Heterogeneous Autoantibody Against Neurofilament Protein in the Sera of Animals with Experimental Kuru and Creutzfeldt-Jakob Disease and Natural Scrapie Infection

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Received 3 May 1982/Accepted 8 June 1982

Heterogeneous autoantibodies against axonal neurofilament proteins of mature mouse neurons grown in vitro were detected by the indirect immunofluorescence technique in 12.7% (9 of 71) of the sera from nonhuman primates infected with kuru, in 14.5% (17 of 117) and 4% (1 of 25), respectively, of the sera from nonhuman primates and laboratory rodents infected with Creutzfeldt-Jakob disease, and in 35% (7 of 20) of the sera from sheep naturally infected with scrapie. Autoantibody titers ranged from 1:16 to 1:512 in Creutzfeldt-Jakob disease-infected animals, 1:32 to 1:512 in kuru-infected animals, and 1:64 to 1:1,024 in sheep with natural scrapie. The sera from 11 monkeys and 17 hamsters infected with scrapie and from 19 chimpanzees inoculated with brain tissues from humans with other neurological diseases did not contain autoantibodies. Of the 41 chimpanzees with Creutzfeldt-Jakob disease, 6 had autoantibodies against neurofilament proteins before experimental inoculation, whereas 6 others developed autoantibodies after inoculation, 4 developed autoantibodies during the asymptomatic phase, and 2 developed autoantibodies during the terminal clinical phase. Of the 48 chimpanzees with kuru, 2 had autoantibodies before inoculation, 6 developed autoantibodies after inoculation, 3 developed autoantibodies during the asymptomatic phase, and 3 developed autoantibodies during the terminal clinical phase. Among the normal nonhuman primate controls, 4.6% (9 of 195) had autoantibodies. In contrast, no autoantibodies were detected in 49 control rodents and 13 control sheep. The increased incidence of autoantibodies against neurofilament proteins in animals with kuru, Creutzfeldt-Jakob disease, and scrapie constitutes the first evidence of an immunological reaction in this group of atypical infections caused by unconventional viruses and suggests that neurofilaments may be involved in pathogenesis.

Using neuron cultures derived from fetal mouse central nervous system tissues (17) and frozen sections of rat spinal cords (3), we previously demonstrated the presence of autoantibodies of the immunoglobulin G (IgG) class against axonal neurofilament proteins in the sera of patients with kuru and Creutzfeldt-Jakob disease (CJD) and in the sera of a smaller percentage of patients with other or no neurological disorders (3, 16). Since these autoantibodies in human sera reacted with neurofilament proteins of neuron cultures prepared from several species of mammalian hosts, they were classified as being heterogeneous (16, 17).

We now report the occurrence of these heterogeneous autoantibodies in the sera of chimpanzees, Old World and New World monkeys, and laboratory rodents experimentally infected with kuru, CJD, and scrapie, as well as in the sera of sheep naturally infected with scrapie. These autoantibodies occurred in the sera of some normal nonhuman primates before experimental inoculation with the viruses that cause subacute spongiform encephalopathies. In other animals, autoantibodies appeared during the course of disease. The biological and pathogenetic significance of heterogeneous autoantibodies against neurofilament proteins in experimental kuru and CJD and natural scrapie infection remains to be determined.

MATERIALS AND METHODS

Neuron cultures. Primary neuron cultures were derived from mechanically disrupted cephalic tissues of NIH Swiss white mouse fetuses taken on day 11 of

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gestation as previously described (17). Briefly, cells were grown on glass cover slips in Dulbecco modified medium containing 10% fetal bovine serum, 10% horse serum, dextrose (10 g/liter) crystalline insulin (80 
U/liter), and NaHCO₃ (1.5 g/liter) at 37°C and 10% CO₂ atmosphere. Maintenance medium contained 20% horse serum and arabinosylcytosine (10 mg/liter) but did not contain fetal bovine serum.

Sera. Before testing, all sera had been stored frozen at -20 to -70°C for periods ranging from several months to several years after collection from animals being maintained for long-term transmission experiments. Pre- and post-inoculation serum specimens from 108 chimpanzees were divided into three groups: (group 1) 41 chimpanzees that developed CJD after inoculation with suspensions of CJD-infected brain tissues from human and animal sources; (group 2) 48 chimpanzees that developed kuru after inoculation with suspensions of kuru-infected brain tissues from human and animal sources; and (group 3) 19 chimpanzees that had been asymptomatic for many years after inoculation with suspensions of brain tissues from human patients with other neurological diseases (four with Alzheimer’s disease, three with multiple sclerosis, two with parkinsonism-dementia, two with Parkinson’s disease, two with amyotrophic lateral sclerosis, one with subacute sclerosing panencephalitis, one with Alpers’ disease, one with progressive supranuclear palsy, one with epilepsy partialis continua, one with Friedreich’s ataxia, and one with Huntington’s chorea). Post-inoculation sera from chimpanzees in group 3 were collected after approximately twice the mean incubation periods of experimental CJD (20.3 months) and kuru (22 months) had elapsed. All chimpanzees were approximately 3 to 5 years old when inoculated.

Sera were also obtained from normal monkeys and from monkeys infected with CJD, kuru, or scrapie. Two species of Old World monkeys (African green and rhesus monkeys) and one species of New World monkey (squirrel monkey) were used. There were 25 normal rhesus monkeys and 15, 3, and 1 rhesus monkeys with CJD, kuru, and scrapie, respectively; there were 15 normal African green monkeys and 15 with CJD. There were 49 normal squirrel monkeys and 46, 20, and 10 squirrel monkeys with CJD, kuru, and scrapie, respectively.

Sera from normal rodents (14 Hartley guinea pigs, 12 golden Syrian hamsters, and 11 NIH Swiss white mice) and infected rodents (14 guinea pigs with CJD, 17 hamsters with scrapie, and 11 mice with CJD) and sera from 13 normal sheep and 20 sheep naturally infected with scrapie were also tested.

Rabbit antisera to actin and tubulin were obtained from G. Rutter, H. Pette Institut, Hamburg, West Germany; rabbit antisera to glial fibrillary acidic protein was obtained from A. Bignami, Veterans Administration Medical Center, Boston, Mass.; rabbit antisera to neurofilament proteins (molecular weight, 150,000) was obtained from M. Shelanski, New York University Medical Center, New York, N.Y.

Immunofluorescence technique. An indirect immunofluorescence technique was used to measure antibody titers of serum specimens against neurofilament proteins (16). Sera were initially screened at a dilution of 1:16 (prepared in 0.0067 M phosphate-buffered saline, pH 7.4), and those showing specific fluorescence were titrated in twofold increments. The antibody titer was the highest dilution of serum giving definite fluorescence of one-plus (1+) or greater.

Double-fluorescence staining with fluorescein isothiocyanate (FITC) and rhodamine conjugates was used to demonstrate two antigens in the same fixed culture of cells (19). FITC-conjugated rabbit anti-human IgG, IgA, and IgM, anti-sheep IgG, anti-guinea pig IgG, anti-hamster IgG, and anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, Ind.) were used to detect chimpanzee, sheep, guinea pig, hamster, mouse, and rabbit antibodies, respectively. FITC-conjugated rabbit anti-monkey IgG and rhodamine-conjugated goat

### TABLE 1. Incidence of autoantibodies against neurofilament proteins, as determined by the indirect immunofluorescence technique, in the sera of subacute spongiform virus encephalopathy-infected and control animals

<table>
<thead>
<tr>
<th>Animal</th>
<th>Diseasea</th>
<th>No. tested</th>
<th>No. (%) positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chimpanzees</td>
<td>CJD</td>
<td>41</td>
<td>12 (29.3)</td>
</tr>
<tr>
<td></td>
<td>Kuru</td>
<td>48</td>
<td>8 (16.7)</td>
</tr>
<tr>
<td></td>
<td>Other neurological diseasesb</td>
<td>19</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>106</td>
<td>8 (7.5)</td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>CJD</td>
<td>15</td>
<td>3 (20.0)</td>
</tr>
<tr>
<td></td>
<td>Kuru</td>
<td>3</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Scrapie</td>
<td>10</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>25</td>
<td>1 (4.0)</td>
</tr>
<tr>
<td>African green monkey</td>
<td>CJD</td>
<td>15</td>
<td>2 (13.3)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>15</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Squirrel monkey</td>
<td>CJD</td>
<td>46</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Kuru</td>
<td>20</td>
<td>1 (5.0)</td>
</tr>
<tr>
<td></td>
<td>Scrapie</td>
<td>10</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>49</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>CJD</td>
<td>14</td>
<td>1 (7.1)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>14</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hamster</td>
<td>Scrapie</td>
<td>17</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>None (normal brain)</td>
<td>12</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>12</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Mouse</td>
<td>CJD</td>
<td>11</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>11</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Natural scrapiec</td>
<td>20</td>
<td>7 (35.0)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>13</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

a Sera were from animals experimentally infected with CJD, kuru, or scrapie at the terminal stage of illness, unless otherwise indicated.

b Sera were from chimpanzees which had remained asymptomatic for many years after inoculation with suspensions of brain tissue from patients with neurological diseases other than CJD or kuru. See text for details.
c Sera were from sheep naturally infected with scrapie.
anti-human IgG, IgA, and IgM and anti-rabbit IgG, IgA, and IgM (Cappel Laboratories, Downingtown, Pa.) were used to detect Old World and New World monkey, human, and rabbit antibodies, respectively. To determine the class of immunoglobulins in the chimpanzee sera, FITC-conjugated goat anti-human IgG (γ-chain) anti-human IgM (μ-chain) were used (1). Cells were viewed with a Zeiss microscope equipped with an epifluorescence condenser III RS and exciter filters for FITC and rhodamine.

RESULTS

Anti-neurofilament protein autoantibodies in nonhuman primates. Of the 413 nonhuman primate sera tested, 17 of 117 (14.5%) from animals infected with CJD and 9 of 71 (12.7%) from animals infected with kuru had autoantibodies against axonal neurofilament proteins. Of 195 nonhuman primate control sera, 9 (4.6%) also had autoantibodies (Table 1).

Pre-inoculation serum specimens were available from all but 2 of the 108 chimpanzees tested. Autoantibodies were detected in eight (7.5%) of these sera. During the terminal phase of disease, 12 of 41 (29.3%) chimpanzees infected with CJD and 8 of 48 (16.7%) chimpanzees infected with kuru had autoantibodies against neurofilament proteins. None of the 19 chimpanzees inoculated with brain tissues from human patients with other neurological diseases had detectable autoantibodies in serum specimens taken at intervals after their inoculation that were twice the mean incubation periods of animals that developed CJD and kuru.

Of the 12 CJD-infected chimpanzees with anti-neurofilament protein autoantibodies during the terminal stage of illness, 6 had autoantibodies even before experimental inoculation with infected brain suspensions, and autoantibodies persisted throughout the course of the disease. Of the other six chimpanzees, four developed autoantibodies during the asymptomatic phase, and two developed autoantibodies during the terminal clinical phase of the disease (Fig. 1). Of the eight kuru-infected chimpanzees with autoantibodies in terminal-phase sera, two had autoantibodies before inoculation and throughout the course of the disease, three developed autoantibodies during the asymptomatic phase, and three developed autoantibodies during the terminal clinical phase of the disease (Fig. 2). In all cases the autoantibodies belonged to the IgG class of immunoglobulins, as demonstrated by indirect immunofluorescence with FITC-conjugated goat anti-human IgG (γ-chain) and goat

![Table 1](http://iai.asm.org/...)

**FIG. 1.** Development of autoantibodies against neurofilament proteins in the sera of chimpanzees infected with CJD. Six chimpanzees had autoantibodies in their pre-inoculation sera, and the remaining six chimpanzees developed autoantibodies at various time intervals after inoculation. There was no preserum specimen available for testing on chimpanzee A-165. Chimpanzee A-231 died of an intercurrent infection 55 months after inoculation but had histopathological lesions of experimental CJD in his brain. Symbols: +, positive for autoantibodies by immunofluorescence at a dilution of 1:16 or greater; −, negative for autoantibodies by immunofluorescence at a dilution of 1:16. Open bars, Asymptomatic phase; shaded bars, symptomatic phase.
anti-human IgM (μ-chain). The titers of autoantibodies in the terminal-phase sera ranged from 1:32 to 1:512. There were no significant differences in autoantibody titers among the sera from chimpanzees with CJD and kuru (Fig. 3) or in the rate of occurrence of autoantibodies in the sera of male (12 of 65, 18.5%) and female (8 of 43, 18.6%) chimpanzees during the terminal phase of the disease.

To determine if changes in autoantibody titers occurred during the course of disease, serial serum specimens from four chimpanzees with CJD and four with kuru were tested. In each of the two groups of chimpanzees, two animals had autoantibodies in the pre-inoculation specimens and in the sera collected throughout the course of the disease, and two animals developed autoantibodies during the course of the disease. Once autoantibodies appeared, their titers did not vary significantly during the course of illness in either CJD-infected (Fig. 4a) or kuru-infected animals (Fig. 4b).

Autoantibodies were detected in the terminal-phase sera of 3 of 15 rhesus monkeys with CJD and 2 of 15 African green monkeys with CJD but in neither species infected with kuru or scrapie. Of 25 normal rhesus monkeys, 1 had autoantibodies. Autoantibody titers in the terminal-phase sera of five CJD-infected animals were 1:16, 1:32, and 1:64 in rhesus monkeys and 1:128 and 1:512 in African green monkeys. Only 1 of 20 kuru-infected squirrel monkeys had autoantibodies (titer, 1:32) in the terminal-phase serum. Autoantibodies were not detected in the sera of un inoculated squirrel monkeys or those infected with CJD or scrapie (Table 1).

Anti-neurofilament protein autoantibodies in rodents. Among the 91 guinea pigs, hamsters, and mice tested, only 1 guinea pig clinically affected with CJD had autoantibodies in serum specimens collected during the terminal phase of disease (titer, 1:512). None of the sera from scrapie-infected hamsters, CJD-infected mice, or control rodents had autoantibodies (Table 1).

Anti-neurofilament protein autoantibodies in sheep. In marked contrast were the results of tests of the sera of normal sheep and sheep naturally infected with scrapie (Table 1). Of 20 sheep infected with scrapie, 7 (35%) had autoantibodies, whereas none of 13 normal controls had autoantibodies. Autoantibody titers ranged from 1:64 to 1:1,024 (Fig. 3).

Specificity of autoantibodies. The staining pat-
**a. CJD**

TITERS

MONTHS AFTER INOCULATION

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**b. KURU**

TITERS

MONTHS AFTER INOCULATION

FIG. 4. Titers of autoantibodies against neurofilament proteins in pre- and post-inoculation sera of chimpanzees with experimental CJD (a) and in pre- and post-inoculation sera of chimpanzees with experimental kuru (b).

FIG. 5. Immunofluorescence staining of mouse central neurons in vitro with sera from four different animal species, showing the same staining pattern of neuronal processes. (a) Serum from a chimpanzee (A-20) with kuru diluted 1:16 and stained with FITC-conjugated rabbit anti-human IgG, IgA, and IgM. (b) Serum from an African green monkey (921-T) with CJD diluted 1:16 and stained with FITC-conjugated rabbit anti-monkey IgG. (c) Serum from a guinea pig (no. 54) with CJD diluted 1:16 and stained with FITC-conjugated rabbit anti-guinea pig IgG. (d) Serum from a sheep (G.A. 368) with natural scrapie diluted 1:16 and stained with FITC-conjugated rabbit anti-sheep IgG. Bars, 30 μm.
cytoskeletal proteins, cells. Background goat anti-rabbit antisera to autoantibodies. Bars, background nonneuronal conjugated goat staining specific in terns with 1:40 and terns were cytoskeletal structure, and occurring autoantibodies in axonal processes. Autoantibodies to the host of bodies detected were restricted were pattern was human serum; detected with FITC and rhodamine conjugates was used. As shown in Fig. 7, no differences were noted in the double-staining patterns when autoantibody-positive sera and rabbit antisera to neurofilament proteins were compared: a1 and a2, positive human serum with positive guinea pig serum; b1 and b2, rabbit antisera to neurofilament proteins with positive nonhuman primate serum.

DISCUSSION

Heterogeneic autoantibodies against normal axonal neurofilament proteins, as detected by the indirect immunofluorescence technique, were demonstrated in the sera from chimpanzees, Old World and New World monkeys, and a guinea pig experimentally infected with kuru or CJD. Autoantibodies were found more frequently in the sera obtained during the terminal phase of illness. A significant percentage of

FIG. 6. Immunofluorescence staining of mouse central neurons in vitro with four different rabbit antisera to cytoskeletal proteins, showing specific staining patterns for each antisera. (a) Rabbit antisera to actin diluted 1:40 and stained with FITC-conjugated goat anti-rabbit IgG, showing stress pattern of microfilament staining in nonneuronal background cells. (b) Rabbit antisera to tubulin diluted 1:40 and stained with FITC-conjugated goat anti-rabbit IgG, showing staining of microtubules not only in neuronal processes but also in nonneuronal background cells. (c) Rabbit antisera to glial fibrillary acidic protein diluted 1:50 and stained with FITC-conjugated goat anti-rabbit IgG, showing specific staining of intermediate filaments in the astrocyte. (d) Rabbit antisera to neurofilament proteins diluted 1:40 and stained with FITC-conjugated goat anti-rabbit IgG, showing specific staining of neuronal processes. The pattern of staining is indistinguishable from that of the heterogeneic autoantibodies. Bars, 30 μm.
sheep naturally infected with scrapie also had such autoantibodies. When autoantibody titers of the sera from humans with kuru or CJD (16) and from sheep naturally infected with scrapie are compared with those of the sera from experimentally infected animals, it appears that the natural infections with the subacute spongiform virus encephalopathies are associated with higher titers of autoantibodies.

Autoantibodies against neurofilament proteins have been demonstrated by immunofluorescence and immunoperoxidase staining techniques in the sera of 59% of CJD patients and 27% of kuru patients (16). Recently, autoantibodies have also been found in the sera of 11.2% of patients with other neurological diseases and in the sera from normal controls (8%) (C. J. Gibbs, Jr., J. Sotelo, T. Aoki, M. Wurth, and D. C. Gajdusek, manuscript in preparation). These findings have been subsequently confirmed with immunofluorescence-stained longitudinal sections of frozen rat spinal cords (3).

The characteristic staining patterns of autoantibodies in the sera of experimentally infected nonhuman primates and naturally infected sheep were identical to those in positive human sera. However, the incidence and titers observed in animals experimentally infected with terminal diseases were not as great as those observed in humans with CJD or kuru. Some sera obtained from animals before inoculation with brain tissues showed the same type of antibodies. Thus, autoantibodies cannot be used to determine the success of transmission of the subacute spongiform encephalopathies to animals. In chimpanzees, the occurrence of autoantibodies was not closely associated with clinical signs of illness or time of death, and the titers of autoantibodies, once detectable, did not change significantly during the course of the disease. Thus, heterogeneic autoantibodies against neurofilament proteins cannot serve as clinical markers of disease. Viruses have long been suspected to cause autoimmune diseases, but the precise mechanisms remain obscure (8). After the description of antibodies to smooth muscle in the sera of
patients with chronic active hepatitis (9), autoantibodies against cytoskeletal proteins were reported in patients with various diseases (18). Antimyosin antibodies have been found in viral pericarditis (5), anti-intermediate filament antibodies have been demonstrated in common viral infections of children (20), and antimicrotubule antibodies have been reported in infectious mononucleosis (21). Interactions occurring between viruses and cytoskeletal components include the alteration of actin-containing filaments by Newcastle disease virus and vesicular stomatitis virus (14), the association of adenoviruses with microtubules (4), the interaction of early adenovirus protein with the cytoskeletal lattice (11), and the association of budding viruses with microfilaments (15).

All cytoskeletal filaments, composed principally of microfilaments, intermediate filaments, and microtubules, are normally confined to the cytoplasm and to the inner surfaces of the cell membranes. Recently, actin and tubulin were demonstrated not only on the surfaces of lymphocytes, but also in the growth medium of lymphocyte cultures after mitogenic stimulation, Epstein-Barr virus infection, and malignant transformation, suggesting that cytoskeletal proteins may act as autoantigens in certain viral infections (2). Viruses, then, might induce autoantibodies by lysing or activating subpopulations of lymphocytes (8). The recent observation of infection of splenic lymphocytes with the virus that causes CID (Y. Kuroda, C. J. Gibbs, Jr., H. L. Amyx, and D. C. Gajdusek, manuscript in preparation) suggests such a mechanism for the appearance of autoantibodies in the subacute spongiform encephalopathies.

Alternatively, latent, persistent, or intercurrent infections with conventional viruses (13) may account for autoantibody production. This would explain the finding of autoantibodies against neurofilament proteins in the sera of normal animals and humans. Autoantibodies against intermediate filaments (7, 12, 22) and tubulin (10) have also been detected in the sera of normal preimmunized laboratory animals.

Anti-neurofilament protein autoantibodies previously reported in human sera (16) and now demonstrated in the sera of naturally and experimentally infected animals constitute the first convincing evidence of involvement of the immunological system in the subacute spongiform encephalopathies, in which humoral response was previously unknown (6). Although the overall incidence of autoantibodies in experimentally infected animals is fairly low and although they do not appear to be specific for these diseases, they may provide a clue in studying the pathogenesis of CJD, kuru, and scrapie. Ultrastructural and biochemical studies of neurofilament alterations in subacute spongiform encephalopathies are already underway.

LITERATURE CITED
