Prion Infection of Skeletal Muscle Cells and Papillae in the Tongue

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The presence of the prion agent in skeletal muscle is thought to be due to the infection of nerve fibers located within the muscle. We report here that the pathological isoform of the prion protein, PrPSc, accumulates within skeletal muscle cells, in addition to axons, in the tongue of hamsters following intralingual and intracerebral inoculation of the HY strain of the transmissible mink encephalopathy agent. Localization of PrPSc to the neuromuscular junction suggests that this synapse is a site for prion agent spread between motor axon terminals and muscle cells. Following intracerebral inoculation, the majority of PrPSc in the tongue was found in the lamina propria, where it was associated with sensory nerve fibers in the core of the lingual papillae. PrPSc staining was also identified in the stratified squamous epithelium of the lingual mucosa. These findings indicate that prion infection of skeletal muscle cells and the epithelial layer in the tongue can be established following the spread of the prion agent from nerve terminals and/or axons that innervate the tongue. Our data suggest that ingestion of meat products containing prion-infected tongue could result in human exposure to the prion agent, while sloughing of prion-infected epithelial cells at the mucosal surface of the tongue could be a mechanism for prion agent shedding and subsequent prion transmission in animals.

In prion diseases, the prion agent is primarily found in the lymphoreticular and nervous systems (8, 23). In both natural and experimental prion infections, the prion agent is commonly amplified by replicating in the lymphoreticular system prior to entry into nerve cells and subsequent invasion of the brain (1, 18, 23). Spread within the nervous system can occur by transport along axons and between synaptically linked neurons. Experimental studies have demonstrated that the initial pattern of prion agent spread in the spinal cord and brain follows defined autonomic, sensory, and motor pathways (3, 4, 12, 13, 27).

Modest levels of prion agent replication in skeletal muscle have been reported in a few studies following intracerebral or extraneural inoculation of the prion agent. Prion infectivity in skeletal muscle was first demonstrated in mink with transmissible mink encephalopathy (TME); the amount of infectious agent in skeletal muscle was 10,000-fold less than the amount found in brain (26). Prion infectivity or accumulation of the abnormal form of the prion protein, PrPSc, in skeletal muscle has more recently been described in experimental prion infection of rodents and in sporadic Creutzfeldt-Jakob disease (CJD) of humans (14), but the cellular location of agent deposition and/or replication in muscle has not been identified (3, 6, 40). In natural prion diseases, PrPSc is found in the peripheral nervous system of scrapie-infected sheep and goats, and it has been proposed that it is the peripheral nerves which transverse skeletal muscle that are the source of prion infectivity in muscle (16, 17, 19, 20). Attempts to measure the prion agent in muscle of goats infected with the scrapie agent (18, 20) and

cattle infected with the bovine spongiform encephalopathy (BSE) agent (10, 42) have not detected prion infectivity. However, a murine bioassay was used to measure prion infectivity in these studies, and this assay cannot detect below $10^{4.1}$ lethal median doses (LD_{50}) of the BSE agent (10, 42). Therefore, low levels of prion agent that could be present in muscle tissue of domestic livestock would not be found using this bioassay. Routine testing for PrPSc in brains from cattle in Europe and in lymph nodes from deer and elk in the United States has been implemented to reduce human exposure to BSE and chronic wasting disease by removing infected animals from the food supply. However, human consumption of skeletal muscle from a prion-infected animal is currently considered a low risk for contracting a prion disease.

In this study, we report that in hamsters infected with the TME agent, by either an intralingual or intracerebral route, PrPSc can accumulate in skeletal muscle cells of the tongue. Localization of PrPSc to the neuromuscular junction (NMJ) indicates that this synapse can be a site for prion agent replication and may provide a path by which the prion agent spreads between skeletal muscle cells and axon terminals. PrPSc was also found in the lingual papillae of the tongue, specifically in nerve fibers of the lamina propria and in the stratified squamous epithelium of the tongue surface. These findings have public health implications and suggest that (i) ingestion of prion-infected tongue could be a source of prion exposure for humans, since livestock tongue is not included in the specified risk material; and (ii) normal shedding of the outer layer of the tongue epithelium can release the prion agent into saliva, which may be a source for prion agent transmission in livestock and cervids.

MATERIALS AND METHODS

Animal inoculations. All procedures involving animals were approved by the Creighton University Institutional Animal Care and Use Committee and were in

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compliance with the *Guide for the Care and Use of Laboratory Animals* (31). Hamsters were inoculated in the lingual muscles of the tongue or the cerebrum with 20 μl of a brain homogenate from a TME-infected hamster which contained $10^{7.5}$ intracerebral LD_{50} per ml of the HY TME agent. Following inoculation with the HY TME agent, hamsters were observed three times per week for the onset of clinical symptoms. Hamsters were euthanized at either specific intervals postinfection or after the onset of disease. In the latter case, animals were culled at 75 to 85 days postinfection for the intracerebral inoculation group and at 95 to 105 days for the intratongue inoculation group. Tongue and brain were collected for PrP^{Sc} analysis by Western blotting and immunohistochemistry. At least two independent mock-infected and HY TME-infected tissue samples were used for each of these analyses, but three or more tissue samples were typically used in each study. Western blotting and immunohistochemistry results were similar among individual animals within each control and experimental group.

Tissue preparation for PrP^{Sc} Western blotting. PrP^{Sc} was enriched from tongue prior to Western blotting by tissue extraction in detergent and differential ultracentrifugation as previously described (3, 4). PrP^{Sc}-enriched samples were digested with proteinase K and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer. Five-percent brain homogenates were also digested with proteinase K and prepared for Western blotting as previously described (4). SDS-PAGE and Western blot analysis were performed using monoclonal antibody 3F4 hybridoma supernatant (22) (a gift of Victoria Lawson, NIH Rocky Mountain Labs, Hamilton, Mont.) as previously described. Quantification of PrP^{Sc} signal in Western blot assays was performed using ImageQuant software as previously described (4).

PrP^{Sc} immunohistochemistry, immunofluorescence, and confocal microscopy. Immunostaining for PrP^{Sc} in tongue was performed as previously described (3). Briefly, animals were intracardially perfused with paraformaldehyde-lysine-periodate fixative followed by postfixation in paraformaldehyde-lysine-periodate for 5 h. Paraffin-embedded tissue sections (4 μm) were subjected to antigen retrieval by pretreatment with formic acid for 20 min. A minimum of 10 tissue sections per animal was examined for each antibody staining procedure. PrP^{Sc} was detected by incubation with monoclonal 3F4 hybridoma supernatant (1:600 dilution) and the ABC-horseradish peroxidase Elite staining method (Vector Laboratories, Burlingame, Calif.). For PrP^{Sc} staining in the double immunofluorescence procedure, the ABC-horseradish peroxidase incubation step in the ABC method was replaced by incubation with an Alexa Fluor 488 streptavidin conjugate (Molecular Probes, Portland, Oreg.) at a 1:400 dilution. PrP^{Sc} immunofluorescence staining was combined with rabbit polyclonal antibody staining for either desmin (1:50 dilution; DAKO Corp., Carpinteria, Calif.), synaptophysin (1:50 dilution; DAKO Corp.), S100 (1:800 dilution; DAKO Corp.), cytokeratin (1:800 dilution; DAKO Corp.), or calcitonin growth-related peptide (CGRP; 1:125 dilution; Peninsula Labs, Inc., San Carlos, Calif.). This latter group of primary antibodies was visualized by incubation with anti-rabbit antibody conjugated to Alexa Fluor 568 (Molecular Probes). The nuclear counterstain TO-PRO3 (Molecular Probes) was applied to some tissue sections at a concentration of 0.25 μM for 10 min. Tissue sections were mounted with Vectashield Antifade (Vector Laboratories). Images were visualized with epifluorescence using a Bio-Rad Radiance 2000 confocal system attached to a Nikon Eclipse 800 microscope.

RESULTS

Inoculation of the HY TME agent into the lingual muscles of the tongue and the cerebrum resulted in incubation periods of 83 ± 3 days and 62 ± 2 days, respectively, in Golden Syrian hamsters (mean ± standard error of the mean). At the onset of clinical symptoms, both groups had evidence of PrP^{Sc} deposition in the tongue (Fig. 1). Quantification of the PrP^{Sc} polypeptide bands using ImageQuant software indicated that the amount of PrP^{Sc} in the tongue was approximately fourfold higher following intratongue inoculation than that following intracerebral inoculation. However, the amount of PrP^{Sc} in the brain was approximately 1,000-fold higher than that found in comparable amounts of TME-infected tongue following either route of inoculation (Fig. 1). Immunohistochemistry revealed that PrP^{Sc} was located in three regions of the tongue, including the skeletal muscle (Fig. 2B), the lamina propria of the papillae (Fig. 2C), and the stratified squamous epithelium of the tongue mucosa (see below).
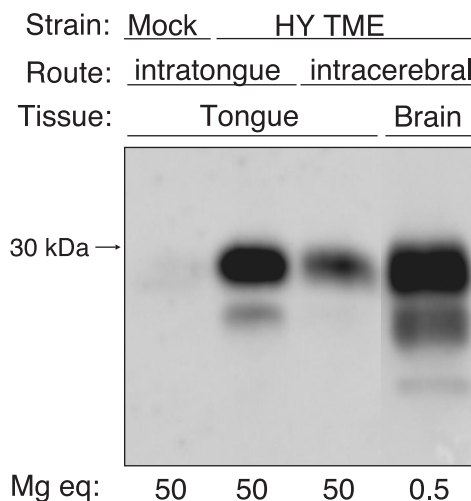

FIG. 1. PrP^{Sc} deposition in tongue and brain following inoculation of the HY TME agent. Hamsters were inoculated with the TME agent or a normal brain homogenate (mock) by either the intratongue or intracerebral route. Tongue and brain tissues were collected from animals that were sacrificed after the onset of clinical disease as described in the text. PrP^{Sc} in brain homogenates or PrP^{Sc}-enriched preparations from tongue digested with proteinase K were prepared as described in Materials and Methods and analyzed by SDS-PAGE and PrP Western blotting. The amount of tissue analyzed in each lane is indicated in milligram tissue equivalents (mg eq), and the molecular masses of polypeptides in kilodaltons are indicated to the left of the panel.

Confocal microscopy was used to localize PrP^{Sc} to specific cell types in the tongue. Following intratongue inoculation of the HY TME agent, the majority of PrP^{Sc} staining was found within skeletal muscle cells. This was demonstrated in the tongues of clinically ill hamsters at greater than 95 days postinfection by colocalization of desmin staining (i.e., an intermediate filament protein in skeletal muscle cells) in PrP^{Sc}-positive cells (Fig. 3A to C). While PrP^{Sc} was widely distributed within skeletal muscle cells, it was often present in higher amounts at the periphery of the cell (Fig. 3A). At 1 week postinoculation of the tongue, PrP^{Sc} staining was found in less than three skeletal muscle cells per tissue section; the number of PrP^{Sc}-positive cells as well as the amount of PrP^{Sc} staining progressively increased in skeletal muscle cells during the course of subclinical TME infection (data not shown). At 95 days postinoculation, when hamsters were clinically affected, skeletal muscle cells throughout the tongue were PrP^{Sc} positive. It is worth noting that PrP^{Sc}-positive muscle cells were located between PrP^{Sc}-negative cells (Fig. 2B and 3A). These results demonstrate that the TME agent can accumulate within skeletal muscle cells and suggest that replication can also occur in these cells. At the clinical stages of TME, a lower amount of PrP^{Sc} staining was found in skeletal muscle cells of the tongue in hamsters that were intracerebrally inoculated compared to those inoculated into the tongue with the HY TME agent (data not shown). This finding was consistent with PrP^{Sc} Western blotting data of tongue from intracerebral- and intratongue-inoculated hamsters (Fig. 1). In mock-infected controls, specific binding of anti-PrP monoclonal 3F4 antibody was not observed (Fig. 2A).
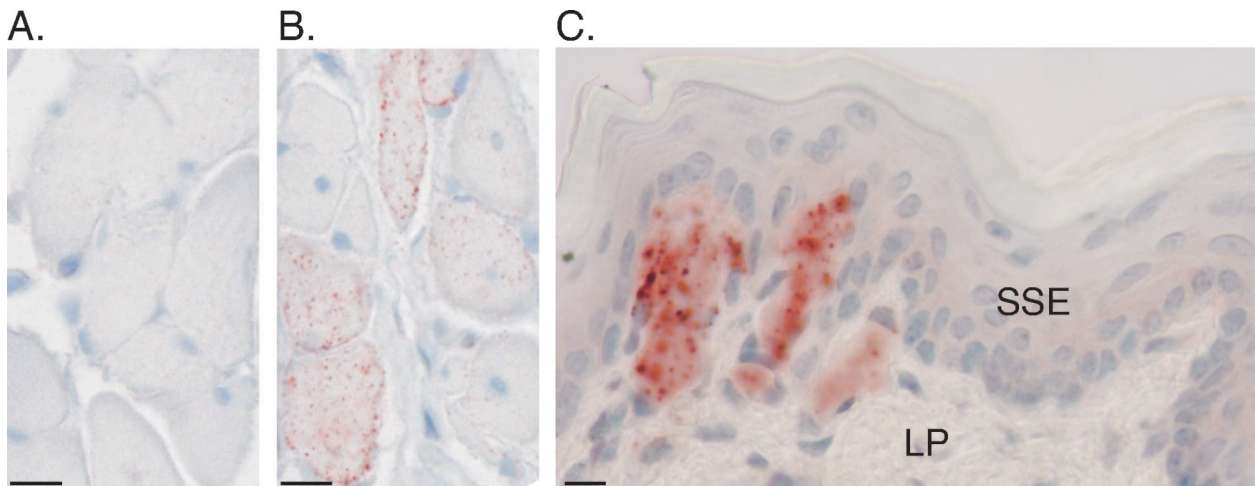
FIG. 2. PrP^{Sc} deposition in tongue following inoculation of the HY TME agent. Hamsters were inoculated with a normal brain homogenate (A) or the TME agent (B and C) by either the intratongue (A and B) or intracerebral (C) route. Tongues were collected from animals that were sacrificed after the onset of clinical disease and prepared for immunohistochemistry as described in Materials and Methods. PrP^{Sc} immunohistochemistry illustrated PrP^{Sc} (red punctate staining) in skeletal muscle cells (B) and the lamina propria (C) but an absence of immunostaining in mock-infected tongue (A). The tissue was counterstained with hematoxylin. The lamina propria (LP) and stratified squamous epithelium (SSE) of the tongue are indicated. Bar, 10 μm.

Previously, we demonstrated that PrP^{Sc} was associated with axons in nerve fascicles in the tongue (3). In the present report, PrP^{Sc} staining in nerve fascicles was demonstrated after 95 days postinfection following intratongue inoculation of HY TME, but it did not colocalize with S100 staining, which was used as a marker for Schwann cells (Fig. 3D to F). This finding suggested that the majority of PrP^{Sc} was located within the axons in this experimental model and was less likely to be
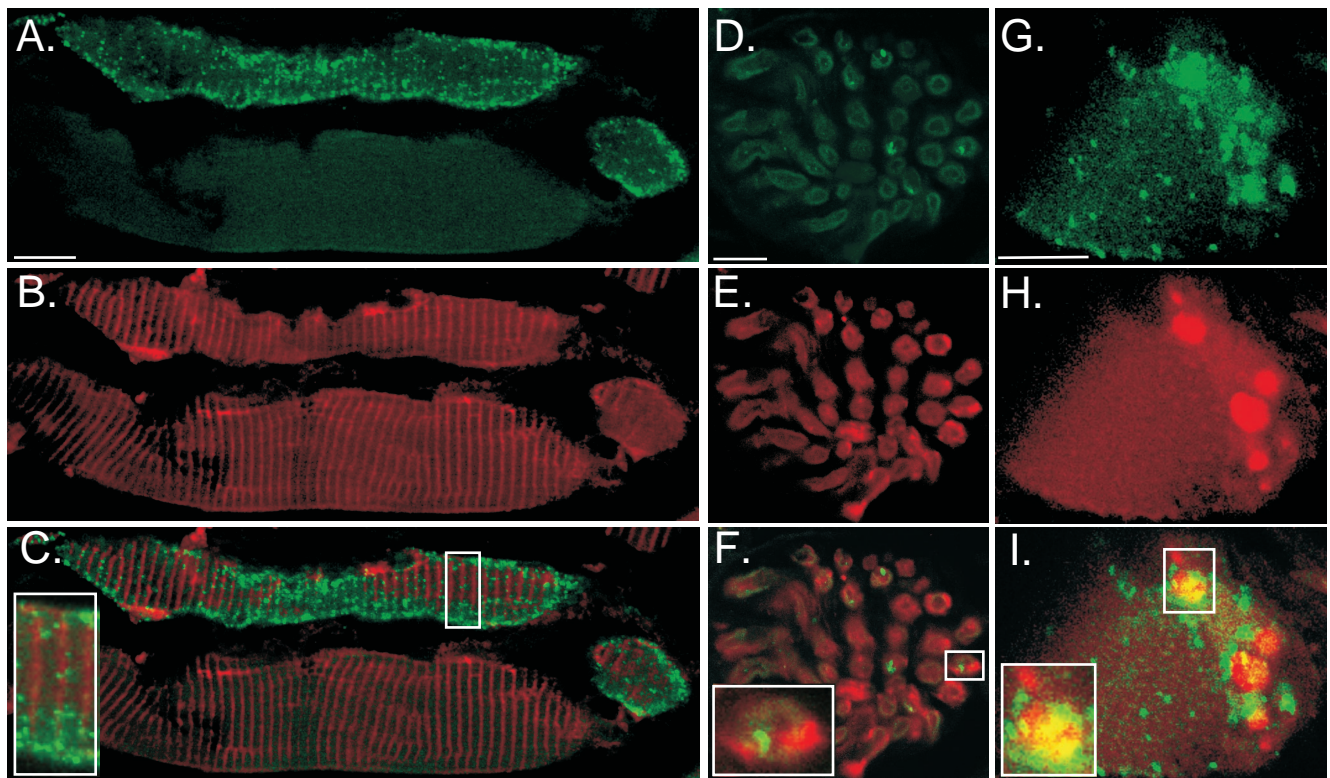

FIG. 3. Confocal microscopy of PrP^{Sc} in skeletal muscle cells and axons. Hamsters were inoculated in the lingual muscles with the HY TME agent. At the onset of clinical symptoms, the tongue was prepared for immunofluorescence staining and confocal microscopy as described in Materials and Methods. Following immunofluorescence staining for identification of PrP^{Sc} with Alexa Fluor 488 (A, D, and G), tissue sections were also stained for either desmin (B), S100 (E), or synaptophysin (H) with goat anti-rabbit immunoglobulin conjugated to Alexa Fluor 568. To investigate the colocalization of PrP^{Sc} with desmin (C), S100 (F), and synaptophysin (I), green and red fluorescent images were merged. Bar, 10 μm. The insets at the lower left corner (C, F, and I) are two- to threefold enlargements of smaller boxes in each of the corresponding panels.

within the Schwann cells that surround axons in the peripheral nervous system. Both large nerve fascicles that travel between skeletal muscle fascicles and small nerve fascicles located in the lamina propria were found to contain punctate PrP^{Sc} staining following intratongue inoculation.

The spatial relationship between PrP^{Sc} and the NMJ was investigated following intratongue inoculation of the HY TME agent, since PrP^{Sc} was found in both skeletal muscle cells and axons in the tongue. An antibody to synpatophysin, a marker for synaptic vesicles in the axon terminal, was used for identification of NMJs in hamster tongue from clinically ill hamsters at 95 days postinfection. Sixty-four percent of synaptophysin-positive skeletal muscle cells were also PrP^{Sc} positive; colocalization of synaptophysin and PrP^{Sc} was found in 25% of these cells (Fig. 3G to I). In the example illustrated (Fig. 3G to I), PrP^{Sc} staining was localized to the NMJ, indicating that this is one potential site for PrP^{Sc} accumulation in skeletal muscle cells and/or axon terminals.

The majority of PrP^{Sc} staining in the lingual mucosa was located in the lamina propria following intracerebral inoculation of HY TME, especially within the connective tissue core of the lingual papillae (Fig. 2C). To investigate the spatial relationship between PrP^{Sc} and nerve fibers in the lamina propria, we examined PrP^{Sc} and CGRP staining in the tongue at 75 to 85 days postinfection following intracerebral inoculation of the HY TME agent. CGRP staining in the lamina propria was used to identify a subset of sensory nerves, since not all sensory fibers are CGRP positive. PrP^{Sc} immunostaining was identified in 35% of CGRP-positive papillae, but colocalization of CGRP and PrP^{Sc} was found in only 5% of CGRP-positive papillae (Fig. 4A to C). In 4% of the lingual papillae, PrP^{Sc} staining was also found in the stratified squamous epithelium of the tongue mucosa following intracerebral inoculation (Fig. 4D). In these examples, the PrP^{Sc} and cytokeratin staining were in close proximity in the papillary epithelium (Fig. 4D to F); cytokeratin immunohistochemistry was used as a marker for the stratified squamous epithelial cells of the tongue. However, there was no direct overlap of the two staining patterns (Fig. 4F, inset). A greater amount of PrP^{Sc} staining was found associated with tongue papillae following intracerebral inoculation compared to inoculation of the HY TME agent into the lingual muscles. No PrP^{Sc}-specific staining was observed in the tongue of mock-infected hamsters (Fig. 4G to I).

PrP^{Sc} staining in the tongue mucosa did not appear in cell groups that had the morphology of the lingual tonsils. However, we cannot exclude the possibility that PrP^{Sc} was associated with diffuse lymphoid tissue that is found in the tongue of some species.

DISCUSSION

In this study we report that the prion agent is located within skeletal muscle cells following inoculation of the HY TME agent into either the tongue or the cerebrum. While the TME agent was distributed throughout the individual muscle cells, PrP^{Sc} was also localized to the NMJ in 25% of synaptophysin-positive muscle cells. These findings do not distinguish whether the PrP^{Sc} staining was present in the presynaptic axon terminal, postsynaptic skeletal muscle, or in both locations in the

NMJ. PrP^{Sc} was also identified in sensory axons of the lamina propria, mainly in the core of the lingual papillae, but also within the stratified squamous epithelium. This tissue distribution of the prion agent in skeletal muscle and oral mucosa has not been previously reported.

Prior studies have demonstrated PrP^{Sc} accumulation in the tongue (3, 40) or prion infectivity in skeletal muscle (6, 26, 40), but PrP^{Sc} staining by immunomorphological methods has only been reported in nerve fibers that transverse skeletal muscle (3). In the present study, PrP^{Sc} deposition was found in nerve fibers and skeletal muscle cells of the tongue. The normal isoform of the prion protein, PrP^{C}, has been demonstrated to spread within nerve fibers by axonal transport (5) and is located in synapses, including subsynaptic areas in both the presynaptic and postsynaptic cells of the NMJ (2, 15). Since PrP^{C} is required for PrP^{Sc} formation (7), we propose that the NMJ is a site for PrP^{Sc} formation and/or PrP^{Sc} spread between skeletal muscle cells and axon terminals. In this case, PrP^{Sc} spread to, and accumulation in, skeletal muscle cells in the tongue following intracerebral inoculation could be due to TME agent replication in the brain and subsequent anterograde transport within the hypoglossal nerve, which provides motor innervation to the lingual muscles. PrP^{Sc} may spread across the NMJ and enter skeletal muscle cells, where additional TME agent replication can occur, since PrP^{C} is also expressed in skeletal muscle cells (15, 29). This hypothesis is supported by studies that describe (i) the spread of the prion agent in both the anterograde and retrograde directions between synaptically linked nerve cell groups in the central nervous system (4, 12, 13, 27), (ii) the retrograde transport of the HY TME agent from the tongue to the brain in the hypoglossal nerve following intratongue inoculation (3), and (iii) prion infectivity in skeletal muscle of transgenic mice that express PrP^{C} in myocytes (6). Although the NMJ is morphologically distinct from synapses in the central nervous system, our findings are consistent with this peripheral synapse serving as a pathway for intercellular spread of the prion agent between motor axon terminals and muscle cells.

PrP^{Sc} deposition in the lamina propria below the mucosal epithelium was associated with sensory nerve fibers; however, we cannot exclude the possibility that other types of cells or axons are infected with the TME agent, since only a portion of PrP^{Sc} staining colocalized with CGRP staining. Given that CGRP staining only labels a subset of sensory fibers, it is possible that other types of sensory fibers located in the lamina propria also contain PrP^{Sc}. Based on the location of PrP^{Sc} staining and morphology of the adjacent tissue, there was no evidence that PrP^{Sc} was present in the lingual tonsils, which are also a potential target for TME agent replication. However, from these studies we cannot exclude that the lingual tonsils are a site of TME agent replication. PrP^{Sc} staining in the lamina propria was often found in the connective tissue in the core of papillae; this area has a high concentration of sensory fibers that innervate mechanoreceptors and taste buds in the tongue (30, 35). PrP^{Sc} staining in the lamina propria and tongue epithelium was more pronounced following intracerebral inoculation compared to intralingual inoculation. This finding could be due to TME agent replication in the brain and subsequent transganglionic transport within the tongue-associated sensory cranial nerves. PrP^{Sc} that was localized to cy-
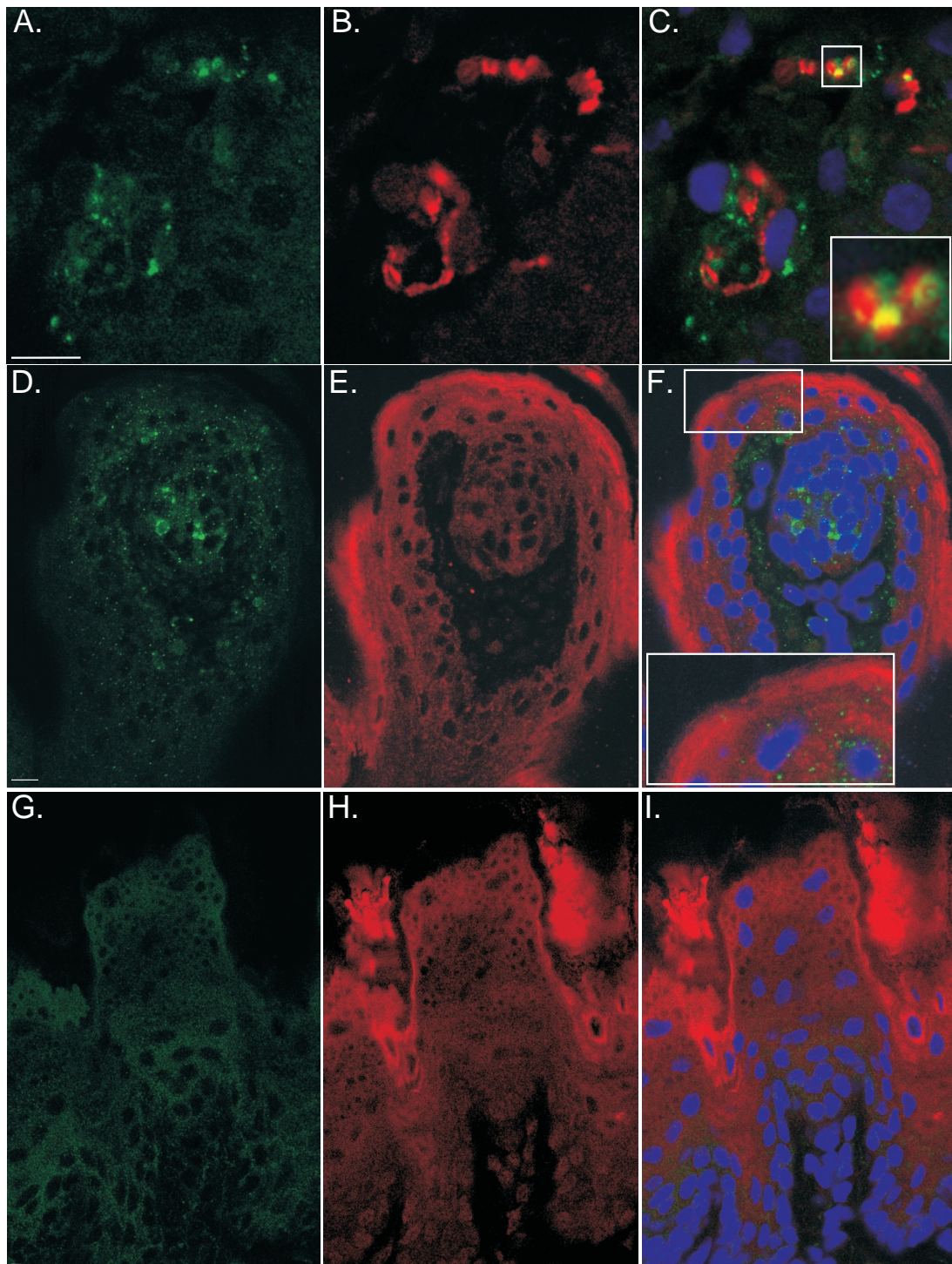
FIG. 4. Confocal microscopy of PrP^{Sc} in sensory nerve fibers and the stratified squamous epithelium. Hamsters were inoculated in the cerebrum with the HY TME agent (A to F) or a normal brain homogenate (G to I) (mock infected). At the onset of clinical symptoms, the tongue was prepared for confocal microscopy as described in Materials and Methods. Following immunofluorescence staining for PrP^{Sc} with Alexa Fluor 488 (A, D, and G), tissue sections were stained for either CGRP (B) or cytokeratin (E and H) with goat anti-rabbit immunoglobulin conjugated to Alexa Fluor 568. The stain TO-PRO3 was used to visualize nuclei under blue fluorescence. To investigate the colocalization of PrP^{Sc} with CGRP (C) and cytokeratin (F and I), green and red fluorescent images were merged. Bar, 10 μm. The insets at the bottom (C and F) are two- to threefold enlargements of smaller boxes in each of the corresponding panels.

tokeratin-positive cells could also be explained by TME infection of sensory nerve fibers that project into the epithelial cell layers of the tongue (11, 25, 32, 38). Alternatively, prion agent could spread to basal keratinocytes from sensory nerve endings and lead to PrP^{Sc} formation, since keratinocytes also express PrP^{C} (33, 39). This proposed pathway for prion agent spread to the mucosa along sensory fibers is analogous to the previous reports of spread of the CJD agent from the brain to the cornea and olfactory mucosa in sporadic CJD (24, 45; P. Duffy, J. Wolf, G. Collins, A. G. DeVoe, B. Streeten, and D. Cowen, Letter, N. Engl. J. Med. **290:**692-693, 1974). The low level or absence of the prion agent in the lymphoreticular system in sporadic CJD (14, 21) suggests that the optic and olfactory cranial nerves, respectively, transport the CJD agent from the brain to these mucosal or exposed surfaces. Ultrastructural studies will be necessary to further define the location of PrP^{Sc} in the lamina propria and sensory nervous system in the tongue and its spatial relationship to epithelial cells of the tongue.

In natural prion diseases, prion agent transport from the brain to the tongue could have implications for human food safety and animal prion transmission. In sheep with scrapie, cattle with BSE, and deer with chronic wasting disease, the brain stem regions containing the tongue-associated cranial nerve nuclei have been reported to be targets for prion infection. In one or more of these diseases, PrP^{Sc} has been found in the hypoglossal nucleus (9, 37, 43), the nucleus of the solitary tract (9, 34, 37, 43), and several of the trigeminal nuclei (9, 34, 37, 43, 44). The ability of the prion agent to establish infection in brain nuclei that contribute to, or synapse with, the four cranial nerves that innervate the tongue suggests that this could be a pathway for centrifugal prion agent spread. The tongue is one of the most densely innervated extraneural tissues in the human body, with a very high concentration of both motor and sensory axons (28, 30, 35, 36, 41). Anterograde prion transport within the hypoglossal nerve would be expected to result in prion infection of the lingual muscles, while transganglionic transport within the trigeminal, facial, and glossopharyngeal nerves could result in prion spread to the sensory fibers in the tongue. It is noteworthy that prion infectivity has been found in the trigeminal ganglia of sheep with scrapie (19) and cattle with BSE (42), indicating that centrifugal spread of these prion agents within the trigeminal nerve can occur and could result in spread to peripheral sites including the tongue. Since livestock and cervid tongues are not banned for human consumption, it is possible that humans could be exposed to animal prion diseases by ingestion of prion-infected tongue. Attempts to identify prion infectivity in bovine tongues have not been successful. However, these studies have used an infectivity bioassay that cannot detect below $10^{4.1}$ LD$_{50}$ of BSE prions, which may not detect low levels of the prion agent (10, 42). Our findings also have implications for intraspecies transmission of animal prion diseases. Establishment of prion infection in the stratified squamous epithelial cells of the tongue, as suggested by our findings, could result in sloughing of the prion agent into the saliva, since this tissue is undergoing continual cell turnover. In this case, animal behaviors resulting in the exchange of saliva between hosts may play a role in prion transmission.

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