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Oral transmission and early lymphoid tropism of chronic wasting disease PrP^{res} in mule deer fawns (*Odocoileus hemionus*)

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Mule deer fawns (*Odocoileus hemionus*) were inoculated orally with a brain homogenate prepared from mule deer with naturally occurring chronic wasting disease (CWD), a prion-induced transmissible spongiform encephalopathy. Fawns were necropsied and examined for PrP^{res}, the abnormal prion protein isoform, at 10, 42, 53, 77, 78 and 80 days post-inoculation (p.i.) using an immunohistochemistry assay modified to enhance sensitivity. PrP^{res} was detected in alimentary-tract-associated lymphoid tissues (one or more of the following: retropharyngeal lymph node, tonsil, Peyer's patch and ileocaecal lymph node) as early as 42 days p.i. and in all fawns examined thereafter (53 to 80 days p.i.). No PrP^{res} staining was detected in lymphoid tissue of three control fawns receiving a control brain inoculum, nor was PrP^{res} detectable in neural tissue of any fawn. PrP^{res}-specific staining was markedly enhanced by sequential tissue treatment with formic acid, proteinase K and hydrated autoclaving prior to immunohistochemical staining with monoclonal antibody F89/160.1.5. These results indicate that CWD PrP^{res} can be detected in lymphoid tissues draining the alimentary tract within a few weeks after oral exposure to infectious prions and may reflect the initial pathway of CWD infection in deer. The rapid infection of deer fawns following exposure by the most plausible natural route is consistent with the efficient horizontal transmission of CWD in nature and enables accelerated studies of transmission and pathogenesis in the native species.

Introduction

Chronic wasting disease (CWD) is a fatal prion disease affecting mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*) and Rocky Mountain elk (*Cervus elaphus nelsoni*). This transmissible spongiform encephalopathy (TSE) has been reported in captive and free-ranging deer and elk from north-eastern Colorado and south-eastern Wyoming (Spraker *et al.*, 1997; Williams & Young, 1980, 1982, 1992). Although the pathology of CWD is well-described (Williams & Young, 1993), little is known about CWD transmission. Epidemiological evidence from captive animals suggests that horizontal transmission may occur at a level apparently unparalleled in other prion diseases (Miller *et al.*, 1998; Williams & Young, 1992). Other non-familial TSEs, such as kuru, transmissible mink encephalopathy and bovine spongiform encephalopathy (BSE) appear to be transmitted via ingestion of PrP^{res}-infected tissue (Cervenakova *et al.*, 1998; Marsh & Bessen, 1993; Wells *et al.*, 1998).

Few studies of early preclinical TSE infections have been performed in natural hosts or using probable natural routes of

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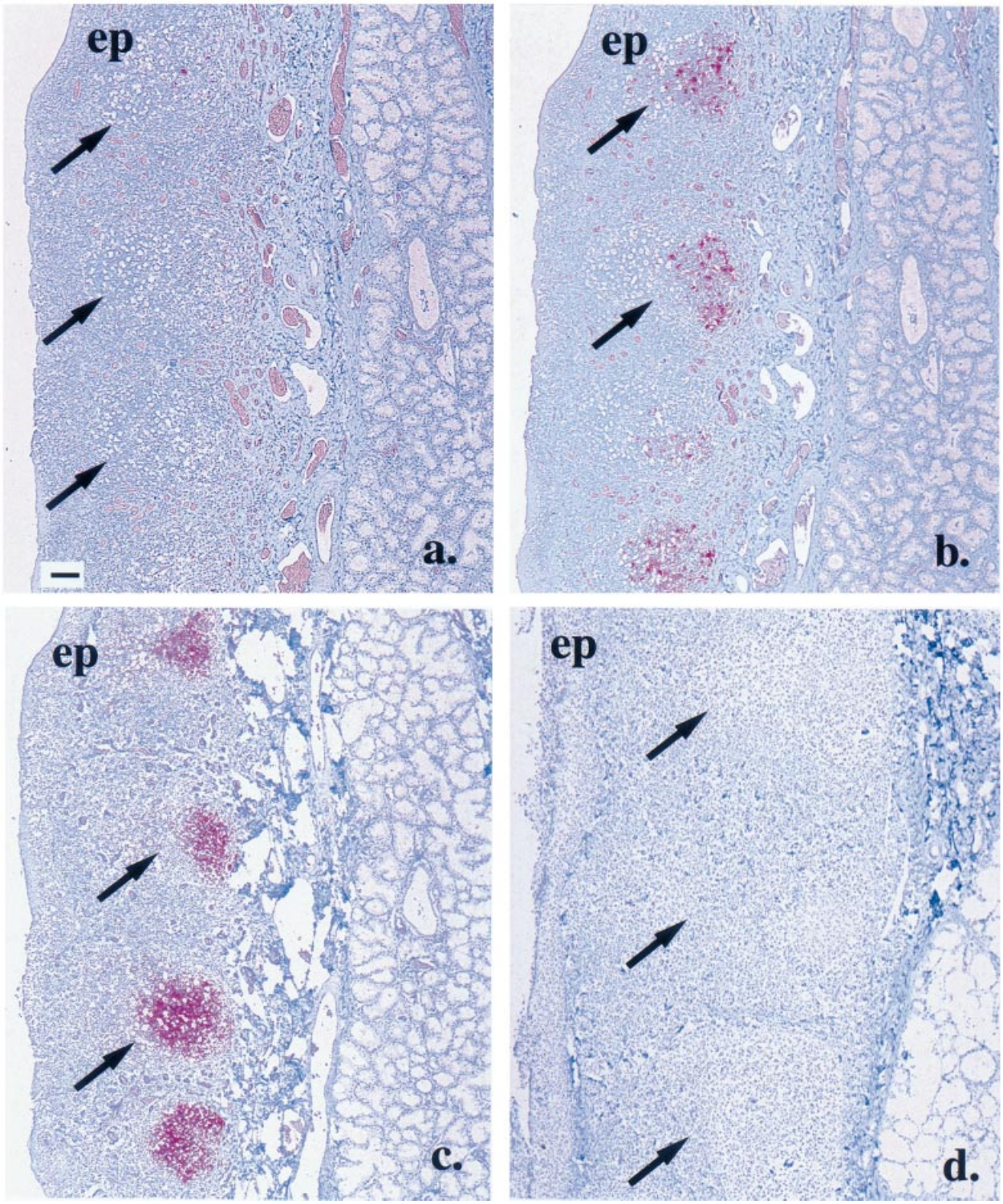

Fig. 1. Enhanced immunohistochemical detection of PrP^{res} (red, arrows) within tonsillar lymphoid follicles of a known CWD-positive deer. Tissue was treated with either: (*a*) hydrated autoclaving only, (*b*) formic acid + hydrated autoclaving, or (*c*) formic acid + proteinase K + hydrated autoclaving prior to IHC using MAb F89/160.1.5 and an alkaline phosphatase-based system. Best staining was achieved with protocol (*c*). No PrP^{res} staining was present in CWD-negative control deer tonsil (*d*). ep, Epithelium. Bar, 100 μm.

Table 1. Immunohistochemical detection of PrP^{res} in fawn lymphoid tissue

Results are expressed as number of positive follicles over total number of follicles counted. Positive IHC staining follicles are in bold.

Deer no.	Days p.i.	Retropharyngeal node*	Tonsil	Peyer's patches	Ileocaecal node	Mesentaric node	Sublumbar node	Popliteal node	Prescapular node	Submandibular node	Parotid node	Ruminal node	Abomasal node	Conjunctiva	Bone marrow	Thymus	Spleen
4	10	0/9	0/14	0/194	0/26	0/37	0/5	0/3	0/29	0/6	0/50	0/3	0/4	0/0	0	0/34	0/58
5	42	**8/119 (6·7%)**†	0/165	**1/190 (0·53%)**	**1/62 (1·6%)**	0/15	0/3	0/0	0/1	0/45	0/25	0/0	0/30	0/2	0	0/29	0/27
1	53	**6/104 (5·8%)**	0/128	0/535	0/108	0/81	0/4	0/16	0/35	0/46	0/64	0/27	0/50	0/0	0	0/118	0/15
7	77	**3/111 (2·7%)**	0/208	**3/549 (0·55%)**	0/72	0/177	0/52	0/45	0/136	0/112	0/56	0/33	0/34	0/0	0	0/26	0/20
2	78	**49/179 (27·3%)**	**1/205 (0·49%)**	**3/611 (0·49%)**	0/57	0/201	0/61	0/46	0/95	0/49	0/79	0/37	0/39	0/3	0	0/48	0/42
8	80	**5/99 (5·1%)**	**7/300 (2·3%)**	0/542	0/102	0/227	0/214	0/76	0/233	0/126	0/103	0/38	0/97	0/22	0	0/57	0/22
9	27	0/219	0/72	0/245	0/17	0/19	0/9	0/5	0/23	0/81	0/6	0/2	0/27	0/0	0	0/97	0/52
3	70	0/135	0/179	0/83	0/40	0/70	0/5	0/54	0/56	0/96	0/48	0/1	0/35	0/0	0	0/120	0/8
6	74	0/136	0/304	0/385	0/22	0/37	0/11	0/42	0/188	0/118	0/19	0/2	0/4	0/1	0	0/20	0/31

* Deer were orally inoculated with CWD-positive (deer nos 4, 5, 1, 7, 2, 8) or negative control (deer nos 3, 6, 9) brain tissue.

† Multiple cross-sections of each lymphoid tissue were examined; low follicle count reflects diffuse lymphocyte distribution and fewer formed follicles.

exposure; however, the results have been intriguing. BSE has been orally transmitted to cattle with infectivity detectable in the ileum of calves at 26 weeks post-inoculation (p.i.) (by mouse bioassay) (Wells *et al*., 1994). In another study, scrapie agent infectivity was first detected in the prescapular lymph nodes of goats at 24 weeks post-subcutaneous inoculation (Hadlow *et al*., 1974). However, mice inoculated intragastrically with scrapie had detectable infectivity in Peyer's patches and cervical lymph nodes as early as 1 week p.i. (Kimberlin & Walker, 1989). Thus, it appears that prions can cross the mucous membranes of the digestive tract to initiate infection in lymphoid tissue prior to invasion of the central nervous system and development of clinical disease.

Oral exposure is the most plausible pathway by which the CWD prion may be introduced to deer in nature. Consequently, we chose this means of inoculation in an attempt to demonstrate the feasibility of CWD transmission by this route and to study early lymphoid tissue tropism of the PrP^{res} in deer. Each deer was repeatedly exposed to a known infectious CWD inoculum over a 5-day-period because recent results with scrapie in hamsters indicate repeated oral exposure increases the incidence of infection (Diringer *et al*., 1998). Because mice are relatively resistant to CWD (M. Bruce, personal communication) precluding bioassay, and because several studies have shown that PrP^{res} strongly correlates with disease (McKinley *et al*., 1983; Race *et al*., 1998), we employed an enhanced immunostaining method (formic acid, proteinase K and hydrated autoclaving) to detect PrP^{res} *in situ*. Formic acid and hydrated autoclaving have been previously described for PrP^{res} epitope exposure prior to immunohistochemistry (IHC) (Miller *et al*., 1994; van Keulen *et al*., 1995). Using these methods, we demonstrate PrP^{res} in regional lymph nodes as early as 6 weeks after oral exposure of deer fawns to the CWD agent.

Methods

■ **Animals.** Nine free-ranging mule deer fawns (*Odocoileus hemionus*) were acquired from 100 km outside the CWD endemic area. Control and inoculated animals were housed in separate indoor rooms which had not previously held deer. All fawns were bottle-fed evaporated milk throughout the study and had free access to calf manna, alfalfa hay, mineralized salt and water as per a protocol previously established for raising deer (Wild & Miller, 1991; Wild *et al*., 1994). Animals received *Clostridium* bacterin-toxoid and iron dextran injections.

■ **Oral inoculation of deer.** Six fawns were inoculated orally with 5 ml of a 40% (w/v) brain homogenate (2 g brain) daily for 5 days using a small syringe inserted into the diastema of the oral cavity. Fawns typically licked and swallowed the material. The homogenate was prepared in normal saline solution from brains of 26 captive mule deer naturally infected with CWD. These deer had characteristic clinical signs and histological lesions of CWD in the brain. The homogenate had characteristic PrP^{res} bands by Western blot and scrapie-associated fibrils by negative stain electron microscopy (E. Williams, personal communication). Using the same protocol, three control fawns were inoculated in a like manner with a 40% brain homogenate from free-

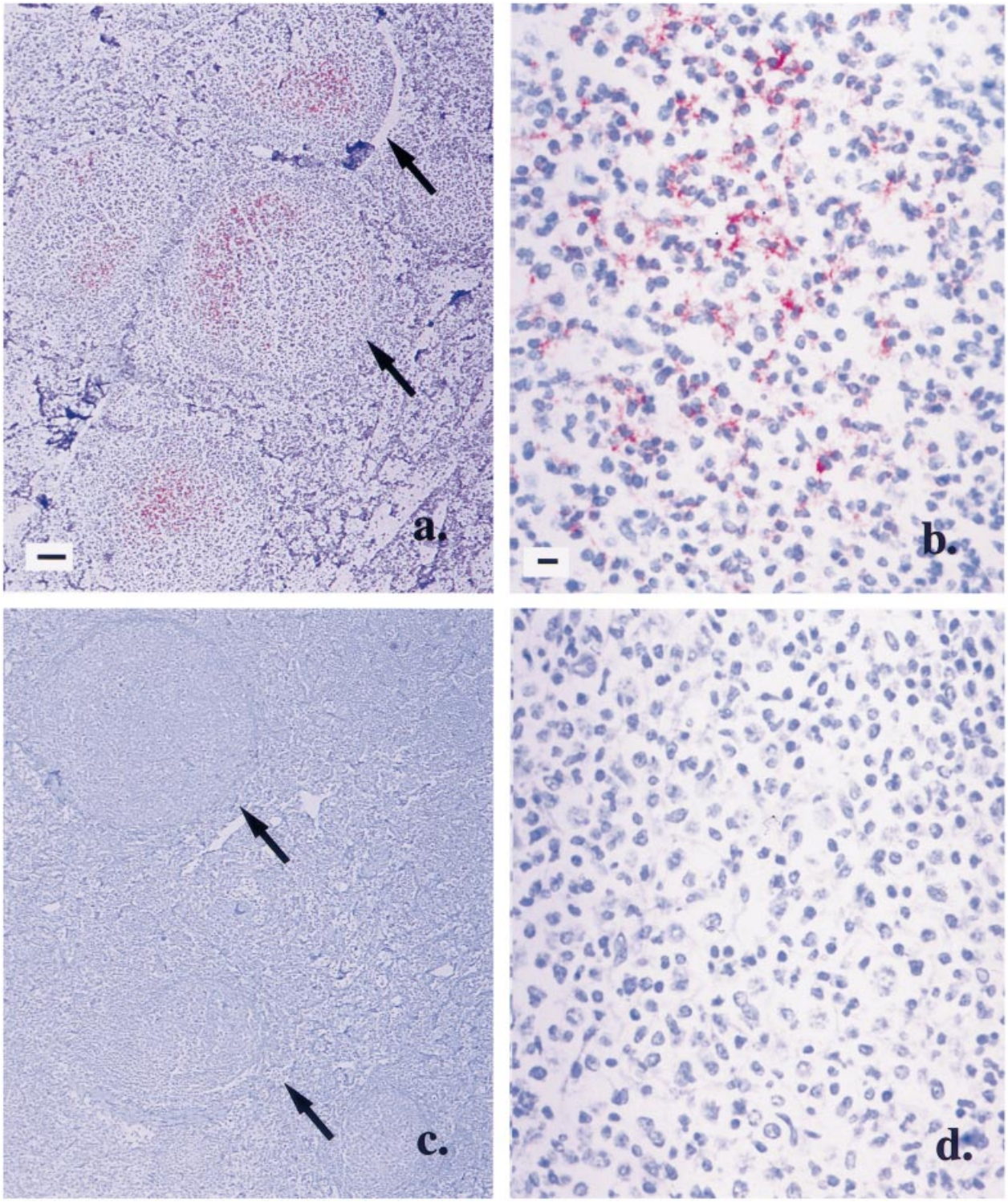

Fig. 2. Immunohistochemical detection of PrP^{res} in retropharyngeal node lymphoid follicles (red, arrows) of a fawn exposed orally to CWD-positive brain inoculum (*a*, *b*). No PrP^{res} staining was detected in the retropharyngeal node follicles (arrows) of fawns exposed to CWD-negative brain inocula (*c*, *d*). Bar, 100 μm (*a*, *c*) or 10 μm (*b*, *d*).

ranging mule deer outside the CWD endemic area; these deer were collected from a heavily monitored herd with no immunohistochemical or histological lesions of CWD (M. W. Miller, unpublished data).

■ **Necropsy and tissue collection.** Infected deer were euthanized with sodium pentobarbital given intravenously and necropsied sequentially at 10, 42, 53, 77, 78 and 80 days p.i. ($n = 6$). Control deer were

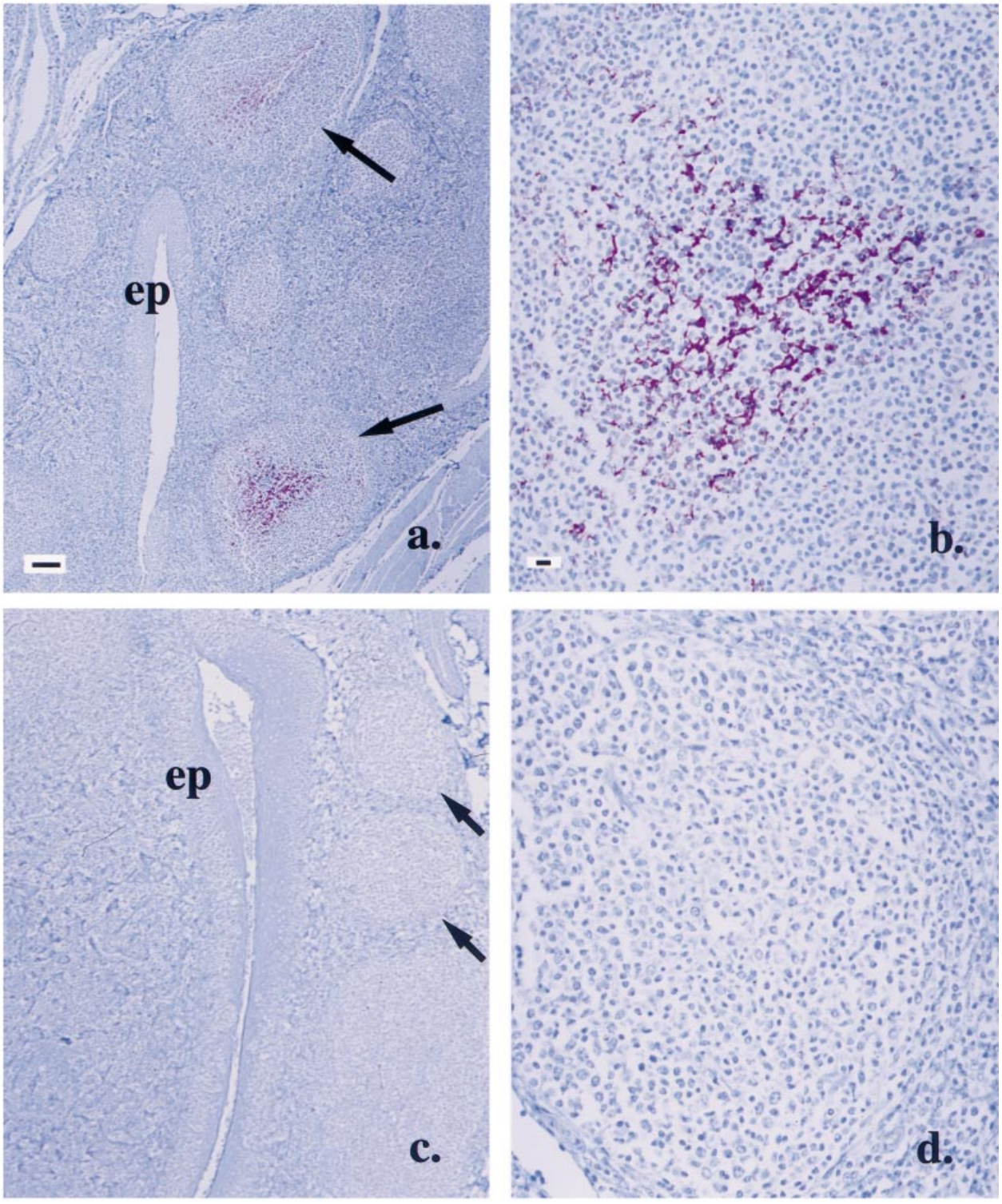

Fig. 3. Immunohistochemical detection of PrP^{res} in tonsillar lymphoid follicles (red, arrows) of a fawn exposed orally to CWD-positive brain inoculum (*a*, *b*). No PrP^{res} staining was detected in the tonsillar follicles (arrows) of fawns exposed to CWD-negative brain inocula (*c*, *d*). Bar, 100 μm (*a*, *c*) or 10 μm (*b*, *d*).

necropsied at 27, 70 and 74 days p.i. ($n = 3$). Days p.i. were calculated from the last day of exposure. Numerous tissues were collected, including ten lymph nodes (mesenteric, ileocaecal, sublumbar, popliteal, prescapular, retropharyngeal, submandibular, parotid, ruminal and abomasal nodes), spleen, bone marrow, thymus, Peyer's patches, tonsil, conjunctiva, spinal cord and brain. Tissues were preserved in neutral-buffered 10% formalin and then trimmed, processed and embedded in paraffin blocks within 7 days.

■ **Immunohistochemical staining.** Prior to staining the fawn tissues, various pre-treatments were tested on tissue sections of obex and tonsil from a positive control CWD mule deer to produce optimal stain enhancement. This was done to maximize staining sensitivity to detect anticipated early accumulation of PrP^{res} in tissues. Sections were treated as follows: (1) hydrated autoclaving at 121 °C for 20 min, (2) immersion of slides in 88% formic acid for 30 min followed by hydrated autoclaving for 20 min, (3) immersion in 25 μg/ml proteinase K for 10 min at 26 °C followed by hydrated autoclaving, (4) immersion in 12·5 μg/ml proteinase K for 10 min followed by hydrated autoclaving, and (5) immersion in 88% formic acid for 30 min, then 25 μg/ml proteinase K for 10 min followed by hydrated autoclaving for 20 min. Immunohistochemical staining on the treated sections followed immediately. Staining intensity and specificity was determined by light microscopy. Of these, protocol no. 5 resulted in the greatest PrP^{res} staining.

Tissue sections were mounted onto positively charged glass slides, deparaffinized and hydrated in preparation for IHC. Tissue treatment performed prior to IHC consisted of slide immersion in 88% formic acid solution for 30 min followed by a rinse in water and immersion in 25 μg/ml proteinase K solution at 26 °C for 10 min. Tissue sections were then autoclaved for 20 min at 121 °C in Tris buffer solution and cooled for 30 min. The treatments were extensive in order to maximally expose epitopes and enhance staining.

IHC employed an automated immunostainer (Ventana Medical Systems) and PrP^{res} monoclonal antibody (MAb) F89/160.1.5, a biotinylated secondary antibody, an alkaline phosphatase–streptavidin conjugate, a substrate chromagen (Fast Red A, naphthol, Fast Red B) and a haematoxylin and bluing counterstain (Ventana Medical Systems). MAb F89/160.1.5 recognizes a conserved epitope on the prion protein of mule deer, elk, sheep and cattle (O'Rourke *et al.*, 1998). Positive and negative control tissue sections were included in each run.

Several IHC controls were performed on lymphoid tissues with MAb F89/160.1.5. Lymphoid tissues from 50 deer (collected outside the CWD endemic area) were immunostained using the same methodology as performed on the fawn tissues. IHC on known positive and negative deer tonsil sections was done using MAb F89/160.1.5 substituted by mouse serum or an irrelevant isotype-matched MAb diluted to the same protein concentration as MAb F89/160.1.5. In addition, IHC was performed on a retropharyngeal node section from each fawn with an irrelevant MAb substitution.

Results

Enhanced immunostaining

We assessed five tissue pre-treatment protocols (see Methods) in an attempt to maximize immunohistochemical staining sensitivity yet preserve sufficient histological detail to permit localization of PrP^{res}. Using positive control tissue from deer with naturally occurring CWD, we found that detection of PrP^{res} was markedly enhanced by slide immersion in either formic acid or proteinase K prior to hydrated autoclaving. Maximal staining was achieved using sequential pre-treatments with formic acid and proteinase K followed by hydrated autoclaving (Fig. 1).

Deer tonsil sections from known positive and negative CWD cases immunostained with an irrelevant antibody or with mouse serum substituted for the primary antibody were uniformly negative. No immunostain was detected in lymphoid sections from 48 CWD-negative deer originating from non-CWD endemic geographical regions (MAb F89/160.1.5) or in fawn retropharyngeal nodes (irrelevant MAb substitution). In two of the negative deer control cases, a small focus of greyish pink stain was observed in less than five follicles. The CWD-positive control tissue had strong positive staining in the follicular areas when stained with MAb F89/160.1.5.

Earliest detection of PrP^{res} in orally exposed fawns

PrP^{res} was not detectable in any tissue of the fawn necropsied at 10 days p.i. However, in the fawn necropsied at day 42 p.i., PrP^{res} was detected in follicular germinal centres of the retropharyngeal lymph nodes, Peyer's patches and ileocaecal nodes. Of 119 follicles examined in the retropharyngeal nodes, eight (6·7% of follicles) were PrP^{res}-positive. PrP^{res} also was detected in the retropharyngeal node follicles of all infected fawns examined at later time intervals p.i. (53, 77, 78 and 80 days) (Table 1).

Tissue distribution of PrP^{res}

In six fawns examined between days 10 and 80 p.i., PrP^{res} was detected in the retropharyngeal lymph node follicles of five, Peyer's patches of three, tonsil of two and ileocaecal node of one (Table 1). PrP^{res}-specific staining consistently appeared as bright granular deposits (red using Fast Red A substrate) arranged in patterns suggestive of dendritic cells within germinal centres of well-developed secondary follicles. Staining often occurred in clusters of adjacent follicles (Fig. 2). In all fawns, the quantity of PrP^{res} estimated by subjective evaluation of stained product was substantially less than that seen in symptomatic cases of CWD, consistent with early foci of formation.

PrP^{res} was detected in 2·7% to 27·3% of the retropharyngeal lymph node follicles in fawns necropsied between days 42 and 80 p.i. (Table 1). At 42 days p.i., PrP^{res} was visible in 0·53% of follicles in Peyer's patches. As in lymph nodes, the stain deposits were localized to the germinal centres of the lymphoid aggregates. In tonsil, stain was only seen at the two final time-points (78 and 80 days p.i.), in 0·49% and 2·3% of follicles, respectively (Fig. 3).

PrP^{res} was not detected in brain (obex region), spinal cord or salivary gland examined from the inoculated animals. No PrP^{res} staining was detected in any tissue of the sham-inoculated control fawns (Figs 2 and 3).

Clinical signs

No clinical signs of CWD occurred in any of the inoculated deer throughout the course of the study. One fawn incidentally developed severe laryngeal swelling which was resolved completely with antibiotic therapy, and two fawns developed mild diarrhoea; otherwise fawns remained healthy.

Discussion

These results indicate that mule deer fawns develop detectable PrP^{res} after oral exposure to an inoculum containing CWD prions. In the earliest post-exposure period, CWD PrP^{res} was traced to the lymphoid tissues draining the oral and intestinal mucosa (i.e. the retropharyngeal lymph nodes, tonsil, ileal Peyer's patches and ileocaecal lymph nodes), which probably received the highest initial exposure to the inoculum. Hadlow *et al*. (1982) demonstrated scrapie agent in the tonsil, retropharyngeal and mesenteric lymph nodes, ileum and spleen in a 10-month-old naturally infected lamb by mouse bioassay. Eight of nine sheep had infectivity in the retropharyngeal lymph node. He concluded that the tissue distribution suggested primary infection via the gastrointestinal tract. The tissue distribution of PrP^{res} in the early stages of infection in the fawns is strikingly similar to that seen in naturally infected sheep with scrapie. These findings support oral exposure as a natural route of CWD infection in deer and support oral inoculation as a reasonable exposure route for experimental studies of CWD.

Cells associated with PrP^{res} were within germinal centres of lymphoid follicles. The staining pattern was morphologically consistent with that of follicular dendritic cells. Experimental inoculation of mice with scrapie or Creutzfeldt–Jakob disease prions resulted in similar localization of PrP^{res} to follicular dendritic cells (Kitamoto *et al*., 1991; McBride *et al*., 1992). In sheep Peyer's patches are chiefly concentrated in the terminal ileum (Reynolds & Pabst, 1984). Assuming the immunobiology of deer is similar to sheep, it seems probable that initial uptake and propagation of PrP^{res} could occur in the ileal Peyer's patches and tonsils, and within dendritic cells emigrating via the lymphatic system to the ileocaecal and retropharyngeal lymph nodes.

Studies in mice show rapid accumulation of dendritic cells bearing antigen within regional lymph nodes hours after the skin was painted with contact allergens (Cumberbatch & Kimber, 1990). By analogy PrP^{res} from inoculum would be expected in draining lymph nodes by 10 days p.i. In that PrP^{res} was not detected in the lymphoid tissue of the day 10 fawn, the PrP^{res} staining in fawns examined at later time-points probably represented accumulating PrP^{res} versus residual inoculum. Interestingly, and in contrast to the sequence postulated above, PrP^{res} was visible in the tonsil only in the two fawns with the longest p.i. intervals, 78 and 80 days. This may indicate lower initial quantities of PrP^{res} in tonsil as compared with retropharyngeal node, perhaps due to the migration route of initially infected dendritic cells, resulting in a longer lag before PrP^{res} accumulates in the tonsil to levels detectable with IHC.

We detected PrP^{res} by IHC as early as 6 weeks p.i. – an extraordinarily brief period. Detection of PrP^{res} stain in lymphoid tissues by 6 weeks p.i. suggests that PrP^{res} accumulates at early disease stages. In goats experimentally infected with scrapie, infectivity was not detected until $\geqslant 3$ months p.i. (Hadlow *et al*., 1974). Given the repeated exposure to a relatively large amount of inoculum over 5 days, it seems logical to presume that infection in these orally inoculated fawns may be accelerated, enabling earlier PrP^{res} detection compared to naturally infected deer. Nevertheless, the present study provides proof in principle that CWD PrP^{res} is detectable after oral exposure. Although the present study design precluded the development of clinical disease, the presence of PrP^{res} has been shown to be strongly correlated with infectivity with other TSEs (Race *et al*., 1998).

CWD in deer is similar to scrapie in that the PrP^{res} is disseminated throughout lymphoid tissues (T. Spraker, unpublished data). This disseminated lymphoid infection is unlike some other TSEs, such as BSE, in which PrP^{res} is detected only in the ileal Peyer's patches or not at all (Wells *et al*., 1998). Kimberlin & Walker (1989) and Williams & Young (1992) have made an association between infection of the lymphoreticular system and the high transmissibility of scrapie among sheep, similar to the findings described in deer and elk. It is possible that localization of PrP^{res} to lymphoid tissues adjacent to mucosal surfaces promotes prion shedding into the environment via fluids such as saliva or faeces, although the pathway of CWD shedding and potential contagion requires further study.

The exact mode of CWD transmission in nature remains unknown. Scrapie in sheep has been demonstrated in experimental studies to be transmissible via ingestion of foetal membranes from scrapie-positive ewes (Pattison *et al*., 1972). Nevertheless, scrapie transmission in nature remains incompletely understood (Detwiler, 1992). Understanding mechanisms of shedding and transmission will be important in management of CWD and in providing insights into the pathogenesis of other TSEs.

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