Immunohistochemical Localizations of Class II Antigens and Nerve Fibers in Human Carious Teeth: HLA-DR Immunoreactivity in Schwann Cells*

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Summary. Nerve fibers and class II major histocompatibility complex (MHC) antigen-expressing dendritic cells have been known to gather in the dental pulp beneath carious lesions. Significant functional interactions presumably occur between the neural and immune elements. The present study analyzed the morphological relationship between class II-expressing cells and nerve fibers in human carious teeth, visualized by a HLA-DR monoclonal antibody and a protein gene product 9.5 (PGP 9.5) polyclonal antibody; a confocal laser scanning microscope (CLSM) and an electron microscope were used. In pulps affected by early caries, HLA-DR-positive dendritic cells aggregated mainly in the cell-free zone associated with bundles of PGP 9.5-immunoreactive nerve fibers. In pulps affected by advanced caries, the accumulated HLA-DR-positive cells and PGP 9.5-immunoreactive nerve fibers showed close association with each other especially beneath the odontoblast layer: the cells even embraced the nerve fibers. Intriguingly, class II molecules were recognized not only in dendritic cells but also in the Schwann cells of non-myelinated nerves in the pulp. Using immunoelectron microscopy, class II molecules were localized on the surface of the non-myelinating Schwann cells and also within some vesicles, whereas myelinating Schwann cells lacked this immunoreactivity. PGP 9.5-immunoreactive nerve fibers were also observed densely in the odontoblast layer, and CLSM revealed an intimate association of the nerve fibers and dendritic cells. The immunoreactivity for HLA-DR in Schwann cells depended upon the severity of the carious lesion. Class II-expressing Schwann cells are suggested to function as antigen-presenting cells in addition to dendritic cells.

The genes of the major histocompatibility complex (MHC) code for two major groups of antigens, class I and II. In humans, the class II antigens consist of membrane bound glycoproteins HLA-DP, -DQ and -DR (FOULIS, 1986.) Most immune reactions begin with the presentation of antigen to T cells by cells bearing class II molecules. The existence of class II MHC antigen-expressing cells has been reported in the dental pulp (JONTELL et al., 1988; OKJI et al., 1992; OHSHIMA et al., 1994, 1995; YOSHIBA et al., 1996). Our previous study showed that the distribution of class II-expressing cells in human dental pulps of both unerupted and erupted normal teeth as well as teeth at various stages of the carious process (YOSHIBA et al., 1996). HLA-DR positive dendritic cells are distributed mainly in and around the odontoblast layer from the time of unerupted, developing teeth. In carious teeth, the aggregation of class II-expressing dendritic cells has been reported to occur primarily beneath carious lesions (YOSHIBA et al., 1996).

Nerve fibers also become concentrated beneath carious lesions in human teeth (PLACKOVA, 1966; ARAI, 1991). Silver impregnation has revealed drastic changes in the distribution pattern of nerve fibers with the progress of pulpitis (ARAI, 1991). In hyperemia of the pulp, a gathering of fine nerve fibers has been recognized around the reparative dentin, and in serious pulpitis, numerous nerve fibers beaded in appearance concentrate around there. This change in the distribution of nerve fibers seems to correlate with the above-mentioned aggregation of class II-

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expressing dendritic cells in carious teeth (YOSHIBA et al., 1996). Recent studies have suggested an interaction between nervous and immune systems (LOTZ et al., 1988; NONG et al., 1989; McGILLIS et al., 1992; HOSOI et al., 1993), and it seems reasonable to presume that significant interactions might occur between nerve fibers and class II-expressing cells in human carious teeth.

The present investigation aims to analyze the morphological relationship between class II MHC antigen-expressing cells and nerve fibers in the human dental pulp beneath carious lesions, using HLA-DR monoclonal and protein gene product 9.5 (PGP 9.5) polyclonal antibodies whose usefulness has been reported in the identification of fine nerve fibers in human teeth (RAMIERI et al., 1990; MAEDA et al., 1994).

MATERIALS AND METHODS

Samples
Ten third molars at various stages of decay were extracted from patients exhibiting no clinical symptoms, of ages ranging between 20 and 25 years and undergoing orthodontic treatment. Immediately after extraction, the apical third of the roots of the teeth was cut with a water-cooled air turbine to facilitate the penetration of the fixative solution. The teeth were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 24 h, then washed in PBS, and demineralized in 10% EDTA (Ph 7.4) for 6-8 weeks at 4°C. They were then sliced into 80 μm sections with a microslicer (DTK-1000, Dosaka EM Co., Kyoto, Japan). The depth of the carious lesion was determined by the pigmentation and the thickness of the remaining dentin on the sections.

Immunofluorescence
Double immunofluorescence staining was performed to analyze the distributional relationship between class II molecules and nerve fibers. The sections were pre-incubated for 60 min with PBS containing 1% bovine serum albumin (BSA), and thereafter incubated with the mouse monoclonal antibody to human HLA-DR (DAKO, Glostrup, Denmark) diluted 1:100 in PBS for 24 h at 4°C, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulins (DAKO) diluted 1:100 in PBS. The adjacent section was incubated with the rabbit anti-human PGP 9.5 polyclonal antibody diluted 1:200 in PBS for 24 h at 4°C, followed by HRP-conjugated swine anti-rabbit immunoglobulins (DAKO) diluted 1:100 in PBS. After 12 h incubation at 4°C, these sections were rinsed with PBS, and then re-fixed with 2.5% glutaraldehyde. Following a PBS rinse, they were immersed in a mixture of diamobenzidine (0.05%) and H2O2 (0.008%) in 0.05 M TRIS-HCl buffer (pH 7.6) for 4 min. They were post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h at 4°C, dehydrated in ethanol, and embedded in Epon 812. For light microscopic observation, 2 μm sections were counterstained with toluidine blue. Ultrathin sections of about 80-90 nm thickness were cut with a diamond knife, stained with lead citrate, and observed under a transmission electron microscope (H-500, Hitachi, Tokyo, Japan).

Controls
Control sections were stained using normal mouse serum, normal rabbit serum, or PBS as the primary antibody.

RESULTS

Control sections showed no specific labeling (photographs not shown).

In pulp affected by early caries, PGP 9.5-immunoreactive nerve fibers were more densely gathered under the carious lesion than elsewhere (Fig. 1a). Immunoreaction was also recognized on odontoblasts. Observation using double channels indicated that HLA-DR-positive dendritic cells accumulated between the odontoblast layer and the bundles of PGP
Fig. 1. Dental pulp affected by early caries. The extent of carious pigmentation is about 0.5 mm from the enamel-dentin junction. *Broken lines* in a indicate the direction of the dentinal tubules corresponding to the carious lesion. a. A confocal laser scanning micrograph indicates the PGP 9.5-immunoreactive nerve fibers (shown in green), which are evident under the carious lesion (*arrowheads*). The immunoreactivity is also seen on the odontoblasts. b. Higher magnification of the asterisk area in a observed by double-channel. HLA-DR-positive dendritic cells (shown in red) are accumulated closely beneath the odontoblast layer (O). c. A magnified view of the area beneath the odontoblast layer, showing contact (*arrowheads*, yellow spots) between PGP 9.5-immunoreactive nerve fibers and HLA-DR-positive dendritic cells. d and e. The area corresponding to c, demonstrated by immunoperoxidase labeling for HLA-DR and PGP 9.5 respectively. d. HLA-DR-positive dendritic cells (*arrows*) are located in the cell-free zone. Some are observed along the pulp-dentin border (*arrowheads*). Note the lack of any specific reaction for HLA-DR to be recognized on the bundles of nerve fibers (*double arrows*). e. The PGP 9.5-labeled nerve fibers (*arrows*) in the cell-rich layer comprise myelinated and non-myelinated nerves. P pulp, O odontoblast layer, D dentin. Scale bars=500 μm (a), 100 μm (b), 50 μm (c), 30 μm (d, e)
Fig. 2. Legend on the opposite page.
9.5-immunoreactive nerve fibers (Fig. 1b). The nerve fibers, beaded in appearance, were seen subjacent to the odontoblast layer, where they were closely associated with the HLA-DR-positive dendritic cells (Fig. 1c). The section labeled with immunoperoxidase showed the HLA-DR-positive dendritic profiles mainly dispersed in the cell-free zone, and partly lying along the pulp-dentin border extending cytoplasmic processes into dentinal tubules (Fig. 1d). The PGP 9.5-labeled nerve fibers in the cell-rich layer comprised myelinated and non-myelinated nerves (Fig. 1e).

In pulp affected by advanced caries where pigmentation extended about four-fifths the thickness of the dentin from the enamel-dentin junction, a dense distribution of PGP 9.5-immunoreactive nerve fibers was evident close to the carious lesion (Fig. 2a). The same section, which was observed under another channel, indicated a distribution of the HLA-DR-positive cells similar to that of the nerves, especially in the subodontoblast layer (Fig. 2b). A number of PGP 9.5-immunoreactive elements with a dotted appearance were observed in the odontoblast layer beneath the decayed lesion (Fig. 2c). Subjacent to this layer, the HLA-DR-positive cells and PGP 9.5-immunoreactive nerve fibers were co-distributed (Fig. 2c). Closer observation revealed that the HLA-DR-positive cells enveloped the PGP 9.5-immunoreactive elements (Fig. 2d). In the odontoblast layer, CLSM showed intimate associations of the PGP 9.5-immunoreactive nerve fibers with the HLA-DR-positive dendritic cells (Fig. 2e-g), which were located in the odontoblast layer and extended their cytoplasmic processes into dentinal tubules (Fig. 2f).

Immunoperoxidase labeling for HLA-DR showed, besides the above described dendritic cells, a mass of the immunoreactive elements under the carious lesion (Fig. 3a). These labelings appeared to extend along the nerve fibers (Fig. 3b). On the section labeled with PGP 9.5 antibody, bundles of labeled nerve fiber were found densely under the carious lesion (Fig. 3c). Examination at high magnification revealed that the immunoreaction for HLA-DR was localized only along non-myelinating Schwann cells, while myelinating Schwann cells lacked the immunoreactivity (Fig. 3d). PGP 9.5-immunoreactive axons were seen in the bundles of nerve fibers (Fig. 3e). The labeled nerve fibers were also observed densely in the odontoblast layer close to the carious lesion (Fig. 3f). In intact parts of the same section, none of the immunoreactivities for HLA-DR were recognized in Schwann cells (Fig. 3g). Bacterial invasion into subodontoblastic blood vessels could occasionally be observed (Fig. 3b).

Under the electron microscope, HLA-DR immunoreactivity was demonstrated on the surface of non-myelinating Schwann cells (Fig. 4a). On the other hand, myelinating Schwann cells showed no specific labeling on their surface (Fig. 4a). The HLA-DR immunoreactive products were also recognized within some vesicles of the Schwann cells (Fig. 4b). The labeled Schwann cells enveloped the non-myelinated axons, which contained mitochondria and neurofilaments (Fig. 4c). In the odontoblast layer, PGP 9.5-immunoreactive naked axons were seen to contact odontoblasts (Fig. 4d).

**DISCUSSION**

This is the first finding that Schwann cells express class II antigen in human dental pulps under a carious condition. Furthermore, the particular immunoreactivity for class II molecules has been recognized not only on the surface membrane of non-myelinating Schwann cells but also within some vesicles in those...
Fig. 3. Legend on the opposite page.
cells.

In normal tissue, the expression of class II (Ia HLA-DR, DQ, DP) MHC molecules generally is confined to macrophages, dendritic cells, B cells, activated T cells and some epithelial and endothelial cells (WINCHESTER and KUNKEL, 1979). Neurons and Schwann cells, the component cells of the peripheral nervous system, do not normally express class II molecules (LISAK et al., 1983). The induction of class II molecules in some Schwann cells has been shown in patients with inflammatory demyelinating neuropathy of acute or chronic types, Guillain-Barre syndrome (GBS), and chronic inflammatory demyelinating polyneuropathy (CIDP) (CADONI et al., 1986; POLLARD et al., 1986, 1987; SCARPINI et al., 1990). In addition, interaction between CD4+ T-cell lines and the Schwann cells has been known (ARGALL et al., 1992), providing evidence for a role by Schwann cells as antigen-presenting cells.

Our immuno-electron microscopic study in carious teeth has demonstrated the specific staining for class II molecules on the surface membrane of non-myelinating Schwann cells and within some vesicles in those cells. Antigen-presenting cells process and partially degrade antigens by lysosomal proteases, and transport back the resultant immunogenic peptide to the cell surface (GERMAIN, 1986). The phagocytic function of Schwann cells has been reported (BIGBEE et al., 1987), and immunoelectron microscopic characteristics observed in the present study would support the function of Schwann cells as antigen-presenting cells in carious infection. Intriguingly, class II molecules have not been recognized in the myelinating Schwann cells, even if they are located close to the immunopositive non-myelinating Schwann cells. The reason for this difference is not clear at present. The lack of class II molecules on myelinating Schwann cells is in accord with a previous report on sural neuropathies (SCHRODER et al., 1988). In human carious teeth, Schwann cells labeled with HLA-DR antibody were confined to an advanced carious lesion. In the intact part of those teeth, or in a slightly decayed lesion, no labeling for class II molecules was seen in Schwann cells. These results suggest that the immunoreactivity for HLA-DR in Schwann cells correlates with the severity of the carious lesion. The Schwann cells presumably would function as antigen-presenting cells under the carious infection, in addition to dendritic cells.

Double-labeled sections observed under CLSM revealed intimate associations of HLA-DR-positive dendritic cells with nerve fibers beneath the carious lesion. Sensory nerve fibers, containing calcitonin gene-related peptide (CGRP) and substance P, represent a major component of dental innervation (OL-GART et al., 1977; UDDMAN et al., 1986; GAZELIUS et al., 1987; WAKISAKA et al., 1987). In models of experimentally induced pulpitis either in innervated or denervated teeth, the possible participation of those nerve fibers in the inflammatory defense reaction has been investigated (FRISTAD et al., 1995a, b, KIMBERLY and BYERS, 1988). The association of CGRP-containing nerve fibers with Langerhans cells has been recognized especially during inflammation (HOSOI et al., 1993); the functional significance is reportedly to inhibit the Langerhans cells' antigen presentation by those nerve fibers (HOSOI et al., 1993). Recently, the sensory nerve fibers have been suggested to have an influence on the pulpal immunodefense (OKIJI et al., 1997). The present findings also support the ideal of functional interactions between nerve fibers and HLA-DR-positive dendritic cells.

A dense innervation was observed in the odontoblast layer corresponding to the carious lesion, and, under the electron microscope, naked axons were observed in contact with the odontoblasts. Cavity preparation or pulp exposure has been known to induce considerable sprouting of sensory nerve fibers (KIMBERLY and BYERS, 1988; TAYLOR et al., 1988; SATO, 1989; BYERS et al., 1990; TAYLOR and BYERS, 1990). A role for reparative dentin formation and pulp survival by neuropeptides has been reported (TAYLOR

**Fig. 3.** Immunoperoxidase labelings for HLA-DR (a, b, d and g) and PGP 9.5 (c, e, f and h) in the same carious tooth observed in Figure 2. a. A mass of labelings for HLA-DR (arrows) are observed under the carious lesion. b. Higher magnification of a. Immunoreactivity for HLA-DR is recognized along nerve fibers. Bacterial invasion is seen in blood vessels (arrowheads). c. A lesion vicinal to the area indicated in b. Bundles of PGP 9.5-immunoreactive nerve fibers (arrows) are observed under the carious lesion. d. A magnified view showing that the immunoreactivity for HLA-DR is present on the non-myelinating Schwann cells (arrows), whereas myelinating Schwann cells lack the immunoreaction (arrowheads). e. Bundles of nerve fibers immunostained for PGP 9.5. Axons (arrowheads) are labeled. f. The odontoblast layer corresponding to the carious lesion. PGP 9.5-immunoreactive nerve fibers are densely observed between the odontoblasts. g and h. The nerve fibers (arrows) in intact parts of the same tooth stained for HLA-DR (g) and PGP 9.5 (h). g. No specific reaction for HLA-DR is seen on the nerve fiber. Endothelial cells show positive staining (arrowheads). O odontoblast layer. Scale bars=50 μm (a), 30 μm (b, c, g, h), 10 μm (d, e), 20 μm (f)
Fig. 4. Immuno-electron micrographs. a. An image corresponding to Figure 3d. HLA-DR molecules are evident on the surface of non-myelinating Schwann cells (arrows). Myelinating Schwann cells show no specific labeling (arrowheads). b. High-power electron micrograph of a. HLA-DR immunoreactive products are also recognized within some vesicles (arrows) of the labeled Schwann cells. c. The labeled Schwann cells envelope the non-myelinated axons, which contain mitochondria and neurofilaments. d. An image corresponding to Figure 3f. A PGP 9.5-immunoreactive naked axon (arrow) is observed in contact to an odontoblast (OB). A axons, N nuclei of Schwann cells, M myelin sheaths. Scale bars=5 μm (a), 2 μm (b), 1 μm (c, d)
and Byers, 1990; Byers and Taylor, 1993). Furthermore, recent studies in vitro have shown that neuropeptides, such as substance P, CGRP or neurokinin A, stimulate the proliferation of pulp fibroblasts in mice (Bongenhielm et al., 1995) or in humans (Trantor et al., 1995). In other tissues, epidermal denervation has been shown to exert a great influence on both class II positive Langerhans cells and keratinocytes, causing a reduction in the thickness of the epidermis (Hsieh et al., 1996). The restricted formation of reparative dentin beneath the carious lesion has been long known, although the exact mechanism is unclear. The nerve fibers aggregated in the odontoblast layer beneath the carious lesion would contribute to this process.

REFERENCES


