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Spontaneous Neurodegeneration in Transgenic Mice with Mutant Prion Protein

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Transgenic mice were created to assess genetic linkage between Gerstmann-Sträussler-Scheinker syndrome and a leucine substitution at codon 102 of the human prion protein gene. Spontaneous neurologic disease with spongiform degeneration and gliosis similar to that in mouse scrapie developed at a mean age of 166 days in 35 mice expressing mouse prion protein with the leucine substitution. Thus, many of the clinical and pathological features of Gerstmann-Sträussler-Scheinker syndrome are reproduced in transgenic mice containing a prion protein with a single amino acid substitution, illustrating that a neurodegenerative process similar to a human disease can be genetically modeled in animals.

GERSTMANN-STRÄUSSLER-SCHEINKER syndrome (GSS) is a rare human neurodegenerative disease that is vertically transmitted, apparently as an autosomal dominant trait (*1, 2*), and can often be horizontally transmitted to primates and rodents through intracerebral inoculation of brain homogenates from patients with the disease (*3*). Patients with GSS develop ataxia and dementia in the third to seventh decades of life and deteriorate until they die, in 1 to 10 years. The protease-resistant isoform of prion protein (PrP) is implicated in the pathogenesis and transmission of GSS and in scrapie, a similar animal disease (*4*). Enriching scrapie-infected hamster brain fractions for infectivity led to the discovery of PrP 27-30 (*5*), which was later recognized to be derived from a larger protein, PrP^{Sc}, by limited proteolysis (*6*). PrP^{Sc} is derived from cellular PrP (PrP^{C}) by a post-translational process; both PrP isoforms are encoded by a single copy gene (*7*).

A leucine substitution at codon 102 of the human PrP gene (*PRNP*) (*2, 8*) on the short arm of chromosome 20 (*9*) is genetically linked to GSS (*2*) and is present in families with GSS from different ethnic backgrounds (*2, 10, 11*). Other mutations in *PRNP* segregate with disease in families with other varieties of prion diseases (*11, 12*), providing evidence for a strong but not absolute correlation between specific PrP mutations and different clinical forms of familial prion diseases (*13*). Thus, the codon 102 mutation in *PRNP* is more than a linked genetic marker and may cause the disease or at least directly influence its pathogenesis.

Studies showing that Syrian golden hamster (Ha) PrP transgenes modify virtually all aspects of experimental scrapie in mice suggested that the dominantly inherited GSS phenotype might be manifest in transgenic mice expressing mutant PrP (*14, 15*). To test this hypothesis, we microinjected mouse (Mo) PrP genes containing a codon 101 leucine substitution (homologous to codon 102 in *PRNP*) into fertilized oocytes to produce transgenic mice (*14*). The microinjected DNA consisted of a chimeric murine cosmid constructed by exchanging the Bam HI–Sal I fragment containing the open reading frame (ORF) from a recombinant NZW genomic clone (*16*) for the corresponding fragment in an I/Ln mouse cosmid (*17*) (Fig. 1). We modified a ~100-nucleotide region flanking the codon 101 leucine to yield DNA sequence homology to HaPrP while retaining amino acid homology to MoPrP, thus enabling tail DNA of weanling mice to be screened with a ~60-nucleotide probe derived from this region. We detected mice containing $MoGSSPrP_{Leu^{101}}$ transgenes by comparing intensities of the radiolabeled probe hybridized to approximately 20 μg of denatured tail DNA from weanling mice or control hamsters.

Three transgenic founder lines were initially created—Tg(GSSPrP)174, Tg(GSSPrP)180, and Tg(GSSPrP)196. All animals were housed in a room in which no animals inoculated with scrapie prions had ever been kept; all second- and third-generation transgenic mice were kept in new cages and drank from new water bottles not previously exposed to scrapie-infected animals. The haploid transgene copy numbers in each line were determined by comparison of the intensities of a radiolabeled 0.6-kb Bst EII–Eco RI fragment near the 3′ end of exon III (containing the ORF) (*16, 17*) hybridized to mouse DNA of varying dilutions. Tg(GSSPrP)174, 180, and 196 mice harbored approximately 64, 9, and 6 haploid transgene copies, respectively. The male Tg(GSSPrP)174 founder mouse readily produced offspring, but the Tg(GSSPrP)180 founder is sterile and the female Tg(GSSPrP)196 founder has only recently produced progeny. PrP^{C} expression in Tg(GSSPrP)174 brains was > eightfold higher than in controls as determined by intensities of immunoblots on nitrocellulose of varying dilutions of brain extracts. The ~60-nucleotide hamster-biased probe (Fig. 1A) hybridized to 0.1 μg of transgenic brain RNA but not to 20 μg of nontransgenic brain RNA, thus confirming expression of the transgene. The mutant transgenes were inherited in a manner consistent with a single autosomal site of insertion, because this site was present in approximately half (87/176) of Tg(GSSPrP)174 progeny and could be transmitted by males.

Thirty-five Tg(GSSPrP)174 mice appeared healthy until symptoms of ataxia, lethargy, and rigidity developed between 7 and 39 weeks of age (Fig. 2). Mouse 0.1 in Fig. 2 is the nontransgenic littermate of the transgenic founder 0.2 and is now more

Table 1. Transgenic mice expressing *Prn-p*^{b} mouse and wild-type Syrian hamster PrP do not develop spontaneous neurologic dysfunction.

Mouse	Transgene	Copy number	*n*	Mean age (days)
NonTg*		0	24	225 (175–312)‡
Tg 94†	MoPrP-B	>10	16	468 (332–589)
Tg 117†	MoPrP-B	>10	4	488 (438–542)
Tg 71†	HaPrP	~4	15	364 (303–389)
Tg 81†	HaPrP	~30	26	400 (395–415)
Tg 7†	HaPrP	>60	14	313 (284–351)

*A representative sample of nontransgenic mice that are littermates of Tg(GSSPrP)174 mice. †Tg 94 and Tg 117 brain PrP^{C}, relative to nonTg, are fourfold higher. Tg 71, Tg 81, and Tg 7 brain $HaPrP^{C}$ are one- to twofold, two- to fourfold, and four- to eightfold higher, respectively, as determined by ELISA and immunoblot dilutions of brain extracts (*14, 15, 17, 20*). ‡The range is given in parentheses.

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than 1 year old. Because both mice were derived from the same batch of microinjected, fertilized oocytes, this argues against contamination with scrapie prions. The mean age of the 35 animals that developed neurologic dysfunction was 166 ± 6 days (±SEM). To date, the oldest Tg(GSSPrP) mouse to develop disease was 272 days of age (mouse I.3), and the youngest animal (mouse III.1) was 57 days old. Mouse III.1 is the offspring of two transgenic mice and may be homozygous for the MoGSS $PrP_{Leu^{101}}$ transgenes. Once affected, the animals deteriorated over 3 to 36 days until death. At the time of writing, all of the Tg(GSSPrP)174 mice that have not yet developed neurologic symptoms are younger than the oldest mouse to develop disease. In the first, second, and third generations, none of the nontransgenic littermates have developed neurologic symptoms. Although it is possible that either the site of Mo-$PrP_{Leu^{101}}$ transgene insertion or elevated expression levels alone cause spontaneous neurodegeneration in Tg(GSSPrP)174 mice, this has not been observed in other lines of transgenic mice harboring many copies of the variant *Prn-p*^{b} gene of the I/Ln mouse (*17*) or wild-type HaPrP (*14, 15*) (Table 1).

Five clinically sick Tg(GSSPrP)174 mice, both immersion-fixed and perfusion-fixed, had the same pathological features. Numerous 5- to 15-μm vacuoles (spongiform degeneration) were present in both the gray and white matter (Fig. 3). Vacuoles in perfusion-fixed animals appeared to contain a pale-staining, amorphous substance (Fig. 3D) that was absent from most of the vacuoles in immersion-fixed animals; however, some vacuoles in the latter also contained this material (Fig. 3B). The vacuoles were found in most gray and white matter structures of the cerebral hemispheres and brainstem. There was a mild to moderate degree of reactive astrocytic gliosis in a patchy distribution in both gray and white matter. A prominent Bergmann radial gliosis was present in much of the molecular layer of the cerebellar cortex (Fig. 3F). The brains of two perfusion-fixed, asymptomatic, 8-week-old Tg(GSSPrP)174 mice were also examined. One was normal; the other exhibited multiple filled vacuolar structures <5 μm in diameter. These structures were seen most readily in the gray matter of the hippocampus and cerebellum and were not found in the white matter.

No amyloid plaques were found in the brains of affected Tg(GSSPrP) mice by the Gomori trichrome method or with PrP antiserum R073 (*18*). PrP amyloid plaques are a constant feature of GSS in humans, but

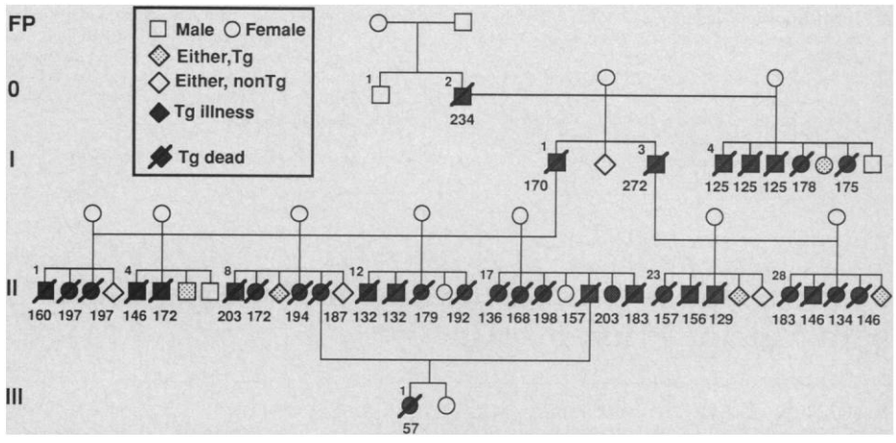
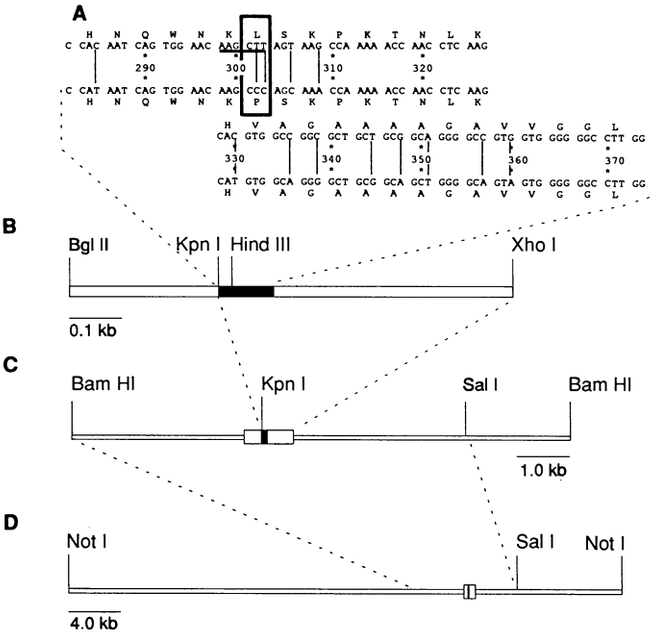


Fig. 2. Inheritance of a neurodegenerative disorder in Tg(GSSPrP)174 mice. The founding parents (FP) were both (C57BL/6 × SJL)F1 mice. Superscripts denote symbol numbers for each generation. Each of the filled symbols represents a transgenic (Tg) mouse, and the numbers below these symbols denote the age in days at which neurologic dysfunction was first observed. Stippled symbols represent transgenic mice who have not yet developed disease. Open symbols represent nontransgenic littermates. To compress the data, many stippled and open symbols represent multiple mice. Those denoting more than one mouse are as follows: I.2, n = 8; I.7, n = 3; II.3, n = 7; II.6, n = 2; II.7, n = 3; II.9, n = 3; II.11, n = 2; II.16, n = 2; II.22, n = 5; II.26, n = 3; II.27, n = 2; and II.31, n = 3.

Fig. 1. Construction of the $MoGSSPrP_{Leu^{101}}$ transgene. (**A**) Comparison of nucleotide and deduced amino acid sequences of $MoGSSPrP_{Leu^{101}}$ (upper) with wild-type MoPrP (*Prn-p*^{a}) (lower). The sequence from the Kpn I cleavage site at nucleotide 282 and the Bgl I site at 374 was reconstructed with Pro^{101}→Leu substitution at the boxed position identified by the presence of a unique Hind III site (AAGCTT) polymorphism, which is underlined. Silent nucleotide substitutions are biased toward HaPrP and are designated by vertical bars. We synthesized MoGSSPrP-specific probes by annealing two partially overlapping complementary oligonucleotides, MOMOD3 (AGCTTAGTAAGCCAAAAACCAACCTCAAGCACGTGGC) and MOMOD6 (CACCACGGCCCCTGCCGCAGCAGCGCCGCCACGTG), and then performing primer extension in the presence of radiolabeled nucleotide triphosphates (*16*). Because of the nucleotide sequence bias in the substituted region of the MoGSS gene, these probes hybridize to MoGSSPrP and HaPrP ORFs but not to wild-type MoPrP ORFs. (**B**) The $MoGSSPrP_{Leu^{101}}$ ORF is depicted as a filled white box. A 0.8-kb MoPrP ORF cassette was constructed containing the leucine substitution with a Bgl II site proximal to the initiation codon and an Xho I site distal to the ORF. The sequence in (A) is depicted as a black box. (**C**) A 9-kb Bam HI mouse genomic DNA clone containing the *Prn-p*^{a} allele of the MoPrP ORF (*16*) was subcloned in pBR322 and partially digested with Stu I. One of the two sites for Stu I cleavage within this fragment lies immediately distal to the MoPrP ORF. This DNA was then completely digested with Kpn I and the 12-kb Kpn I–Stu I fragment lacking MoPrP sequences between the Kpn I and Stu I sites was isolated by agarose gel electrophoresis. We prepared a replacement fragment by filling in the overhanging end obtained after cleavage of the $MoGSSPrP_{Leu^{101}}$ cassette with Xho I followed by digestion with Kpn I, and the 0.5-kb fragment was ligated to the Kpn I–Stu I acceptor fragment. (**D**) The 40-kb insert of a MoPrP *Prn-p*^{b} genomic DNA cosmid clone (*17*) was partially digested with Bam HI and completely digested with Sal I. Molecules with a single cleavage at the Bam HI site nearest the 5′ end



of the ORF were isolated by agarose gel electrophoresis and ligated to the 7-kb Bam HI–Sal I fragment obtained after cleavage of the 9-kb $MoGSSPrP_{Leu^{101}}$ genomic clone described above.

they are infrequent in familial Creutzfeldt-Jakob Disease (*1–3, 12*). The absence of PrP plaques in Tg(GSSPrP) mice may be due to the small amounts or absence of PrP^{Sc} in these animals, as discussed below, or to the nonamyloidogenic nature of wild-type PrP^{Sc}-A molecules (*15, 16*).

The amounts of proteinase K–resistant prion proteins in brain extracts of six affected Tg(GSSPrP)174 mice (mice II.1, II.12, and II.7, corresponding to Fig. 4A, lanes 2, 4, and 6; and mice 0.2, II.13, and II.25, data not shown) were considerably lower than those found in extracts from scrapie-infected rodents (Fig. 4A, lane 9; Fig. 4B, lanes 6 and 9). Similar proteinase K–resistant prion proteins were also present in two asymptomatic, weanling Tg(GSSPrP)174 mice at 7 weeks of age (Fig. 4B). Because Tg(GSSPrP)174 mice express high concentrations of $MoPrP_{Leu^{101}}$, some or all of the protease-resistant PrP detected on protein immunoblots may be residual PrP^{C} rather than PrP^{Sc}. With Tg(HaPrP) mice, uncertainties about low concentrations of protease-resistant HaPrP on immunoblots were resolved by enzyme-linked immunosorbent assay (ELISA) with HaPrP monoclonal antibodies (MAbs) (*15*), but no MoPrP MAbs are currently available. Another possibility is that leucine at codon 101 may increase the susceptibility of PrP to protease digestion irrespective of whether it has been converted to PrP^{Sc}.

To assess whether brains of affected Tg(GSSPrP)174 mice contained infectious prions, we injected 10% (w/v) brain homogenates intracerebrally into Swiss CD-1, Tg(HaPrP)7, Tg(HaPrP)81, and Tg(MoPrP-B)117 mice (*15, 17*) and Syrian golden hamsters. After ~220 days, none of the inoculated animals has exhibited signs of neurologic disease. The absence of demonstrable infectivity after prolonged incubation periods does not necessarily indicate the absence of prions in Tg(GSSPrP)174, because the codon 101 leucine might prevent transmission to rodents lacking homologous PrP^{C} molecules (*14, 15, 19*). Alternatively, an inborn error of PrP metabolism could produce neurologic disease without the generation of infectivity (*3, 13, 20*).

Our clinical and neuropathological observations on spontaneous disease in Tg(GSSPrP)174 animals support the hypothesis that the PrP codon 101 leucine change is the cause of their neurologic disease and, by inference, that the *PRNP* codon 102 leucine mutation is responsible for GSS in humans. Although the neurologic disorder in Tg(GSSPrP)174 mice is clinically and pathologically similar to scrapie, the low concentrations of protease-resistant PrP and the lack of transmission to date distinguish these animals from those developing scrapie after inoculation with prions.

The broad range in the age of onset and the duration of disease in Tg(GSSPrP)174 mice is in contrast to the narrow range in the incubation period and disease duration observed in mice inoculated with prions (*21, 22*). It resembles that observed in human GSS (*2, 3, 13*), however, and supports the argument that PrP primary structure is not the sole determinant of age of onset and disease duration. Genes both linked and

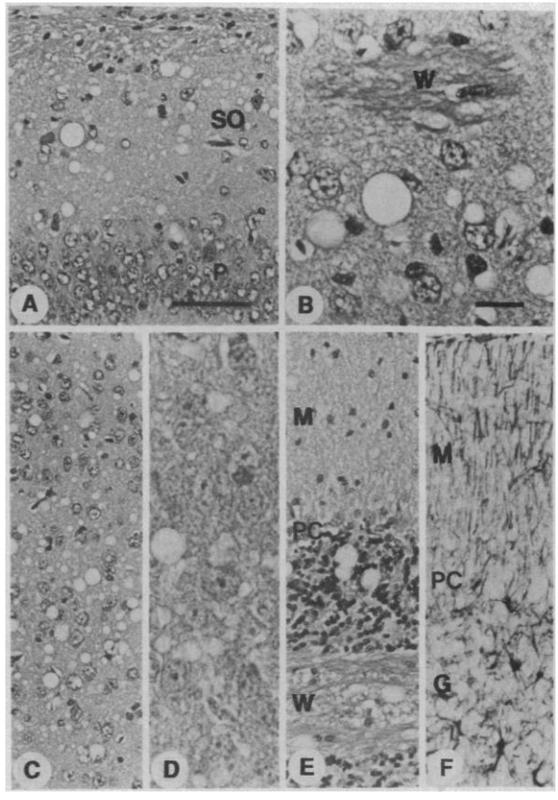


Fig. 3. Neurohistological examination of brain tissue sections from Tg(GSSPrP) mice. (**A**) The stratum oriens (SO) of the CA 4 region of the hippocampus. Nuclei of Ammon's horn pyramidal cells (P) are seen in the lower part of the micrograph. (**B**) The gray and white matter tracts (W) of the putamen. (**C** and **D**) Layers 3 and 4 of the dorsomedial frontal cortex. (**E**) The white matter, granule cell layer, and molecular layer (M) of the cerebellar cortex (PC, Purkinje cell layer). Vacuoles in the molecular layer are smaller and more numerous. (A), (B), (C), and (E) are from animals that were immersion-fixed with formalin and stained with hematoxylin and eosin. (D) is from a perfusion-fixed animal and was stained by the Gomori trichrome method. (**F**) Immunohistochemistry for glial fibrillary acidic protein revealed a moderate degree of reactive astrocytic gliosis in the granule cell layer (G) and Bergmann radial gliosis in the molecular layer of the cerebellum of an immersion-fixed animal. The bar in (A) is 50 μm and also applies to (C), (E), and (F). The bar in (B) is 15 μm and also applies to (D).

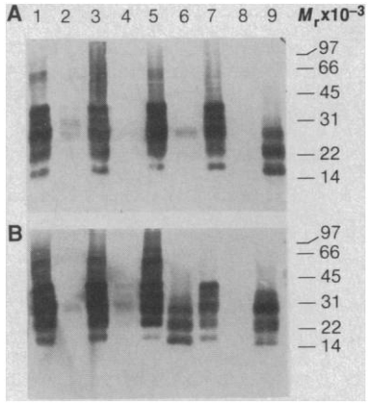


Fig. 4. Immunoblots of brain extracts from Tg(GSSPrP)174 mice. Brain tissue from transgenic mice, nontransgenic control mice, and hamsters was disrupted in 0.32 M sucrose by passing it through a 20-gauge needle five times and through a 22-gauge needle ten times. The 10% (w/v) homogenate was centrifuged at 1600*g* for 5 min at 4°C. Typically, 500 μg of supernatant protein measured by BCA dye binding (*27*) was present in 30 to 50 μl. It was diluted to 200 μl with 50 mM tris-acetate, pH 8.2, buffer containing 150 mM NaCl and 2% *N*-lauroyl-*N*-methylglycine (sarkosyl), and it was centrifuged at 1600*g* for 5 min at 4°C. Proteinase K (1 mg/ml) from a freshly prepared stock was added to selected samples (final concentration, 20 μg/ml; 1 hour at 37°C). Limited digestions were terminated by 2 mM phenylmethylsulfonyl fluoride (lanes 2, 4, 6, 8, and 9). All samples were precipitated with the addition of 5 volumes of ethanol (−20°C), incubated for at least 1 hour at −20°C, and centrifuged at 16,000*g* for 15 min at 4°C. Pellets were resuspended in 50 μl of polyacrylamide gel electrophoresis (PAGE) sample buffer containing 2% SDS (*28*). A 10-μl aliquot was boiled for 5 min and loaded onto a 12.5% polyacrylamide slab gel 1.5 mm thick. After SDS-PAGE, proteins were electrotransferred for 1.5 to 2 hours to Immobilon (Millipore) membranes. PrP was detected with a rabbit antiserum (R073) (*18*). Molecular weights (M_r) are indicated at the right side of the immunoblots. (**A**) Protease-resistant PrP in brains of Tg(GSSPrP)174 mice exhibiting neurologic dysfunction. Three transgenic mice are shown in the pedigree (Fig. 2): mouse I.1, lanes 1 and 2; mouse II.12, lanes 3 and 4; and mouse II.17, lanes 5 and 6. The variable amounts of protease-resistant PrP in Tg(GSSPrP)174 mice are shown in lanes 2, 4, and 6; lane 4 contained the lowest levels of protease-resistant PrP. NonTg174 littermate control, 128 days old, lanes 7 and 8. A Swiss CD-1 mouse with clinical signs of scrapie inoculated with the Chandler scrapie isolate, lane 9 (*29*). (**B**) Protease-resistant PrP in brains of asymptomatic Tg(GSSPrP)174 mice. Two transgenic mice, 49 days old, lanes 1 to 4; Swiss CD-1 mouse with scrapie, lanes 5 and 6; nonTg174 littermate control, lanes 7 and 8; and Syrian golden hamster with scrapie, lane 9.

unlinked to PrP can also have a major influence on incubation times (*21, 22*). Because Tg(GSSPrP)174 mice were not inbred, being derived from (C57BL/6 × SJL)F2 fertilized oocytes (*23*), some of the variation may be due to modifier genes (*24, 25*).

The development of spontaneous spongiform neurodegeneration in Tg(GSSPrP)-174 mice establishes that a neurodegenerative process similar to a human disease can be genetically modeled in an animal. The argument that the leucine PrP variants in humans and Tg(GSSPrP)174 mice are potent susceptibility genes for a ubiquitous but unidentified pathogen remains a formal interpretation of our results (*26*). However, multiple lines of investigation on the physical and biological properties of the infectious agent consistently converge on PrP^{Sc} and fail to reveal additional essential components (*4*). Studies of neurodegeneration in Tg(GSSPrP) mice may provide new insights into the pathogenesis of some more common genetic and sporadic central nervous system degenerative disorders such as Huntington's and Alzheimer's diseases (*4, 24*).

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Broadly Neutralizing Antibodies Elicited by the Hypervariable Neutralizing Determinant of HIV-1

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The principal neutralizing determinant (PND) of human immunodeficiency virus (HIV)–1 resides within the V_3 loop of the envelope protein. Antibodies elicited by peptides of this region were able to neutralize diverse isolates. Serum from one of three animals immunized with the human T cell lymphoma virus (HTLV)–III_{MN} PND peptide, RP142, neutralized MN and the sequence-divergent HTLV-III_B isolate. Serum from one of three animals immunized with a 13–amino acid III_B PND peptide (RP337) also neutralized both of these isolates. Characterization of these sera revealed that the cross-neutralizing antibodies bound the amino acid sequence GlyPro-GlyArgAlaPhe (GPGRAF) that is present in both isolates. This sequence is frequently found in the PNDs analyzed in randomly selected HIV-1 isolates. Sera from two rabbits immunized with a peptide containing only the GPGRAF residues neutralized divergent isolates, including III_B and MN.

A LARGE NUMBER OF HIV-1 ENVELOPE reagents have been tested as immunogens to elicit neutralizing antibody. Commonly, resultant sera neutralize isolates related to the immunogen but not to divergent HIV-1 isolates. This isolate-restricted response is largely due to recognition of a variable domain of the virus envelope referred to as the V_3 loop or the principal neutralizing determinant (PND). It is contained within a hypervariable, 36–amino acid, disulfide-crosslinked loop in the large envelope subunit (amino acids 303 to 338) (*1, 2*). Synthetic peptides consisting of sequences within this region induce isolate-restricted neutralizing antibody (*1–6*) and monoclonal antibodies that bind the PND neutralize virus infectivity (*7–9*). The sequences of two peptides corresponding to the PND of the III_B isolate (RP337) and the MN isolate (RP142) are shown in Table 1. Three guinea pigs were immunized separately with each of these peptides, and the immune sera were assayed for the ability to neutralize the III_B and MN isolates and to prevent fusion of uninfected CD4 cells with cells infected with these isolates (Table 2). As expected on the basis of experience with these immunogens, immune sera from all of the animals immunized with RP142 neutralized and inhibited fusion of the MN isolate. Surprisingly, sera from one of these (animal g89) also neutralized and inhibited fusion of the III_B isolate. Similarly, one of

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