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Quantitative Detection of Hepatitis C Virus (HCV) RNA in Saliva and Gingival Crevicular Fluid of HCV-Infected Patients

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The search for hepatitis C virus (HCV) in body fluids other than blood is important when assessing possible nonparenteral routes of viral transmission. However, the role of oral fluids in HCV transmission remains controversial. Here we quantitatively determined HCV RNA in saliva and gingival crevicular fluid (GCF) of anti-HCV-positive patients. Most patients (14 of 18; 78%) whose saliva specimens were negative had HCV RNA in their GCF. Most patients (20 of 26; 77%) had higher HCV RNA levels in their GCF than in their saliva. Although there was not a statistically significant correlation between the serum viral load and HCV level in saliva or GCF, patients with low serum HCV loads were less likely to have detectable HCV in their saliva. These findings have important implications for medical personnel and suggest that epidemiological studies designed to understand the significance of the oral route of transmission of HCV are warranted.

Hepatitis C virus (HCV) infection represents a major public health problem in the world today. The infection primarily causes liver disease; however, HCV infection has also been associated with extrahepatic abnormalities, including mixed cryoglobulinemia, malignant lymphoma, Sjögren's syndrome, and oral lichen planus (2, 12, 18, 19, 34, 39). Lymphotropism of HCV has been observed, and several laboratories have detected the virus in blood mononuclear cells (BMC) (16, 22, 26, 28, 35, 38). Common risk factors for HCV infection include blood transfusion from unscreened donors as well as injection drug use. Although sexual and vertical transmissions have also been reported, there remain a large number of HCV carriers in whom no route of infection has been identified.

Epidemiological surveys demonstrate that body fluids other than blood, including saliva, might be potential sources of HCV infection. Experimental inoculation of saliva obtained from chronic HCV carrier chimpanzees has been reported to transmit hepatitis to recipient animals (1). Several studies have demonstrated HCV RNA in the saliva of hepatitis C patients by reverse transcription (RT)-nested PCR. However, the detection rates of viral RNA within saliva have varied widely, and some groups have failed to demonstrate HCV RNA within saliva (6–11, 14, 17, 23, 25, 27, 29–33, 36–38). A potential source of HCV RNA within saliva includes gingival crevicular fluid (GCF), which might contain HCV-infected BMC in the setting of periodontal inflammation. To our knowledge, only one study has qualitatively identified HCV in GCF; HCV RNA was detected in 59% of GCF specimens from hepatitis C patients in the study (20). Since the efficiency of HCV transmission is likely related to its viral load, it is important to quantitate viral RNA levels within body fluids in order to properly evaluate possible nonparenteral routes of HCV infection.

Thus, we examined the presence of HCV RNA in the saliva and GCF of anti-HCV antibody-positive patients using real-time quantitative RT-PCR.

MATERIALS AND METHODS

Sample collection. Twenty-six dental patients attending the hospital of Nippon Dental University at Tokyo were studied. All of the patients were anti-HCV antibody seropositive on the basis of screening using a second-generation enzyme immunoassay (Abbott HCV PHA, Abbott Diagnostics, Abbott Park, IL). This study protocol was approved by the Ethics Committee of the hospital and was conducted according to *Ethic Guideline for the Studies on Human Genome and Gene Analysis*. Written informed consent was obtained from each patient participating in the study.

Blood samples were collected and centrifuged for 20 min at 5,000 rpm to separate the serum. Patients spit into a cup to obtain saliva samples. Whole saliva samples (approximately 2 ml) were then transferred into sterile containers. None of the samples were macroscopically observed to contain blood. GCF specimens were collected by first drying the gingival surface with sterile cotton, after which the area was isolated in order to prevent contamination with saliva. A paper strip (2 by 5 mm) was then subgingivally inserted for 30 s to collect specimens (approximately 50 μl). If there was visible contamination of the sample with blood, another sample without macroscopic blood contamination was taken from another site. The depth at gingival crevices was then measured by a periodontal probe, and the presence of bleeding on probing was examined. Serum, saliva, and GCF samples were collected simultaneously and were stored at −80°C before use.

RNA extraction. Total RNA was extracted from 100 μl of serum or saliva specimens and from paper strips with collected GCF using a QIAamp viral RNA kit (QIAGEN, Valencia, CA). In preliminary experiments using various amounts of serum, saliva, and GCF samples in the presence or absence of paper strips, we confirmed that (i) sample volumes of >40 μl yielded the same efficiencies of RNA extraction from each specimen and (ii) inclusion of a paper strip described above in the lysis buffer did not influence the efficiency of RNA extraction.

Quantitation of HCV RNA. To determine the quantity of HCV RNA, real-time RT-PCR involving single-tube reactions was performed using TaqMan EZ RT-PCR Core reagents (PE Applied Biosystems, Foster City, CA), as previously described (3). Briefly, the reaction mixture contained 1× TaqMan EZ buffer, 500 nM concentrations of each primer from the HCV 5′ noncoding region (5′-GAG TGT CGT GCA GCC TCC A-3′ and 5′-CAC TCG CAA GCA CCC TAT CA-3′), a 200 nM concentration of fluorogenic probe [5′-(6-carboxyfluorescein)

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TABLE 1. Clinical and virological characteristics of 26 patients examined in this study[a]

Age	Gender	ALT level (IU/liter)	AST level (IU/liter)	HCV antibody titer (2^n)	Genotype	Oral disease(s)
68	F	30	48	>12	1b	
64	M	115	103	>12	1b	Periodontitis/BOP
71	F	14	23	12	2b	Periodontitis/BOP
71	M	124	71	12	1b	Periodontitis/BOP
71	M	47	55	12	1b	
63	M	14	19	11	1b	SCC
66	F	59	67	11	1b	OLP
61	F	61	35	11	1b	
73	F	48	40	10	2a	Periodontitis/BOP
70	F	18	25	10	1b	Periodontitis/BOP, OLP
72	F	15	20	8	1b	Periodontitis/BOP
61	M	7	12	8	ND	Periodontitis
66	F	19	30	8	1b	OLP
69	F	31	39	7	ND	SCC
73	M	16	24	6	ND	Periodontitis/BOP, SCC
72	F	20	22	6	ND	
67	M	12	18	4	ND	Periodontitis/BOP, SCC
70	F	5	17	4	ND	Periodontitis/BOP, SCC
69	M	13	20	4	1b	Periodontitis/BOP, SCC
60	M	22	23	4	1b	Periodontitis
71	F	15	30	4	ND	SCC
56	F	11	17	4	ND	Periodontitis/BOP, OLP
71	F	12	21	4	ND	Periodontitis/BOP
67	F	9	24	4	ND	
58	M	26	25	4	ND	
79	F	22	21	4	ND	

[a] Abbreviations: F, female; M, male; ND, not detected; OLP, oral lichen planus; BOP, bleeding on probing; SCC, squamous cell carcinoma.

CCC GCA AGA CTG CTA GCC GAG TAG TGT TGG (6-carboxytetramethyrhodamine)-3′], 200 μM concentrations of each deoxynucleoside triphosphate, 3 mM $Mn(OAc)_2$, 5 U of *Thermus thermophilus* DNA polymerase, 0.5 U of AmpErase uracil *N*-glycosylase, and template RNA. The primers and probe were designed on the basis of the conserved sequences among HCV genotypes. The RT step was started with a 1-min incubation at 50°C, followed by 50 min at 65°C. Thermal cycling conditions were as follows: a precycling period of 5 min at 95°Cfollowed by 50 cycles of denaturation at 94°C for 15 s and annealing at 55°C for 10 s and extension at 69°C for 1 min. All reactions and analyses of the amplification plots were performed on an Applied Biosystems PRISM 7700 sequence detector (PE Applied Biosystems). Standard curves of the assays were obtained by plotting 10-fold serial dilutions of known concentrations of a synthetic HCV genotype 1b transcript. HCV RNA copy numbers of the synthetic transcript were calculated from the quantity and its molecular weight. Using a standard curve, the Sequence Detector software calculated automatically the concentration of RNA copies in the experimental samples. We found that results obtained from our in-house real-time RT-PCR method were well correlated with those from the COBAS AMPLICOR HCV MONITOR Test, version 2.0 (Roche Diagnostics, Tokyo, Japan) (15), and that 1 HCV RNA copy/ml in our method corresponded to approximately 1 international unit/ml by the above-mentioned commercial assay (data not shown).

HCV genotyping. HCV genotype was determined by RT-PCR of the core region sequence with genotype-specific primers for determination of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a, as described previously (24).

PCR amplification of β-globin DNA. Total DNA was extracted from saliva samples using a QIAamp DNA Mini kit (QIAGEN) according to the manufacturer's instructions. To characterize the degree of cell contamination in saliva, isolated DNA was subsequently used as a template to amplify the human β-globin gene fragment of 268 bp with the following primers: 5′-GAA GAG CCA AGG ACA GGT AC-3′ and 5′-CAA CTT CAT CCA CGT TCA CC-3′ (21).

Statistical analysis. The Spearman rank test was used for evaluating the correlation between variables: anti-HCV antibody levels and viral loads in serum, saliva, and GCF.

RESULTS

The clinical and virological characteristics of 26 patients are presented in Table 1. The study group consisted of 10 males (38%) and 16 females (62%) with a mean age of 69 years (range, 56 to 79 years). Their mean liver enzyme values were as follows: 30 IU/liter for alanine aminotransferase (ALT) and 33 IU/liter for aspartic aminotransferase (AST). HCV RNA levels in the serum of 20 patients (77%) were determined by real-time RT-PCR assay, which showed a detection limit of 10^2 copies/ml and a linear range over 5 logs. Four of six serum samples whose HCV RNA levels were below the detection limit in this measurement were found to have detectable HCV RNA by the qualitative nested RT-PCR (4). We found no difference in efficiency and specificity of HCV cDNA amplification among genotypes 1b, 2a, and 2b in the real-time RT-PCR assay (data not shown).

Figure 1 summarizes viral loads in the serum, saliva, and GCF specimens of the patients. A mean serum HCV RNA level of 5.1×10^5 copies/ml was observed among samples with viral loads greater than 10^2 copies/ml. As expected, serum viral RNA levels were significantly correlated with anti-HCV antibody levels ($r = 0.80$, $P < 0.0001$) (Fig. 2A). In a number of cases (20 of 26; 77%), the viral load of the GCF was greater than that of the saliva. HCV RNA was detected in 31% of the saliva samples and 85% of the GCF specimens using real-time RT-PCR. Mean viral RNA levels were 1.9×10^4 (saliva) and 3.1×10^4 (GCF) copies/ml in these samples. It should be noted that most (seven out of eight) of the saliva samples contained 1.4×10^2 to 8.2×10^3 copies/ml of HCV RNA, with a mean value of 2.0×10^3 copies/ml among these seven samples (Fig. 1).

Among the 18 patients with HCV RNA-negative saliva, 10^2 to 10^3 copies/ml of viral RNA were detected in the GCF of 3 patients, 10^3 to 10^4 copies/ml of viral RNA were detected in the GCF of 2 patients, and $>10^4$ copies/ml were detected in

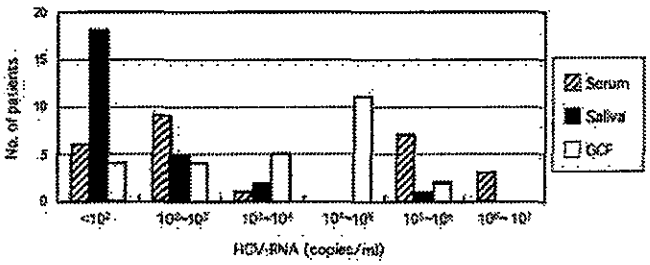

FIG. 1. HCV viral load in the serum, saliva, and GCF of anti-HCV-positive patients. Numbers of patients within each range of the viral load are indicated.

the GCF of 9 patients. No significant association was observed between viral RNA levels in the serum and viral RNA levels in the saliva (Fig. 2B) or GCF (Fig. 2C). However, relatively high serum viral loads ($>10^5$ copies/ml) were observed in five out of eight patients with HCV RNA-positive saliva, while serum viral loads were 1.5×10^3 copies/ml or less in most of the patients whose saliva specimens were negative (13 out of 18). Four patients with HCV RNA-positive saliva and/or GCF had no detectable serum HCV RNA by real-time RT-PCR (Fig. 2B and C); however, viral RNA was detectable in their sera by qualitative nested RT-PCR. Although no visible contamination of the saliva and GCF with blood was observed, there may be a small amount of cells or lysed cells in the fluids. To determine the degree of cell content in samples, total DNA was extracted from three saliva specimens, which contained $>10^3$ copies/ml of HCV RNA (Fig. 2B), and tested for the presence of cellular DNA by amplifying a human β-globin gene. A certain amount of cellular DNA was detectable in the saliva specimens (data not shown), suggesting some salivary HCV RNA may be derived from HCV-infected cells, such as BMC and mucosal epithelial cells, as discussed below. Various amounts of HCV-infected cells in the saliva and GCF may, in part, account for differences in the viral loads.

HCV RNA was detectable in most GCF and/or saliva specimens obtained from patients with clinical evidence of oral diseases: HCV RNA was detected in all 14 (100%) patients with periodontitis, 6 of 7 (85%) patients with squamous cell carcinoma, and 3 of 4 (75%) patients with lichen planus. Three out of four patients with HCV RNA-negative GCF, however, also had some oral epithelial lesions. On the other hand, among seven patients without oral diseases, HCV RNA was detected in the GCF and saliva of six and three patients, respectively. There was a trend toward increased viral loads in the oral fluids, especially GCF, among patients with bleeding on probing compared to those without the bleeding. The viral RNA levels in the GCF and saliva had no correlation with age, gender, or serum levels of ALT or AST. It also seems that their viral RNA levels were not correlated with HCV genotype, although the viral genotypes in 12 of 26 patients were not determined.

DISCUSSION

Identification of HCV in body fluids other than blood is important in order to evaluate possible nonparenteral routes of transmission. The role of oral fluids in HCV transmission remains controversial. Although the presence of HCV RNA in saliva has been reported by several research groups (6–11, 14, 17, 23, 25, 27, 29–33, 36–38), only one study has attempted to quantify HCV RNA in saliva, in which patients coinfected with HCV and human immunodeficiency virus were examined using a branched DNA assay (27). Moreover, limited information exists regarding the prevalence of HCV in the GCF of patients with hepatitis C, apart from one study in which a qualitative RT-PCR method was used to detect HCV in 59% of GCF and 35% of saliva specimens from patients with HCV viremia (20).

To the best of our knowledge, this study is the first to quantitate HCV loads within the saliva and GCF of anti-HCV antibody-positive patients using real-time RT-PCR. To search for a possible oral route of HCV transmission, whole saliva and GCF containing cell fractions were used to determine the viral

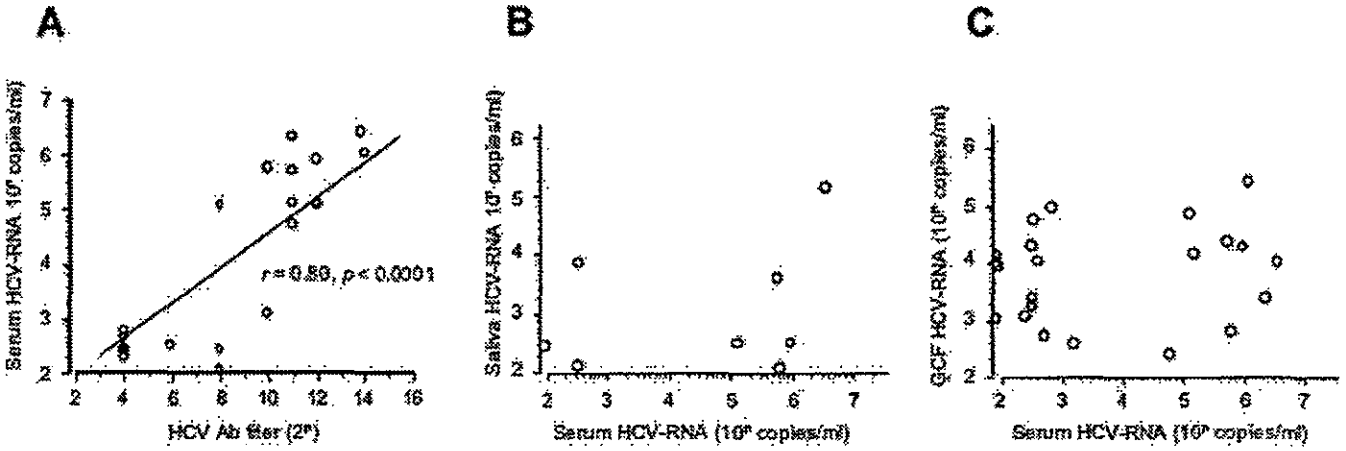

FIG. 2. (A) Correlation between anti-HCV antibody levels and HCV RNA levels in serum. The Spearman rank test was used for testing the correlation between variables. There is a significant positive correlation ($r = 0.80, P < 0.0001$) between the serum levels of HCV antibody detected by the passive hemagglutination assay and those of HCV RNA determined by real-time RT-PCR. (B) Correlation between viral loads in the serum and those in saliva specimens. Results for patients whose HCV RNA levels in saliva were $\geq 10^2$ copies/ml are plotted. No significant correlation was observed. (C) Correlation between viral loads in serum and those in GCF specimens. Results for patients whose HCV RNA levels in the GCF were $\geq 10^2$ copies/ml are plotted. No significant correlation was observed.

loads in this study. Although any saliva and GCF samples tested were not macroscopically observed to contain blood, we cannot rule out the possible effect of a small amount of bleeding as a source of HCV RNA. Here we observed HCV more commonly in the GCF than the saliva of HCV-seropositive patients. We further found viral loads of 10^2 to 10^4 copies/ml and 10^3 to 10^5 copies/ml in saliva and GCF, respectively. This result may be partially due to the presence of PCR inhibitors in saliva. An internal control to measure the possible effect of PCR inhibitors was not included in our real-time RT-PCR. Although the mean viral load within the GCF was approximately 10-fold lower than that in the serum, GCF samples from 12 of 26 patients (46%) had viral titers similar to or greater than those observed in the sera. No significant correlation was observed between the serum viremia levels and viral levels in the saliva or GCF. However, there was a trend that patients with HCV RNA-positive saliva showed higher viral loads in sera than patients with HCV RNA-negative saliva. These findings suggest that GCF might be one of the sources of HCV RNA within the saliva.

Although HCV is a hepatotropic virus, convincing evidence of HCV lymphotropism has been demonstrated in tissue culture (13). HCV has been widely detected in BMC in patients with chronic HCV infection, and differences in quasispecies identification within serum and BMC suggest that viral replication occurs within BMC (16, 22, 26, 28, 35, 38). HCV-infected BMC might allow HCV to infiltrate the GCF and saliva, since BMC migrate from dentogingival vessels into gingival crevices. There also might be transudation of HCV-containing serum into the mouth. Generally, periodontal inflammation increases the excretion of BMC-rich GCF. There is also a possibility that HCV exists within mucosal epithelial cells. HCV has been identified in the mucosal tissue, as well as salivary glands, of anti-HCV-positive patients with oral lichen planus using various techniques, including in situ hybridization, strand-specific RT-PCR, and immunohistochemistry (5, 32). Thus, it is likely that several possible sources discussed above are involved in HCV penetration into the saliva and GCF. Whatever the sources or mechanisms are, the findings obtained provide important implications for medical personnel regarding HCV transmission in health care settings as well as for HCV epidemiology, as the origin of the viral infection remains unclear in up to 40% of cases.

In this study, although the numbers of specimens were limited, we quantitatively determined HCV RNA in oral fluids from dental patients, including some patients with oral diseases, and demonstrated frequent detection of HCV in the saliva and GCF. Further large-scale epidemiological studies employing real-time RT-PCR assays are required to clarify the clinical significance of HCV in the saliva and GCF, including the potential for viral transmission through exposure to these fluids.

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研究報告の概要

フランスでのヤギの BSE 確認が、小反芻動物が飼料を介した経時的な曝露により BSE を維持するのかという議論をヨーロッパで活発化させている。TSE フリーの生後 6 ヵ月の雌の子ヒツジ 30 匹に BSE 汚染ウシ脳が経口投与された。約 6 ヵ月後に BSE 投与のヒツジの群れの中に同様の BSE 非投与の子ヒツジ 20 匹が加えられ、TSE フリー飼料を用いて自然に成熟するまで同じグループとして厳格に飼育された。最初に投与された 30 匹中 24 匹が 655～1,056 日の間に BSE に感染し、これらの雌ヒツジから産まれた 2 匹も BSE で生後 73 日目、親ヒツジへの投与から数えて 655 日後に死亡した。このことから実験的なヒツジの群れの間での BSE 汚染飼料による BSE 伝播及びヒツジの子宮又は分娩による BSE 伝播が確認された。なお、後に加えられた 20 頭については現在まで（群れに入れてから 3 年間）感染は認められていないが、水平感染はないと結論するのは時期尚早である。

使用上の注意記載状況・その他参考事項等

現在販売していないため、添付文書を作成していない。

報告企業の意見	今後の対応
ヒツジを用いた実験の結果、BSE垂直感染が示唆された報告である。 本剤はヒトフィブリノゲン、ヒトトロンビン、塩化カルシウム及びウシアプロチニンを主成分とする生理的組織接着剤であるが、このうちウシアプロチニンはウシ肺から製造される。2003年12月、米国のウシにBSEが発生した際に、「ウシ等由来原材料を使用した医薬品、医療用具等の一部変更承認申請等におけるリスク評価等の取り扱いについて」（平成15年8月1日付薬食審第0801001号、薬食安第0801001号）の別添「カナダでのBSE発生の確認を踏まえた医薬品等のBSEリスク評価の考え方について」（平成15年7月8日付 伝達性海綿状脳症調査会資料）に基づき、アプロチニン液（ウシ肺由来）のリスク評価を行った結果、一定の安全性は確保されていると評価した。	本製品は、発売する予定はなく、承認整理する方針である。

Natural transmission of BSE between sheep within an experimental flock

SIR,– The recognition of bovine spongiform encephalopathy (BSE) in a French goat (Eloit and others 2005) has heightened the debate in Europe as to whether BSE has been maintained in small ruminants following historical exposure via feed. Key to the debate and associated risk assessments, especially in the UK, is whether BSE can transmit naturally between infected sheep. Here, we report preliminary evidence that natural transmission can take place between sheep in an experimental flock.

Thirty six-month-old ewe lambs of the PrP ARQ/ARQ genotype from transmissible spongiform encephalopathy (TSE)-free sources were dosed orally with 5 g of BSE cattle brain inoculum. This genotype has previously been shown to be fully susceptible to this inoculum. Approximately six months after infection, the BSE-dosed sheep were mixed with 20 matched undosed animals of the same age and genotype, and kept as a single group under strict biosecurity. Normal intensive commercial practices were followed as far as possible, while avoiding iatrogenic spread of infection.

The ewes were bred from 18 months of age by natural mating using breed/genotype-matched sires from the same TSE-free flocks that had been introduced to the unit. Placental cotyledons were collected at birth. All sheep had unrestricted access to the lambing area to maximise the potential for transmission of disease. Clinical observation, weight recording and tonsil and third eyelid biopsies were used to monitor disease progression. At clinical end point, the sheep were euthanased and examined postmortem, and tissues were collected for a range of immunohistochemical (IHC) and biochemical tests.

Twenty-four of the original 30 dosed sheep reached clinical end point between 655 and 1056 days postinfection (dpi), with a mean (sd) incubation of 797 (105) dpi. Two of the lambs born in 2003 also died of BSE. The first clinical disease in the flock occurred in a dosed ewe, the dam of lamb 2, just 73 days after the birth of its lamb, with clinical end point at 655 days after dosing. The dam of lamb 1 reached clinical end point 198 days after its birth. The first positive tonsil biopsies occurred in these dosed ewes at 369 dpi and in lambs 1 and 2 at 546 days of age. IHC examination of tissues from lambs 1 and 2 using monoclonal antibodies R145 and P4 showed the typical reduction in intracellular labelling with P4 in the obex previously associated with BSE (Fig 1). Lymphoid tissues also showed the typical BSE-associated pattern of reduced labelling of tingible body macrophages with the P4 antibody (Fig 2).

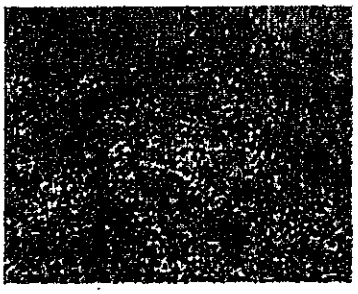

FIG 1: Vagus nucleus in the obex of lamb 1 stained with P4, showing abundant extracellular labelling with very little or no labelling of neurons or microglia. x 230

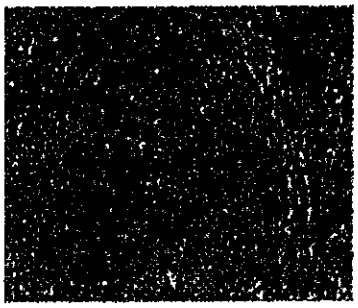

FIG 2: Secondary follicle in the tonsil of lamb 1 stained with P4, showing immunolabelling of follicular dendritic cells within the light zone but no labelling of tingible body macrophages within the dark or light zones. x 230

To date (June 2005), 22 lambs from the 2003 lamb crop born to both dosed and undosed ewes remain alive at 781 to 786 days of age and there are no clinical cases in the original undosed ewes three years after their introduction.

Although scrapie is known to transmit between sheep under natural and experimental conditions, there have been no previous reports of BSE being transmitted naturally between sheep. Foster and others (2004) failed to demonstrate transmission, but this may be explained by lower infection pressures in their experimental design and the use of less susceptible genotypes. At this stage in our study, it is impossible to determine whether infection was acquired from the dam in utero or during the perinatal period, but the incubation period of the affected lambs suggests infection occurred at or just before birth. Previous studies in experimentally infected sheep of the same genotype resulted in incubation periods of 628 to 1132 dpi in animals dosed orally with 5 g of the same inoculum at six months of age (Bellworthy and others 2005) and 525 to 723 dpi in lambs dosed orally with 1 g of the inoculum at two weeks of age (S. J. Bellworthy, unpublished data). The incubation period in the lambs in the present study would preclude the extremely unlikely potential of iatrogenic infection associated with tonsil biopsy. The absence of disease in unrelated, but susceptible, lambs introduced both before and after the first lambing period suggests that transmission may have been restricted to mother and lamb, rather than also horizontally, but it would be premature to conclude at this stage of the study that horizontal transmission had not occurred. Age and closeness of contact may play critical roles in determining likelihood of transmission, although in studies with scrapie we have demonstrated that adult sheep do become infected following introduction to an infected flock, albeit with longer incubation periods than lambs. Horizontal or vertical transmission is clearly a major factor in the spread of scrapie, and transmission may even occur in the absence of direct sheep-to-sheep contact. It remains to be seen whether this is confirmed also with BSE in sheep.

This is the first confirmation that BSE can transmit either in utero or perinatally in sheep. It indicates that if BSE had entered the sheep population at the start of the BSE epidemic, it could have propagated within the flock if the level of infection was sufficient in the presence of susceptible sheep. However, an extensive survey of the UK flock has shown no evidence of the classic BSE phenotype (Stack and others 2005).

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