

Chronic Wasting Disease Agents in Nonhuman Primates

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Chronic wasting disease, a prion disease of cervids, may infect humans, but this is unproven. Primates from 2 genera were observed for 9–10 years after intracerebral or oral inoculation. Cynomolgus macaques were completely resistant. However, squirrel monkeys were highly susceptible to the pathogen, which adapted more quickly on second passage.

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy that can infect mammals in the family Cervidae, which includes deer, elk, and moose, among other species. Initially detected in a captive deer in 1967, the disease is now widespread in the United States, Canada, and South Korea (1). Because cervids are commonly consumed as food by humans and other mammals, the cross-species potential of the causal pathogen must be determined. CWD prions injected intracerebrally have infected agricultural animals and scavengers (2); however, transgenic mice that expressed human prion protein (PrP) were not susceptible (3–6) through this route. We previously analyzed the susceptibility of 2 genera of nonhuman primates to CWD agents by intracerebral and oral routes (7). The results showed a high attack rate in squirrel monkeys (*Saimiri sciureus*) inoculated intracerebrally but a low attack rate and long incubation periods by those exposed orally. In contrast, no cynomolgus macaques (*Macaca fascicularis*) showed clinical signs of transmissible spongiform encephalopathy (TSE) when exposed by either route. The long incubation periods observed in squirrel monkeys prompted us to observe the remaining monkeys for >4 additional years. Here we provide an update and report results of new experiments showing that squirrel monkey–adapted CWD (SM-CWD) has an accelerated incubation period on second passage.

The Study

Squirrel monkeys were inoculated intracerebrally or orally with CWD inocula (7). We initially reported that

11/13 intracerebrally infected monkeys were euthanized at 41 months postinoculation (mpi) on average, and disease developed in 2/12 orally infected squirrel monkeys on average of 69 mpi (7). Disease developed in the 2 remaining intracerebrally infected squirrel monkeys at 61 and 75 mpi, respectively, changing the intracerebral attack rate to 100% (Figure 1, Table 1). Of the 10 remaining orally inoculated squirrel monkeys, disease developed in 9, bringing the overall oral attack rate to 92% and the average incubation period to 68 mpi (Figure 1, Table 1). Clinical signs were subtle; the most prominent finding was gradual weight loss (Table 1). A final diagnosis of CWD agent infection was made by using immunoblotting and immunohistochemical testing to determine accumulation of abnormal, disease-associated prion protein (PrPres) in brain tissue (wwwnc.cdc.gov/EID/article/20/5/13-0778-Techapp1.pdf).

To compare the neuropathologic changes in intracerebrally and orally infected squirrel monkeys, we analyzed 10 brain regions for spongiform lesion severity and PrPres deposition (Figure 1, panels B, C). No statistically significant differences were noted between the 2 routes of infection ($p < 0.05$). All squirrel monkeys studied had severe spongiform degeneration in the striatum (Figure 1, panel D) and little involvement in cerebellum and occipital lobes (Figure 1, panel E). Spongiform lesions in cortical gray matter were not consistent throughout the brain. Affected areas were commonly observed adjacent to normal regions, most frequently in the frontal, temporal, and parietal lobes (Figure 1, panel F). Except for the striatum, PrPres deposition was generally most prominent in areas that showed severe vacuolation. PrPres deposits appeared in 2 forms: dense punctate extracellular plaques (Figure 1, panel G) and less dense pericellular aggregates. The spleens of CWD agent–infected squirrel monkeys were positive for PrPres in 46% of intracerebrally infected and 60% of orally infected squirrel monkeys (Figure 1, panel H). At least 1 lymph node was positive in 30% of intracerebrally infected squirrel monkeys and in 40% of orally infected squirrel monkeys (Figure 1, panel J; Technical Appendix).

Of the squirrel monkeys under study, 3 *PRNP* genotypes were represented (7). In the group of orally infected squirrel monkeys, 3 had a unique heterozygous genotype that encoded either 4 or 5 octapeptide repeats. Two of these monkeys were the last orally infected monkeys to be euthanized because of clinical disease (80 and 107 mpi), and the third heterozygote was clinically normal at 108 mpi. Heterozygosity within the *PRNP* gene has been shown to delay or prevent prion disease (8) and may play a role in this study.

We inoculated cynomolgus macaques as another nonhuman primate model for cross-species transmission of CWD. Compared with squirrel monkeys, cynomolgus

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macaques are biologically closer to humans, and cynomolgus macaque PrP is more homologous to human PrP (7). Nine cynomolgus macaques were inoculated orally and 6 were inoculated intracerebrally with 1 of 3 CWD pools as described (7). Our first report included negative data from

1 cynomolgus macaque euthanized at 49 mpi (7). Since then, we have euthanized and screened 6 cynomolgus macaques for TSE (Table 2). No evidence of prion infection was detected by immunoblot and immunohistochemical methods (data not shown).

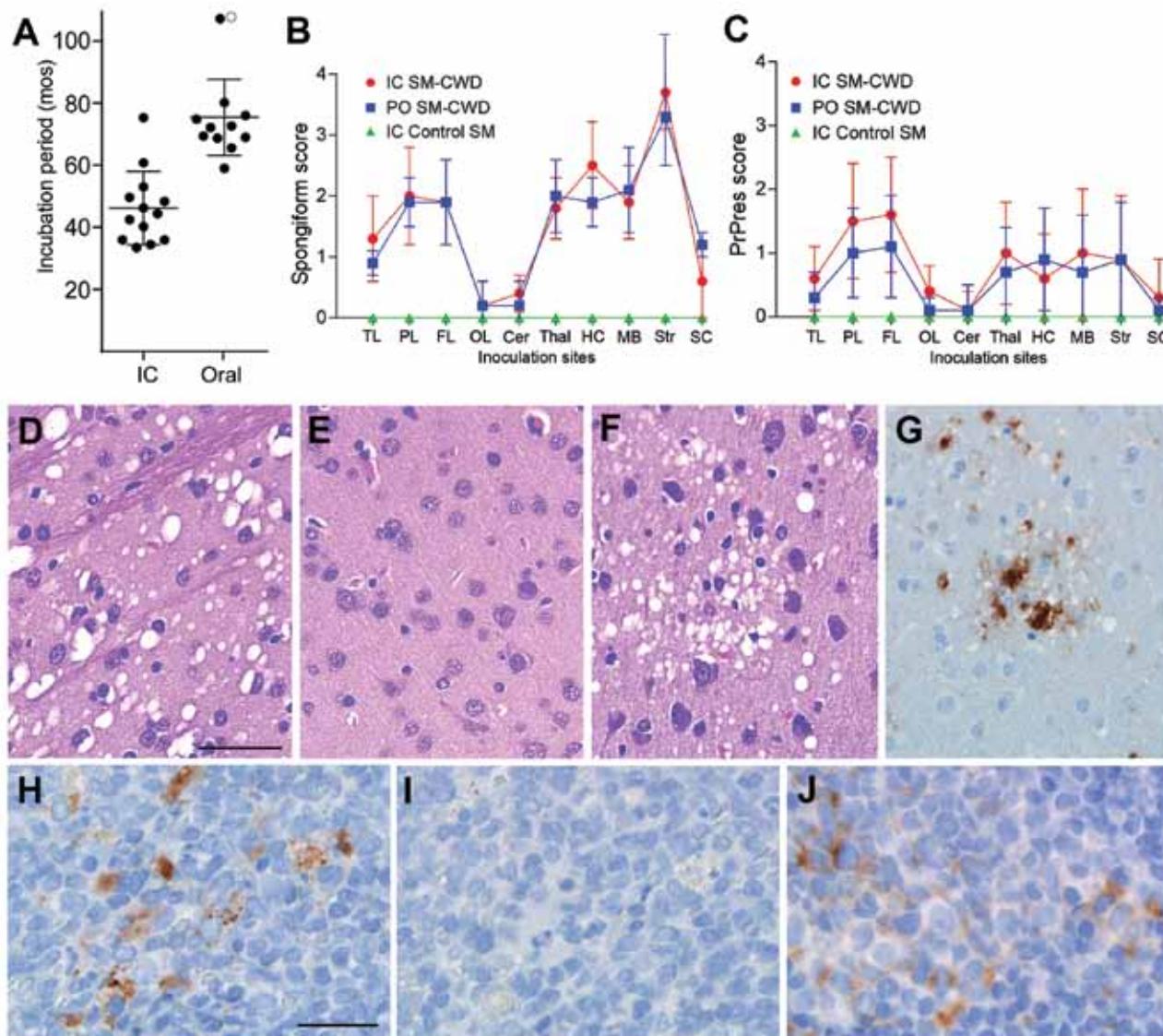


Figure 1. Incubation periods of chronic wasting disease (CWD) and neuropathologic features of CWD agent-infected squirrel monkeys. A) Incubation periods for squirrel monkeys infected with CWD agents by intracerebral (IC) or oral (PO) routes. Solid circles indicate euthanized squirrel monkeys (SM) that tested positive for prion disease. The open circle indicates 1 squirrel monkey that remained clinically normal at 108 months postinoculation (mpi). Lines indicate the mean and standard deviation within each group. B, C) Lesion profiles of CWD agent-infected squirrel monkeys showing spongiform degeneration (B) and PrPres deposition (C) values in 10 gray matter regions of the brain. N values for each group are as follows: IC SM-CWD, 11; PO SM-CWD, 7; IC control SM, 1. TL, temporal lobe; PL, parietal lobe; FL, frontal lobe; OL, occipital lobe; Cer, cerebellum; Thal, thalamus; HC, hippocampus; MB, midbrain; Str, striatum; SC, spinal cord. Error bars show the SD for each group. Panels D–G show brain from a squirrel monkey infected PO with CWD and euthanized at 69 months postinoculation. Panels D–F are stained with hematoxylin and eosin and show D) severe spongiform lesions in the striatum, E) lack of pathology in the occipital lobe, and F) pathology in the parietal lobe. Panels G, H, and J show immunohistochemical staining for PrPres by using anti-PrP antibody D13. G) Adjacent section to the region depicted in F shows the positive correlation of PrPres (brown) with spongiform degeneration. Panels H–J show lymphoid tissue from a squirrel monkey infected PO with CWD and euthanized at 80 mpi. H) PrPres (brown) staining in spleen and J) mesenteric lymph node. I) No primary antibody control of the region shown in H, demonstrating specificity of stain observed in H. The scale bar shown in D applies to panels D–G and represents 50 μ m; the scale bar shown in H applies to H–J and represents 25 μ m.

Table 1. Squirrel monkeys inoculated with CWD or squirrel monkey–adapted CWD agents*

Disease incidence†	Inoculum‡	Route of inoculation	Titer inoculated§	Incubation days, range, (avg)¶	Weight change range, % (avg,%)
13/13	MD-1,2,3 Elk-1,2,3 WTD-1,2	Intracerebral	1.3×10^5 – 1.0×10^7	33–75 (46)	–8 to –43 (–29.5)
11/12#	MD-1,3 Elk-1,2,3 WTD-1,2	Oral	9.6×10^7 – 1.5×10^9	59–107 (68)	–8 to –41 (–28)
2/2	SM-CWD	Intracerebral	NA	23–24 (23.5)	–8 to –21 (–14.5)
0/1	Normal elk	Intracerebral	NA	82 NS	0
0/1	Buffer control	Oral	NA	>108	–6
0/1	Normal elk	Oral	NA	123 NS	+7

*An early version of some of these data is shown in Tables 1, 2 of (7). Since that time more infected animals have been euthanized and the data have been updated. CWD, chronic wasting disease; MD, mule deer; WTD, white-tailed deer; NA, not applicable; NS, no clinical transmissible spongiform encephalopathy signs.

†Number of monkeys in which prion disease developed/number inoculated.

‡Several different inocula were used for this study. Each individual animal was inoculated with 1 inoculum. Detailed descriptions can be found in (7).

§Infectivity titers were determined by using endpoint dilution titer in transgenic deer PrP mice and are listed as 50% infectious dose/gram of brain.

¶The range of incubation periods observed is shown as months postinoculation followed with the average incubation period of the group in parentheses. Monkeys listed as NS did not show any clinical signs compatible with transmissible spongiform encephalopathy.

#Three monkeys from this group are not included in this calculation because they were euthanized before 45 months postinoculation for reasons unrelated to transmissible spongiform encephalopathy disease. The sole remaining animal in this group appeared normal at 108 months postinoculation.

The lack of CWD transmission during >10 years suggests that a substantial species barrier exists between cervids and cynomolgus macaques. In most TSE animal models, PrPres can be detected by 1/3–1/2 of the known incubation periods. If we extrapolate this to the cynomolgus macaques in this study, negative test results at 9 years would suggest that the incubation period would be >18 years. Other prion studies of cynomolgus macaques reported clinical disease within 2–3 years after inoculation with variant Creutzfeldt-Jakob disease agents (9), 3 years after inoculation with bovine spongiform encephalopathy agents (10,11), and 5 years after inoculation with sporadic Creutzfeldt-Jakob disease agents (9,12). In contrast, our findings indicate that CWD is unlikely to develop in cynomolgus macaques.

The cause of susceptibility to CWD agents in squirrel monkeys and resistance to them in cynomolgus macaques is uncertain. *Prnp/PRNP* gene sequence variation has been linked to disease susceptibility (8), and differences in the *PRNP* genes of cynomolgus macaques and the genes of squirrel monkeys could play a major role. Comparison of *PRNP* sequences among cynomolgus macaques and squirrel monkeys showed differences exist at 5 codons (56, 100, 108, 159, and 182) (7). It is not clear which difference or

combination of changes might confer protection to cynomolgus macaques, or if resistance is caused by other factors. Of the 5 codon differences described above, those of cynomolgus macaques and humans are identical at positions 56, 159, and 182.

Two SM-CWD brain samples were inoculated into squirrel monkeys and cynomolgus macaques to verify that SM-CWD was infectious, test for further adaptation, and to see if SM-CWD was infectious to a broader range of nonhuman primates. Two squirrel monkeys inoculated intracerebrally with SM-CWD brain homogenates (SMP2-CWD) were euthanized at 23–24 mpi (Table 1). These incubation periods decreased by >11 months compared with that of the donor squirrel monkey. Neurologic signs in the 2 SMP2-CWD were more pronounced than observed during the first passage; however, weight loss was reduced. Neuropathologic examination and Western blot for PrPres confirmed TSE in both squirrel monkeys. In contrast to SM-CWD infections, the SMP2-CWD-infected brains had spongiform lesions and PrPres deposition in the occipital lobe (Figure 2, panels A, B). Biochemical comparison of glycoform patterns among CWD, SM-CWD, and SMP2-CWD were made by using 3 different anti-PrP antibodies (L42, 6H4, and 3F4) (Technical Appendix).

Table 2. Cynomolgus macaques inoculated with CWD or squirrel monkey–adapted CWD agents*

Disease incidence†	Inoculum‡	Route of inoculation	Titer inoculated§	Screening mpi¶	Current mpi
0/6	MD-1, Elk-1, WTD-1	Intracerebral	3.2×10^5 – 2.5×10^6	49, 79, 88, 94	124
0/8#	MD-1, Elk-1, WTD-1	Oral	2.5×10^8 – 2×10^9	97, 106, 106	124
0/2	SM-CWD	Intracerebral	NA	NA	72
0/1	Normal elk	Intracerebral	NA	96	NA

*An early version of some of these data are shown in Table 3 of (7). CWD, chronic wasting disease; mpi, months post-inoculation; MD, mule deer; WTD, white-tailed deer; SM, squirrel monkey; NA, not applicable.

†Number of monkeys in which prion disease developed over number inoculated.

‡Several different inocula were used for this study. Each individual animal was inoculated with 1 inoculum. Detailed descriptions can be found in (7).

§Infectivity titers were determined by using endpoint dilution titer in transgenic mice expressing deer prion protein (PrPres) and are listed as 50% infectious dose per gram of brain.

¶Several monkeys were euthanized during the course of the experiment for conditions unrelated to prion infection such as diabetes, neoplasia, hypocalcemia, and behavioral issues. Brain, spleen, and lymph nodes from these animals were screened for PrPres by using Western blot and immunohistochemical methods. No PrPres-positive tissues were detected.

#One monkey from the original oral inoculation group was euthanized at 1 mpi because of a colonic torsion and has been removed from this group.

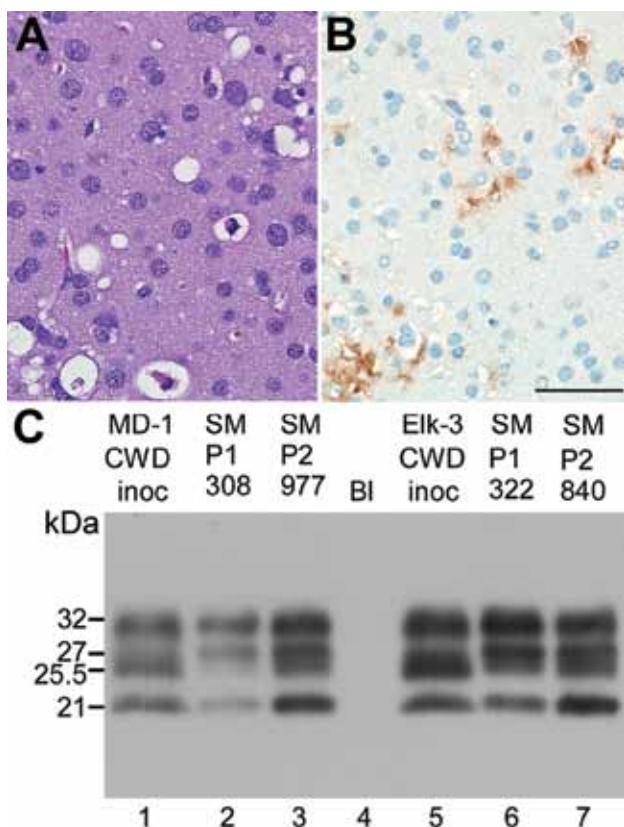


Figure 2. Neuropathologic features and immunoblot results of second-passage squirrel monkeys that had chronic wasting disease (CWD). Scale bar represents 50 μ m and is applicable to panels A and B. Panels A and B show neuropathologic changes in the occipital lobe of SMP2-CWD monkey 977, which was euthanized at 24 months postinoculation. A) Hematoxylin and eosin staining show prominent spongiform changes. B) Immunohistochemical staining for disease-associated prion protein (PrPres) (brown) with anti-PrP antibody D13. C) Results of Western blot for PrPres in brain tissue of cervids and its respective first and second passage in squirrel monkeys. MD-1 was used to infect SM308, and SM308 was used to infect SM977. Lanes 1, 2, 5, and 6, 0.6 mg brain equivalents. Lanes 3 and 7, 0.36-mg brain equivalents to give similar signal intensities to the other samples. Lane 4, blank (BI). Apparent molecular weights (in kDa) are provided on the left side of panel C. Immunoblot was probed with anti-PrP antibody L42. When comparing the 2 central bands, cervid CWD had a more intense band at 25.5 kDa; SM-CWD (nos. 308 and 322) and SM2-CWD (nos. 977 and 840) were more intense at 27 kDa.

In all cases, SM2-CWD had a greater proportion of unglycosylated PrPres and a lower proportion of double glycosylated PrPres than did SM-CWD (Figure 2, panel C). The decreased time of manifestation of disease, differences in glycoform patterns, and distribution of PrPres in brain tissue suggested that the CWD agent was still adapting within the squirrel monkey. However, similar to CWD, SM-CWD had not caused prion disease in cynomolgus macaques by 72 mpi (Table 2).

Conclusion

Our studies have shown that squirrel monkeys, but not cynomolgus macaques, were susceptible to CWD. Although these nonhuman primates are not exact models of human susceptibility, they support the data from transgenic mouse studies (3–6), in vitro experiments (13), and epidemiologic evidence (14,15) that suggest humans are at a low risk of contracting CWD. Nevertheless, it remains sensible to minimize exposure to tissues potentially contaminated with the CWD agent.

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The image shows a screenshot of the CDC's Facebook page. At the top, there is a large advertisement for the 'Solve the Outbreak' game, featuring a tablet displaying the game interface and a sign that says 'New CDC is on Facebook'. Below the advertisement, the CDC profile information is visible, including the name 'CDC', the tagline 'Saving Lives Protecting People', and the description 'Government Organization CDC is dedicated to protecting health & promoting quality of life through prevention and control of disease, injury, and disability. For official CDC info go to www.cdc.gov.' The page also shows a 'Like' button with '263k' likes, and a 'Photos' section with a photo of a person in a blue shirt. Below the photos, there are several posts, including one from Carol Ferguson and one from Thomas Roles. At the bottom of the page, there is a large text overlay that reads 'Find emerging infectious disease information on facebook' with the URL 'http://www.facebook.com' below it.

Chronic Wasting Disease Agents in Nonhuman Primates

Technical appendix

Materials and Methods

Nonhuman Primates

Monkeys were housed individually at the Rocky Mountain Laboratories (RML) in an AAALAC International accredited facility and experimentation followed NIH RML Animal Care and Use Committee approved protocols. Further details were provided (*1*).

CWD, SM-CWD and SM2-CWD brain homogenates used for inoculation of primates

Eight pools of CWD-agent inocula were used in the primary passage studies in SM and CM. Infectivity levels and sources of each pool have been described in detail previously (*1*). For passage experiments using SM-CWD, 2 CWD agent positive squirrel monkeys (SM308 and SM322) were selected. SM308 was originally infected with MD-1 brain homogenate (BH) pool and euthanized at 36 mpi. SM322 was infected with Elk-3 BH pool and euthanized at 34 mpi. Both monkeys had readily detectable levels of PrPres in their brains and severe spongiform lesions. SM-CWD and second passage SM-CWD infected monkey (SMP2-CWD) BHs were prepared from frontal cortex as a 20% (w/v) solution in 0.01 M Tris HCl pH 7.4 and frozen at -80 C. The day of inoculation samples were thawed, vortexed, and sonicated for 1 minute. The 20% BH stock was then diluted to 10% in phosphate buffered balanced salt solution (PBBS) with 2% fetal bovine serum. Samples were cleared of large particulates by centrifuging at 425 x g for 5 minutes.

Inoculation of Monkeys

Methods for the initial oral (PO) and intracerebral (IC) CWD agent and control monkey inoculations were previously described (*1*). For inoculation of SM-CWD BH into SM the animals were anesthetized with ketamine HCL (20 mg/kg) intramuscularly. The hair on the skull was clipped, and the area over the left parietal lobe was scrubbed with betadine solution. A

dremel tool with a 1/16 inch diameter stainless steel sterile drill bit was used to create a small hole through the skull. Two hundred microliters of 10% SM-CWD BH was administered through a 30G ½ inch needle attached to a 1 ml syringe inserted through the predrilled hole into the parietal lobe of the brain. CM were anesthetized with ketamine HCL (10 mg/kg) and prepared as described above for the SM. Four hundred fifty microliters of 10% BH was inoculated using a 1 mL syringe and ½ inch long, 26 gauge needle. The approximate location of the inoculation was near the junction of the left temporal and parietal lobes.

Tissue Processing and Analysis for PrPres by Immunoblot

Tissues (brain, spleen or lymph nodes from infected or uninfected controls) were prepared as 20% (w/v) homogenates in ice cold 0.01M Tris pH 7.4 or sterile PBS pH 7.2 using either an Omni Tissue homogenizer with disposable hard tissue probe (Omni international, Marietta, GA) or Mini-Bead Beater (Biospec Products, Bartlesville, OK). Samples were vortexed and then sonicated 1 minute and frozen until analyzed. Preparation of samples for PrPres analysis was described previously (2). Briefly, mild detergents were added and samples were treated with 50 µg/ml of proteinase K (Roche cat#03115879001) for 45 minutes. The reaction was stopped by adding 2 µl of 0.1M phenylmethylsulfonyl fluoride and placed on ice for 5 minutes. An equal volume of 2X Laemmli sample buffer (Biorad, Hercules, CA, USA) was added, and samples were boiled for 5 minutes. Additional un-treated samples from both infected and uninfected monkeys were used to observe PrPsen and control for non-specific reactivity.

Samples were run on a 16% Tris-glycine gel and proteins were transferred to Immun-Blot PVDF-P membranes (Bio-Rad, Hercules, CA) using an iBlot transfer system (Invitrogen, Carlsbad, CA) set to program 3 with a 7 minute transfer. PrP bands were detected with monoclonal antibody 3F4 diluted 1:3000 (residues 109-112) (3), D13 from cell culture supernatant diluted 1:100 (residues 96-106) (4), 6H4 diluted 1:10,000 (residues 144-152) (5) or L42 diluted 1:5000 (residues 145-163) (rBiopharm) (6). D13 does not react with CM PrP and was not used for this species. Membranes were incubated in primary antibody in TBS-T for 1 hour. Membranes were rinsed with TBS-T buffer and incubated with their appropriate horseradish peroxidase-conjugated secondary antibody (sheep anti-mouse IgG for 3F4, 6H4 and L42, sheep anti-human IgG for D13) at a 1:10,000 dilution in TBS-T for 45 minutes. Bands were detected using enhanced chemiluminescence (ECL) substrate as recommended by the manufacture (Thermo Scientific, Rockford, Illinois). Densitometry studies on the glycoform

ratios were done using a Gel Doc XR and Quantity One software (Bio-Rad). Each sample was analyzed 2-4 times with three different anti-PrP antibodies (L42, 3F4, and 6H4). Results for each monkey with an individual antibody were averaged.

Histology & Immunohistochemistry

Tissues were placed in 3.7 % phosphate-buffered formalin for 3-5 days before dehydration and embedding in paraffin. Serial 4 µm sections were cut using a standard Leica microtome, placed on positively charged glass slides and dried overnight at 56° C. Sections were stained by standard hematoxylin and eosin methods (H&E). Immunohistochemical staining was performed using Ventana automated Nexus stainer (Ventana, Tucson, AZ). Slides were deparaffinized and rehydrated to Tris-HCl buffer, pH 7.5. Anti-PrP staining was done using anti-PrP antibodies D13 and 3F4 as previously described (4,7). Uninfected SM and CM were used to observe PrPsen staining and potential non-specific reactivity. In addition, tissues were stained with the primary antibody omitted to observe non-specific staining due to the secondary antibody.

Brain and Lymphoid Pathology

Each squirrel monkey brain was scored for the degree of spongiform degeneration (H&E slides) and PrPres deposition (antibody D13 slides) in 10 brain regions. Brains were randomized and scored blind by one observer. A scale of 0-4 was used for each parameter. For spongiform degeneration the following method was used: 0, no vacuoles; 1, few vacuoles widely and unevenly distributed; 2, few vacuoles evenly distributed; 3, moderate numbers of vacuoles evenly distributed; and 4, many vacuoles with some confluences. PrPres deposition was scored in the same regions as follows: 0, no deposition; 1, 1–20% of area has visible PrPres plaques; 2, 20–40% of area covered with plaques; 3, 41–60% of area covered with plaques; and 4, >61% of area covered with plaques. Group mean scores were analyzed statistically using Prism 5.0c software (GraphPad Software) and plotted with their standard deviations to show representative lesion profiles.

Spleens and lymph nodes (LN) stained with D13 or 3F4 were also observed for PrPres deposition. No primary controls were used for each tissue to exclude non-specific staining from analysis. In addition, LN and spleens from uninfected squirrel monkeys were used as negative control tissue. A total of 78 lymph nodes (2-9 lymph nodes/monkey) and two sagittal sections of

spleen were observed. Lymph nodes from various anatomic locations were used including (47 mesenteric, 11 cervical, 11 axillary, 4 mandibular, 2 inguinal, 2 brachial and 1 ileocecal lymph node).

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