponsiveness is recognized, but little is known of the innervation of conjunctival follicles. The access and distribution of nerves in follicles of the palpebral conjunctiva were therefore studied and those of trigeminal nerve origin distinguished.

Methods. Serial sections of follicles were prepared for light and selected sections for electron microscopy. Intracranial lesions were made in ophthalamic or both ophthalamic and maxillary nerves several days before fixation in three of the six monkeys used and their distribution in follicles identified by induced degeneration.

Results. Fine nerves penetrated follicles and terminated on arterioles, smaller blood vessels, and rarely on high endothelial venules. Other nerve branches entered the follicle parenchyma, conducted, and terminating in fine reticular fibers. Many terminals were identified as autonomic on morphologic grounds. Few terminals were in direct contact with lymphocytes and none were found in germinal centers. Other fibers terminated in the follicle associated epithelium. A large fraction of the nerve fibers displayed degenerative changes after lesions and epithelial terminals were no longer present.

Conclusions. Nerve distribution is mostly similar to that found in other lymphoid organs with the exception of the epithelial terminals, which are described for the first time in mucosa-associated lymphoid tissue and identified as sensory. Because epithelial terminals virtually were absent from the surrounding unspecialized epithelium, it is likely that those of the follicular epithelium have a specific immune system-related function. They may represent a follicle-alerting mechanism to surface stimuli. Invest Ophthalmol Vis Sci. 1997;38:884-892.

Methodology

All animals were maintained and killed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Six cynomolgus monkeys (Macaca fascicularis), two of them female, weighing 2.6 to 6 kg were prepared for study. Two were colony bred and four were caught wild. The estimated ages were as follows: three animals, 3 to 7 years; two animals, 7 years; and one animal, 8 years. Nerve lesions were made in three, a fourth was not operated on, and in two, wheat germ agglutinin and horseradish taining nerve fibers were noted in monkey lymphoid follicles incidental to a study of Meibomian gland innervation.11 During the course of a recent work by one of us,12 nerve terminals were noted in conjunctival associated lymphoid tissue. These have been examined further, their relation with parenchymal cells and their systemic characteristics noted, and experiments undertaken to seek evidence of a sensory innervation; the results are reported here.

METHODS

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peroxidase had been injected unilaterally into the superior tarsal muscle for the purposes of another experiment. Sides not operated on of the latter two animals and both sides of the others were used.

The ophthalmic nerve was severed in three animals together with the maxillary nerve in one of them, using an intracranial approach. Details of the method used are described in an earlier publication. The lesions were on the left side, and material from the right side was used as control specimens. Animals were killed 3, 7, and 8 days after the lesions were made. The postoperative intervals were selected for another study; otherwise, I week or more would have been chosen for all three animals, but preliminary tests showed that the 3-day interval was adequate for induction of degeneration. Because the maxillary nerve probably contributes to inferior conjunctival innervation in cynomolgus monkeys, only material from the superior conjunctiva after ophthalmic neurotomy (the 7- and 8-day animals) was used. The conjunctiva had a normal appearance in each animal throughout the experimental period.

Animals were sedated with 2 to 3 mg/kg ketamine and anesthetized with 15 to 25 mg/kg sodium pentobarbital delivered through a saphenous vein or intraperitoneally and injected with the anticoagulant heparin sodium (1500 IU). External jugular veins and inferior vena cavae were cut, and 2% glutaraldehyde, 3% paraformaldehyde cacodylate-buffered solution (pH 7.4) was perfused through the left ventricle of the heart. Heads were stored in fixative at 4°C and dissected while immersed in buffered sucrose solution.

The conjunctiva was cut close to the limbus and the bulbar conjunctiva separated from the eye. A circular skin incision was made close to the orbital margin to free the eyelids and their deep attachments cut. The eyelids together with the complete conjunctiva were removed and the canthi cut to separate upper and lower eyelids. The anterior layers of the eyelids were removed by dissection and discarded, retaining the tarsal plates with the conjunctiva. To show follicles adequately, it was found necessary to remove part or all of the superior and inferior tarsal muscles.

Tissues were postfixed in 1% unbuffered osmium tetroxide and dehydrated in graded solutions of ethanol. At this stage, follicles clearly were visible. Most of them were found in the orbital portion of the palpebral conjunctiva, and none was present in the bulbar region. The upper and, in most animals, the lower orbital conjunctiva attached to a narrow strip of adjacent tarsal conjunctiva were dissected free, cleared in xylene, and embedded in Epon 812 (CIBA, Cambridge, UK).

Follicles were cut from blocks either singly or in short rows and interrupted semithin serial sections cut transversely to the plane of the conjunctiva. Intervals between collected sections varied from 4 μm (every fifth section) to 60 μm and, where appropriate, full serials were collected. They were stained with 1% toluidine blue in an equal volume of 2.5% sodium carbonate. At least three thin sections of each follicle, passing through the follicle-associated epithelium, were prepared for electron microscopy, mounted on copper grids, immersed in a saturated solution of uranyl acetate in 70% ethanol for approximately 20 minutes, washed, and then immersed in 0.4% lead citrate in 0.1 N sodium hydroxide for approximately 15 minutes.

RESULTS

Nerve fibers were present in all but one of the 49 follicles examined, one to four small nerves entering at or close to the base (Figs. 1, 2). Nerves contained several small myelinated and unmyelinated fibers averaging four and eight, respectively. They often entered the follicle with an arteriole, dispersing quickly, with myelin sheaths usually terminating shortly after entry. Other nerve fibers entered the follicle opposite the dome (Fig. 1) or from a subepithelial position as single unmyelinated nerve fiber bundles. Varicose nerve fiber terminals containing large aggregates of small granular vesicles and some with small granular vesicles were present in the walls of arterioles (Fig. 3). A large minority of capillaries, with or without a partial covering of pericytes, also received terminals, but none of them contained small granular vesicles (Fig. 4). Varicose nerve fiber bundles occasionally were found in or close to the walls of high endothelial venules (HEV) (Figs. 1, 4), including a few with small granular
vesicles, but most appeared to be uninnervated even when substantial lengths of HEV walls were available for study, as in the example shown in Figure 1.

A majority of nerve fibers were distributed to the parenchyma within fine strands of the follicular reticular system. Reticular fibers were composed of tightly packed collagen enclosed by thin reticular cell processes (Fig. 5). Nerve fiber bundles were therefore generally isolated from the neighboring lymphocytes and other leukocytes (Fig. 5). But the cellular lining of the reticular fibers often was incomplete and occasionally where this occurred, nerve fiber terminals contacted lymphocytes (Fig. 6). Contact with macrophages, plasma cells, and follicular dendritic cells was not observed. Both primary and secondary (with a germinal center) follicles shared this innervation pattern, and all areas of follicles, with the exception of the germinal centers, were innervated, although the distribution of fibers often was uneven. The frequency of parenchymal nerve fibers varied widely, and although full counts were not undertaken, an approximate comparative assessment was made by counting nerve fiber bundles present within grid spaces in the electron microscope. Scores varied at least 12-fold with from two to three bundles each grid space (size, 72 \( \mu \text{m}^2 \)) to one every fourth space. The innervation density was similar in all follicles from individual animals.

Most nerve fiber varicosities contained more or less packed vesicles predominantly of the small agranular type, whereas a minority contained aggregated mitochondria filling most of the space of the varicosity with few or no vesicles (Fig. 7). There usually was little difficulty in assigning a varicosity to one or another category, although there were instances of varicosities...
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FIGURE 5. A large nerve fiber bundle enclosed by collagen and a thin layer of reticular cell processes (arrow) and framed by lymphocytes. One of the terminal profiles (arrow-head) contains separate groups of vesicles and mitochondria. Bar = 1 \( \mu \)m.

A nerve terminal (T) has pierced the reticular wall (arrows) and contacts a lymphocyte (L). Other axons enclosed by a Schwann cell (S) remain within the reticular fiber. Bar = 0.5 \( \mu \)m.

FIGURE 6. A nerve terminal with packed mitochondria, separated from a lymphocyte (L) by thin Schwann cell and reticular fiber processes (arrows). Bar = 1 \( \mu \)m.

Nerve fibers entered the epithelium either from the dome of the follicle or from branches of the nerve fibers entering the follicle from a subepithelial position. They invariably penetrated the follicle-associated epithelium in a zone peripheral to the basement membrane-free central area. Beyond this zone, the epithelium contained a continuous layer of goblet cells (Fig. 1) in which, apart from opposite the first cell or so, no nerve fibers penetrated. Substantial areas of adjacent mucous membrane were available for examination, and although subepithelial nerves were observed occasionally, only a single instance of fiber penetration of the epithelium was found.

Nerve fibers shed their Schwann cells on entering the epithelium, except occasionally where the basement membrane of the epithelium and the Schwann cell remained attached, presumably as the nerve fiber entered the layer or when a fiber lay close to a penetrating leukocyte where the epithelial basement membrane was missing (Fig. 8). The naked axons mostly lay adjacent to the basement membrane, but others penetrated deeper into the epithelium (Figs. 9, 10). None was seen in the superficial layers. All of them lay close to and often enclosed tightly by epithelial cells and never were found in contact with infiltrated basement membrane was missing over a length several epithelial cells in width, and peripheral to the central zone, further small breaches of the basement membrane by single leukocytes occurred. Cellular organization of the epithelium often was interrupted by lacunae, either empty or containing a leukocyte. Only a thin lamellar epithelial cell extension was interposed between the uppermost lacunae and the surface.

Characteristic modification of the epithelium covering the dome of the follicle (the follicle-associated epithelium) consists of a goblet cell-free zone infiltrated by lymphocytes and macrophages (Fig. 1). At its center, where infiltration was most noticeable, the containing separate aggregations of vesicles and mitochondria. An example is indicated in Figure 5.

FIGURE 7. A nerve terminal with packed mitochondria, separated from a lymphocyte (L) by thin Schwann cell and reticular fiber processes (arrows). Bar = 1 \( \mu \)m.
leukocytes. There was an immediately obvious difference in epithelial compared with parenchymal nerve terminals. All the varicosities were of the aggregated mitochondrion type with few or no vesicles (Figs. 8, 11). Only a single exception, in which vesicles formed

the principle content, was found among a total of well in excess of 200 epithelial terminals seen in this study. Between varicosities, the axons contained neurofilaments and a few microtubules.

In most instances, nerve fibers were found on both sides of the basement membrane-free central area in single sections and repeated in consecutive sections of the three or four sets prepared for each follicle. From zero to five axons were present in single sections, two or three being the most common. However, in one follicle from a control preparation and two others from the group not operated on with parenchymal fibers, no epithelial axons were found in any of the sections. Generally, follicles with fewest epithelial axons had the least-dense parenchymal innervation. From these observations, it became clear that the axons were disposed in a ring around the center of the follicle-associated epithelium.
FIGURE 12. Light micrograph of a follicle nerve (arrow) showing disorganized myelin indicating degeneration. Postoperative period, 3 days. Bar = 10 μm.

Operated Material
After ophthalmic or ophthalmic-maxillary neurotomy, inspection of nerves as they entered the base of follicles showed degeneration in most of the myelinated fibers and in a minority of the unmyelinated fibers (Figs. 12, 13). In favorable sections, partly degenerated nerves could be followed into the follicle to their division, where fibers dispersed in the parenchyma (Fig. 14). Deeper within the follicle, where nearly all fibers were unmyelinated, evidence of degeneration was identified less readily because axoplasm was lost and only Schwann cells remained, which often were indistinguishable from reticular cells. Some unmyelinated bundles had a mixed content of axons, and degeneration was observed in a proportion of them. Many nerves of the reticulum were of normal appearance, but vesicles were the predominant organelle of nearly all the varicosities and few were mitochondrion filled. The balance between the two types clearly had changed. The parenchymal cells apparently were unaffected by the lesions.

Epithelial axons were absent from 17 of 18 follicles from the three sides operated on, but a single varicosity was noted in one. The difference in incidence of epithelial axons between sides operated on and control sides was highly significant (Table 1).

DISCUSSION
General Innervation
Lymph follicles appear to be present regularly in the conjunctiva of young adult and adult cynomolgus monkeys and, apart from induced changes in nerves, their cytology was unchanged substantially by the short-term experiments used in this study. An inflammatory response by the conjunctiva and cornea to sensory denervation, noted occasionally in earlier work on monkeys (unpublished data, 1987), was not expressed in the current experimental group.

The results show that conjunctival follicles are innervated regularly with variation in the density of
Animal

P < Student's t-test: 0.0001.

vesicles in terminal varicosities is adequate evidence of distribution of sympadietic terminals. The high incidence functions is expressed in a variety of ways after sympa-

they were mainly 6,7 or exclusively8 referred to blood vessels. Similar fibers were observed in lymph nodes, but in contrast to the current findings, they were mainly identified with the use of a short postoperative interval, which might be effected essentially across the narrow and incomplete barrier of reticular fibers. A similar arrangement of follicle and nerve distribution was reported in axillary lymph nodes4 and tonsils,7 where close contact with lymphocytes also was noted.

The numerous degenerated axon profiles present in reticular fibers are adequate evidence of a parenchymal sensory innervation. Evidence of parenchymal fibers containing neuropeptides associated with the sensory system has been noted in lymph nodes, but in contrast to the current findings, they were mainly23 or exclusively26 related to blood vessels. Similar fibers were observed in conjunctival lymph follicles without reference to the target structures11.

Sensory Innervation

Unequivocal evidence for a sensory (ophthalmic) vascular innervation was not obtained in this study. The presence of degenerated fibers within nerves coursing adjacent to arterioles may be considered consistent with the observation of sensory neuropeptides in vascular nerve fibers in other tissues of the immune system.6,9 However, evidence that they provided vascular terminals was not found despite the use of a short postoperative interval, which might have permitted recognition of degenerated profiles in the vascular wall before the breakdown and dispersal of degenerated material. Nor was the morphology of any of the vascular terminals characteristic of the sensory nerves. In contrast, Kurkowski et al7 used an immunoreactive labeling technique at electron microscopic level to show convincingly that sensory axons terminate on vascular walls in tracheobronchial lymph nodes in the guinea pig.

We conclude that if follicle vessels receive a sensory innervation, it must be rather sparse.

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TABLE 1. Incidence of Follicles With Innervated Epithelium After Ophthalmic Neurotomy

<table>
<thead>
<tr>
<th>Animal Reference</th>
<th>Postoperative Period (days)</th>
<th>Operated Side (number of follicles/number innervated)</th>
<th>Control Side (number of follicles/number innervated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M84</td>
<td>3*</td>
<td>8/0</td>
<td>6/6</td>
</tr>
<tr>
<td>M85</td>
<td>8</td>
<td>6/1</td>
<td>6/5</td>
</tr>
<tr>
<td>M86</td>
<td>7</td>
<td>4/0</td>
<td>3/3</td>
</tr>
</tbody>
</table>

Student's t-test: P < 0.0001.
* Maxillary and ophthalmic neurotomy.
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Epithelial nerve terminals, previously unreported in MALT, are predominantly and perhaps exclusively sensory, as the evidence of this study shows. Practically all epithelial varicosities were of the packed mitochondria type, characteristic of sensory terminals, and were eliminated subsequent to the lesions. Although epithelial terminals could not be found in one of the 15 follicles from control sides and in two others from 16 follicles from animals not operated on, their virtual absence from operated material (17 of 18 follicles) indicates that the lesions were responsible for their absence. The products of degeneration, seen so readily in nerves as they entered follicles, could not be identified with conviction in the epithelium, presumably because axoplasm debris had been removed. Such a result is not unexpected, because all that remains after induced degeneration of unmyelinated nerve fibers are their Schwann cell investments, and these are not present in the epithelium.

The primate conjunctiva is richly innervated at the eyelid margin, at the limbus, and around vessels, including capillaries, yet few nerve fibers penetrate the epithelium, unlike rats, in which evidence of conjunctival goblet cell innervation was reported recently. Epithelial nerve terminals are rather sparse in the bulbar conjunctiva of monkeys, and their absence from the human palpebral conjunctiva is matched by the current results regarding most of the epithelium. Consequently, the regular occurrence of terminals in the orbital part of the conjunctiva was not anticipated and their almost-exclusive association with the modified follicular epithelium discounts the possibility that they serve only the general sensitivity of the conjunctiva and is indicative of a specialized function. Contact exclusively with epithelial cells rather than with leukocytes suggests that they might provide a follicle-alerting system to surface stimuli. The presence of plasma cells in monkey follicles suggests the functional characteristics of other MALT fully has yet to be determined, but there can be little doubt that they contribute to the immunodefense of the ocular surface. The presence of plasma cells in monkey follicles suggests that the conjunctiva might have a role in ocular secretory immune defense. The position in humans is less clear; although plasma cells have been reported in the conjunctival subepithelium in a more recent study, none were found; moreover, the identification of secretory component in human conjunctiva was not confirmed. Given these uncertainties, which also apply to other species, immunocytochemical examination, including testing of plasma cells and secretory component, is needed before the role of the monkey conjunctiva in this aspect of immune defense can be understood properly.

Key Words

Conjunctiva, epithelium, lymph follicles, primate, sensory nerves

References

11. Chung CW, Tigges M, Stone RA. Peptidergic innervation data, 1993). Whether conjunctival follicles share the functional characteristics of other MALT fully has yet to be determined, but there can be little doubt that they contribute to the immunodefense of the ocular surface.
tion of the primate Meibomian gland. Invest Ophthal-


