Passage of chronic wasting disease prion into transgenic mice expressing Rocky Mountain elk (Cervus elaphus nelsoni) PrP\(^C\)

Giuseppe LaFauci, 1 Richard I. Carp, 1 Harry C. Meeker, 1 Xuemin Ye, 1† Jae I. Kim, 1‡ Michael Natelli, 1 Marisol Cedeno, 1 Robert B. Petersen, 2 Richard Kascsak 1 and Richard Rubenstein 1§

1 New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314, USA
2 Case Western Reserve University – Institute of Pathology, 2085 Adelbert Road, Cleveland, OH 44120, USA

INTRODUCTION

Chronic wasting disease (CWD), a neurodegenerative disorder affecting cervids, is classified as one of the transmissible spongiform encephalopathies (TSEs) or prion diseases (Guiroy et al., 1991a, b; Williams & Miller, 2002; Williams & Young, 1980, 1992). CWD is one of only three forms of TSE that occur naturally, the others being Creutzfeldt–Jakob disease in humans and scrapie in sheep. CWD was first recognized in mule deer at research facilities in Colorado (1967) and in Wyoming (1978) (Williams & Young, 1980, 1992). Subsequently, the disease was diagnosed in black-tailed deer, Rocky Mountain elk (Williams & Young, 1982, 1992) and white-tailed deer (Williams & Miller, 2002). Recent data support the presence of CWD among captive and free-ranging cervids in two provinces in Canada, in at least 13 states in the USA and in imported deer in several farms in Korea (Kim et al., 2005b; Kong et al., 2005).

In the laboratory, TSE-affected brain can be used to transmit the disease to other animals of the same species. Passage of the agent from one species to another is usually impeded by a phenomenon known as the ‘species barrier’. This refers to the fact that on passage of TSE from one species to another, the recipient animals exhibit a very long incubation time and frequently few or none of the animals develop disease. The species barrier is eliminated in transgenic mice expressing the cellular form of the prion protein (PrP\(^C\)) of the host species (Buschmann et al., 2000; Castilla et al., 2003; Crozet et al., 2001; Scott et al., 1989, 1993; Telling et al., 1994; Vilotte et al., 2001).
The origin of the CWD agent (PrP^Sc) in cervids is not known (Williams & Miller, 2002, 2003). It has been hypothesized that CWD may have originated from sheep scrapie or from a prion(s) of unknown source, or from spontaneous mutations of the cervid PrP gene. While much is known about the pathogenesis, aetiology and transmission of CWD (Williams, 2005), progress on understanding aspects of CWD biology has been hampered by the fact that the agent cannot be transmitted efficiently to mice (Browning et al., 2004; Bruce et al., 2000). Recently, two transgenic mouse models – expressing either the mule deer or the elk PrP gene – have been created and used to passage CWD from affected cervids (Angers et al., 2006; Browning et al., 2004; Kong et al., 2005).

Here, we describe the construction of transgenic mice homozygous for the Rocky Mountain elk (Cervus elaphus nelsoni) PrP transgene array (TgElk) and report their use in passing prions from cervids affected with CWD. The brains of affected TgElk mice showed histopathological changes, plaques that were immunoreactive with anti-PrP antibodies and PrP^Sc. Western blot profiles that were consistent with the findings observed in CWD-affected cervid brains. Inoculated TgElk mice developed disease with incubation times of 95 days for three elk CWD isolates and 127 days for one mule deer CWD isolate. Upon secondary passage, incubation times were reduced to 90 and 108 days, respectively. The 90 day incubation time reported here is the shortest incubation time observed among the currently available CWD transgenic mice models (Angers et al., 2006; Browning et al., 2004; Kong et al., 2005).

**METHODS**

**Generation of TgElk transgenic mice.** The TgElk mouse line was generated using cosmids cosSHA.ElkPrP, which was constructed by ligating the elk PrP open reading frame (ORF) into the expression vector cosSHA.Tet (Scott et al., 1992). To construct cosSHA.ElkPrP, we modified the elk PrP ORF by adding a consensus Kozak translation initiation site and SalI sites for cloning. This was achieved by PCR using elk DNA as template and the upstream primer 5'-ACGCGtcgacCATGGGTAAAGCCACA-3' carrying the Kozak sequence (bold italic) and downstream primer 5'-ACGCGtgcacCTATCCCTATGAG-3' (SalI sites in lower case). Plasmid pSSelk was obtained by cloning the resulting 808 bp PCR product into pCR2.1 (Invitrogen). Sequencing analysis revealed that the cloned PrP ORF was identical to the published elk PrP sequence (GenBank accession no. AF016227). The insert of pSSelk was released by cutting with SalI and ligated into the SalI-restricted cosmids vector. Cosmid cosSHA.ElkPrP carrying the Elk PrP ORF in the right direction for expression was isolated, restricted with NotI and used to purify the transgene (SHAElkPrP) carrying the hamster PrP enhancer/promoter sequences upstream of the elk PrP ORF. The transgene was then used to generate transgenic mice at the Case Western Reserve University transgenic facility according to published protocols (Scott et al., 1992).

**Prion transmission studies.** Transgenic mice were inoculated intracerebrally (i.c.) with 25 μl 1 % brain homogenate prepared in PBS from three elk and one mule deer affected by CWD. As a control, five TgElk mice were inoculated with 25 μl 1 % normal mouse brain homogenate prepared in PBS. All brain samples were from clinical stage disease cervids and were obtained from Dr Elizabeth Williams (Wyoming State Veterinary Laboratory, University of Wyoming, Laramie, WY, USA). Western blot analysis of serial dilutions of the original CWD brain samples revealed that elk #2 contained a smaller amount of PrP^Sc (10^-3) than the other elk and mule deer brains (10^-4) (data not shown). Starting at 70 days post-inoculation, mice were scored weekly for evidence of prion disease. The screening procedure, described previously (Carp et al., 1984; Meeker et al., 2005), involved placing mice on a grid containing a series of bars (3 mm in diameter) placed 7 mm apart. Mice were deemed positive when they showed a reduced capacity to traverse the bars (ataxia) and a ragged or wobbly gait. The day post-infection when clinical signs were first detected was termed the incubation period. Mice were kept for observation until the third positive clinical score (2 weeks after initial signs), sacrificed by intraperitoneal injection of sodium pentobarbital (Nembutal) and their brains dissected. Half-brain specimens were immersion fixed in 10 % neutral-buffered formalin; the other half of each brain was frozen at -80 °C. The five control TgElk mice did not show any sign of disease and were sacrificed several weeks after all of the experimental animals had shown disease (180 days after injection).

**Histological studies.** Tissue blocks were processed into paraffin blocks and sectioned into 7 μm thick histological sections. For histopathological studies, sections were stained with haematoxylin and eosin (H&E). Nine regions of the brain were H&E stained and examined under light microscopy: olfactory bulb, anterior cortex, posterior cortex, hippocampus, thalamus, hypothalamus, substantia nigra, cerebellum and brain stem. Each of the nine regions was given a score for the amount of vacuolation, with zero for none and four for the maximum level (Kim et al., 1990). For immunohistochemistry, sections were dewaxed in xylene, placed in 100 % ethanol and then rehydrated through graded ethanol solutions prior to being rinsed in PBS. Endogenous peroxidases were quenched with 3 % H2O2 in 100 % methanol for 15 min. Non-specific sites were blocked with normal sheep serum diluted 1:50 in PBS and incubated for 30 min at room temperature. Sections were then incubated overnight at 4 °C with 50 ng anti-PrP monoclonal antibody (mAb) 7A12 ml^-1 (kindly provided by Dr Man-Sun Sy, Case Western Reserve University, Cleveland, OH, USA; Li et al., 2000). Immunostaining was performed using either the peroxidase-antiperoxidase (PAP) or alkaline phosphatase (Zymed Laboratories) technique. To unmask epitopes of the aggregated PrP^Sc, sections were treated with 88 % formic acid for 14 min prior to reaction with mAb 7A12.

**Western blot analysis.** PrP^Sc from frozen half brains of CWD-infected TgElk mice was analysed by Western blotting using a modification of a published protocol (Polymenidou et al., 2002). Briefly, 10 % brain homogenates in 0.5 % sodium deoxycholate, 0.5 % NP-40 in PBS (pH 7.4), were centrifuged at 800 g for 1 min and the supernatants divided into two aliquots of 100 μl each. One of the aliquots was treated with 100 μg proteinase K ml^-1 (Sigma-Aldrich) for 30 min at 37 °C followed by the addition of 2 μl 0.4 % Pefabloc S (Roche). Both samples were then mixed with 300 μl PBS and 500 μl sodium chloride solution [20 % NaCl, 0.1 % Sarkosyl in PBS (pH 7.4)]. These mixtures were kept on ice for 10 min with occasional shaking, centrifuged at 16 000 g for 10 min at room temperature and the pellets resuspended in 2× SDS sample buffer by heating at 99 °C for 5 min. Samples were separated by SDS-PAGE using 12 % Novex pre-cast gels (Invitrogen) and electrottransferred (25 V, 1-5 h) to nitrocellulose membranes. Immunostaining of PrP^Sc was performed using a cheluminescent-based detection scheme involving the anti-PrP mAb 4C4 (diluted 1:20 000) (Kim et al., 2005) and horseradish peroxidase-conjugated goat anti-mouse IgG.
RESULTS

Microinjection of fertilized embryos from FVB/N mice null for PrP (FVB/Prnp 0/0) with DNA from cosmid cosSHa.ElkPrP resulted in the generation of one founder hemizygous for the insertion of an elk PrP transgene array. This mouse was mated to FVB/Prnp 0/0 mice and the hemizygous offspring were mated to each other to generate mice homozygous for the elk PrP transgene array (TgElk). The TgElk transgenic mouse line has been maintained in our laboratory as a homozygous line for more than 20 generations.

The expression of PrPC in TgElk mice was examined by Western blotting with mAb 6D11 which recognizes the epitope QWNK (aa 97–100 of mouse PrP), which is conserved in mice and elk (D. S. Spinner & R. J. Kascsak, unpublished observations; Pankiewicz et al., 2006). The level of expression of PrPC in the brains of TgElk was 2-5-fold higher than the level of PrPC detected in the brains of wild-type FVB/N mice (data not shown). The incubation period following injection of brain homogenates from CWD-affected cervids into TgElk mice was examined in four experiments (Table 1). As shown, incubation periods for the three elk brain homogenates were similar and revealed small standard errors. The first sign of disease was mild incoordination (ataxia), which increased as the incubation period continued. There was, however, one marked difference in the clinical findings between transgenic mice injected with elk and mule deer homogenates. For the latter, mice were relatively calm and did not move rapidly on the grid. This pattern is similar to that seen for most scrapie strain/mouse strain combinations. In contrast, mice injected with the three different elk homogenates moved rapidly and erratically on the grid; their behaviour was sufficiently wild and erratic to warrant concern that the mice would jump from the grid to the floor. To ensure containment, elk-injected mice were maintained under an inverted cage whilst on the grid. This type of behaviour has not been reported previously for TSE-injected mice.

Brain homogenates (25 µl, 1%) prepared from TgElk mice with terminal-stage CWD were injected i.c. into naïve TgElk mice. The incubation period of the elk material was reduced slightly from 94–95 days for passage 1 to 90 days for passage 2 (Table 1). The mule deer data also show a reduced incubation period from 127 days on first passage to 108 days on second passage.

Histopathological changes in the brains of CWD-infected transgenic mice included vacuolation and degeneration of neurons (Fig. 1). In TgElk mice infected with mule deer CWD brain homogenate, the most abundant vacuolation was found in the brain stem and hypothalamus (Table 2). Regions of ample vacuolation were also detected in the hippocampus, thalamus and cerebellum (Table 2). Little or no vacuolation was found in the olfactory bulb (Fig. 1c) and anterior cortex. By contrast, in TgElk mice injected with elk CWD brain homogenate, vacuolation was extensive in the anterior cortex, olfactory bulb (Fig. 1a, b) and hypothalamus, and minimal in the cerebellum (Table 2). The level of vacuolation was less in transgenic mice injected with mule deer CWD homogenate compared with those injected with elk CWD homogenate (Table 2).

In affected mice, anti-PrP immunostaining was localized in both cytoplasm and nuclei of neurons in the cerebral cortex, thalamus, hypothalamus and brain stem. Staining was also seen within the granular layer of the cerebellar cortex, parenchyma cells adjacent to the lateral ventricles and the aqueduct system, as well as in the granular cells of the hippocampus. PrPSc immunostaining was also present in some Purkinje cells of the cerebellum. In addition, many small PrPSc granules were seen in the white matter.

In addition to the accumulation of PrPSc reactivity in the neurons and neuropil of the grey matter, we also detected

<table>
<thead>
<tr>
<th>Table 1. Transmission (i.c.) of CWD agent in TgElk transgenic mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source of inoculum</td>
</tr>
<tr>
<td>From CWD-positive elk and mule deer into TgElk mice</td>
</tr>
<tr>
<td>Elk #1</td>
</tr>
<tr>
<td>Elk #2</td>
</tr>
<tr>
<td>Elk #3</td>
</tr>
<tr>
<td>Mule deer</td>
</tr>
<tr>
<td>From CWD-positive TgElk mice into TgElk mice</td>
</tr>
<tr>
<td>TgElk (elk #1)</td>
</tr>
<tr>
<td>TgElk (elk #2)</td>
</tr>
<tr>
<td>TgElk (mule deer)</td>
</tr>
</tbody>
</table>

ND, Not determined; NA, not applicable.
PrP^{Sc} deposition (single plaques or clusters) in the cortex, hippocampus, thalamus, hypothalamus (Fig. 2b, d), cerebellar cortex and near the lateral ventricles (Fig. 2a, c) in CWD-affected mice. PrP^{Sc} deposits surrounded by spongiform vacuoles (florid plaques) were also detected (Fig. 2b). In the brain areas mentioned above, PrP^{Sc} deposits were more abundant in transgenic mice infected with elk CWD homogenate than in those infected with mule deer CWD homogenate. PrP^{Sc} deposition (plaques or plaque-like structures) has been recognized as a neuropathological hallmark of CWD (Guiroy et al., 1991a, b; Liberski et al., 2001; Williams & Young, 1993) and has also been reported in CWD-infected transgenic mice expressing mule deer PrP^{Sc} (Browning et al., 2004) or elk PrP^{Sc} (Kong et al., 2005).

The brains of TgElk mice inoculated with brain homogenate from CWD-affected elk (elk #1 and #2) and mule deer were analysed by Western blotting using mAb 4C4 (Fig. 3). Proteinase K-resistant PrP^{Sc} bands were detected in all mice showing clinical disease (Fig. 3a). Similar bands were detected in the brains of TgElk mice injected with brain homogenates (second passage) from CWD-affected TgElk mice (Fig. 3b). For all mice affected with CWD (first and second passage), the strongest PrP^{Sc} signal was observed in the uppermost band (30 kDa, diglycosylated) and the weakest signal was observed in the lowest band (22 kDa, non-glycosylated) (Fig. 3). This PrP^{Sc} immunoblot profile is consistent with data reported by others for the brains of CWD-affected mule deer, white-tailed deer and elk (Race et al., 2002) and was also identical to the profiles of the cervid CWD brain homogenates used in our transmissions to TgElk mice (Fig. 4).

**DISCUSSION**

Following passage into TgElk mice, CWD agents maintained the characteristic Western blot profiles seen in CWD-affected mule deer and elk and produced histopathological modifications that were consistent with those observed in the natural disease. For example, florid plaques were seen in CWD-affected mule deer and elk, as well as in affected TgElk mice, and plaques were often in close proximity to areas of vacuolation (Liferski et al., 2001; Williams, 2005). In TgElk mice inoculated with the mule deer CWD brain homogenate, the distribution of vacuolation in different brain regions also corresponded to the spongiform changes described in natural CWD in cervids (Spraker et al., 2002a, b; Xie et al., 2006), e.g. vacuolation was intense in the hypothalamus, thalamus, brain stem and cerebellum, whereas the olfactory bulb and anterior cortex were less affected. By contrast, in the TgElk mice injected with elk brain homogenate, vacuolation was more extensive in the anterior cortex, hypothalamus, olfactory bulb and posterior cortex, whereas the cerebellum was minimally involved. The incubation period for the CWD mule deer brain homogenate (127 days; Table 1) was significantly longer ($P < 0.0001$) than the incubation periods for the elk homogenates (95 days). Upon secondary passage, the incubation time was reduced to 108 and 90 days, respectively. Differences in incubation time were maintained (although slightly reduced) in the second passage, suggesting that the differences were not caused by variation in prion titre in the original elk and mule deer CWD brain samples.

Longer incubation times (225–268 days) have been reported by others (Browning et al., 2004) for primary passage of mule deer and elk CWD prions using Tg(CerPrP)1536^{+/−}
Table 2. Vacuolation intensity in different regions of the brains of CWD-affected TgElk mice

Sections were scored from 0 (no vacuolation) to 4 (intense vacuolation). Results are shown as mean vacuolation score ± SEM.

<table>
<thead>
<tr>
<th>Source of CWD homogenate</th>
<th>Olfactory bulb</th>
<th>Anterior cortex</th>
<th>Posterior cortex</th>
<th>Hippocampus</th>
<th>Thalamus</th>
<th>Hypothalamus</th>
<th>Brain stem</th>
<th>Cerebellum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elk (n=9)</td>
<td>3.0 ± 0.3</td>
<td>3.2 ± 0.3</td>
<td>2.7 ± 0.3</td>
<td>2.2 ± 0.4*</td>
<td>2.3 ± 0.3</td>
<td>3.2 ± 0.3</td>
<td>2.3 ± 0.3</td>
<td>0.6 ± 0.2*</td>
</tr>
<tr>
<td>Mule deer (n=6)</td>
<td>0.5 ± 0.3†</td>
<td>0.6 ± 0.2*</td>
<td>1.2 ± 0.2*</td>
<td>1.8 ± 0.6*</td>
<td>1.8 ± 0.7*</td>
<td>2.2 ± 0.6*</td>
<td>2.3 ± 0.3</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Control (n=5)‡</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*One section was not readable.
†Two sections were not readable.
‡Mice injected with normal mouse brain.

and Tg(CerPrP)1534+/- mice hemizygous for the mule deer PrP transgene array. In one experiment, the same authors infected Tg(CerPrP)1536+/- mice homozygous for the transgene with mule deer CWD prions, producing disease in 160 days (Browning et al., 2004). Similar incubation times (231–283 days) were reported recently using the same transgenic mouse strain (Browning et al., 2004) injected with mule deer brain homogenates (Angers et al., 2006).

Incubation times of 118 days (range 83–143 days; elk 1) and 142 days (range 124–178 days; elk 2) have been published recently for elk CWD prions injected i.c. into Tg12 transgenic mice hemizygous for the elk PrPC gene (Q. Kong, personal communication; Kong et al., 2005). Upon second passage of the elk 1 prion in Tg12 mice (Kong et al., 2005), the incubation time was 125 days (range 115–138 days). In the current study, we have reported comparable incubation periods for the mule deer brain homogenate (Table 1). However, incubation periods obtained with TgElk for the three elk brain homogenates (Table 1) were significantly shorter than the incubation periods reported by other investigators with the two available CWD transgenic models (Angers et al., 2006; Browning et al., 2004; Kong et al., 2005).

The reason(s) for the differences in incubation times observed using TgElk, Tg12 and Tg(CerPrP)1536+/- is not clear. When compared with the amount of wild-type PrPSc detected in the brain of FVB/N mice, the levels of PrP expression in the brains of TgElk and Tg12 transgenic mice were estimated to be 2-5-fold and 2-fold higher, respectively (this paper and Kong et al., 2005). The level of expression of PrPSc in Tg(CerPrP)1536+/- has not been reported; however, in the hemizygous mice [Tg(CerPrP)1536+/-], the level of PrPSc expression was 5-fold higher than that detected in the brains of FVB/N mice (Browning et al., 2004). Therefore, it is unlikely that the level of PrPSc expression per se plays a determined role in the incubation period differences among the different transgenic lines. The elk PrP sequence differs from the mule deer PrP sequence of Tg(CerPrP)1536+/- only at aa 226, which is glutamic acid in the elk and glutamine in the mule deer (Cervenakova et al., 1997). This amino acid difference could be the cause

Fig. 2. PrPSc immunostaining of brain sections of TgElk mice infected with prions from CWD-affected elk (a, b) and mule deer (c, d). (a, c) Periventricular region. (b, d) Hypothalamus. Single PrPSc plaques (arrow) and clusters of plaques (arrowhead) are present in the parenchyma adjacent to the lateral ventricle in both (a) and (c). Plaques located in the proximity of unstained vacuoles (*) are evident in sections throughout the hypothalamus of mice infected with the elk (b) and mule deer (d) CWD prions. In (b), a florid plaque surrounded by vacuoles is indicated by an arrow. In (d), a cluster of plaques is shown (arrowhead). Bars, 100 µm.
of the longer incubation times obtained with the Tg(CerPrP)1536+/+ transgenic line compared with the results using Tg12 (Kong et al., 2005) and our data.

Brain samples from TgElk mice injected with elk and mule deer CWD agents were analysed by Western blotting. The PrPSc profiles were indistinguishable from those observed for the elk and mule deer CWD brain homogenates used as inocula in this study (Fig. 4) and from those reported by others (Race et al., 2002) for brains of CWD-affected mule deer, white-tailed deer and elk. Recently, Western blot profiles reproducing that of the original elk CWD inoculum were also reported by others using the Tg12 transgenic line (Kong et al., 2005). In contrast, Western blot profiles showing a reduced amount of monoglycosylated PrPSc compared with infected cervids has been reported by others (Browning et al., 2004) in CWD-affected Tg(CerPrP).

We observed several differences between TgElk mice injected with mule deer CWD homogenate and those injected with the three different elk CWD homogenates. Differences in clinical manifestations were evident from mouse movement on the grid used for assessment: the elk CWD-injected mice moved in a wild, hyperactive manner, whilst the mule deer CWD-injected mice were slower and calmer. Incubation times were significantly shorter in mice injected with elk CWD samples compared with mule deer CWD samples, both in primary and in secondary passages. More pronounced vacuolation was detected in the brains of TgElk mice injected with elk CWD homogenate than with mule deer CWD homogenate (Table 2). Extensive vacuolation was detected in the olfactory bulb and anterior cortex in TgElk inoculated with elk homogenate, but little or no vacuolation was seen in these regions in mice inoculated with the mule deer homogenate (Table 2). PrPSc staining in samples from elk CWD-infected mice was more intense and extensive than in those infected with the mule deer CWD homogenate.

The above differences in clinical manifestations, incubation times and distribution of vacuolation suggest the intriguing hypothesis that the CWD agent causing disease in the three affected elk might be different from the agent causing disease in the affected mule deer. Recently, it has been reported that venison (semitendinosus and semimembranosus muscles) of mule deer affected by CWD is a source of infectious prions (Angers et al., 2006). The risk of CWD transmission to humans is considered to be low because: (i) TSE transmission is inefficient via the oral route (venison) and (ii) the presence of a species barrier between humans and cervids was reported recently using humanized transgenic mice (Kong et al., 2005). However, people consuming and/or handling venison (e.g. hunters) are at risk of exposure to prions (Angers et al., 2006). The existence of several strains of TSE agent affecting cervids would complicate attempts to assess possible transmission of the CWD agent(s) to humans and other species by dietary exposure (Kong et al., 2005). Data suggesting the existence of two different CWD prion strains were reported previously by others using the Tg(CerPrP) transgenic mouse model (Browning et al., 2004). Of course, because the number of CWD samples analysed in this work was relatively small (three elk and only one mule deer), the hypothesis of the existence of more than one strain of CWD prion is speculative and must be tested by additional passaging experiments with a larger number of cervid CWD specimens.

An important characteristic of the TgElk line is the fact that it is homozygous for the elk PrP transgene array. Therefore,
cumbrous screening of mice for the presence of the transgene prior to experimental protocols is not required. The short incubation time observed on passage from cervids to transgenic mice with both mule deer and elk CWD brain homogenates and the capacity of the mice to propagate CWD agents (mouse to mouse) make the TgElk line a valuable model to determine the titre of infectious agents in infected cervids, to validate the results of diagnostic tests for CWD in bioassays, to study prion distribution in organs of infected animals and to analyse CWD strain variation and origin.

ACKNOWLEDGEMENTS

This work was supported in part by the New York State Office of Mental Retardation and Developmental Disabilities and in part by grants from the National Heart, Lung and Blood Institute (RO1 HL63837), the US Department of Defense (DAMD17-03-1-0368) and the National Institutes of Health (contract N01-NS-01-2327). The authors appreciate the technical support of Ms Ewa Karpinska and thank Dr Daryl S. Spinner for a review of the manuscript, Ms Joanne Lopez for her excellent effort in preparing this manuscript and Dr Robert Freedland, Head of NY State IBR GRAMS, for the preparation of figures.

REFERENCES


