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Autoantibodies Against Axonal Neurofilaments in Patients with Kuru and Creutzfeldt-Jakob Disease

Abstract. *The serums of some patients with subacute spongiform encephalopathies contain an autoantibody in high titer against a normal fibrillar protein within the axon of mature central neurons in culture. The morphological features of this neurofilament, as demonstrated by immunofluorescence and immunoperoxidase staining, and the partial characterization of the antibody are described. The detection of this hetero-specific autoantibody is the first evidence of an immune reaction in the spongiform encephalopathies.*

Autoantibodies against normal filament protein of mature central neurons in culture from several species of laboratory rodents have been found in high titer in serum samples of 59 percent of patients with Creutzfeldt-Jakob disease (CJD), in 27 percent of patients with kuru, and, more rarely, in serums from patients with other chronic neurological diseases and from normal subjects. This antibody has been detected by indirect immunofluorescence and immunoperoxidase techniques, the substratum consisting of fixed cultures of large numbers of mature central neurons on coverslips prepared from fetal mice, hamsters, or rats as reported (*1*). Serum samples from a total of 22 patients with CJD, 28 patients with kuru, and 25 "normal" healthy control subjects have been studied for the presence and characterization of this autoantibody. We have also conducted a blind study of additional serum samples from patients with CJD, kuru, and other chronic neurological disorders.

All the serum samples and cerebrospinal fluids were stored at −70°C; they were tested at twofold dilutions in phosphate-buffered saline (PBS) starting at 1:16. The 22 CJD patients were verified both clinically and pathologically; from nine of them the disease has been experimentally transmitted to monkeys. The 28 kuru patients were all classical kuru victims who are now dead.

Neurons from the brains of rats, mice, or hamsters were grown in vitro as described (*1*) and tested after 20 days in culture when they were morphologically mature. The neurons and cell lines were tested by indirect immunofluorescence (*2*) and immunoperoxidase (*3*) techniques after they had been fixed for 10 seconds in cold acetone (−20°C) and washed in buffered saline. For demonstrating two different antigens in the same fixed culture we used rhodamine and fluorescein conjugates in a double fluorescence test (*4*). Indirect immunofluorescence staining was also done on sections cut from composite blocks of frozen tissues (rat liver, kidney, and stomach) (*5*), on frozen sections of brain from 7-week-old white Lewis rats, and on cultured fibroblasts from fetal rat lung. The serums were immunoabsorbed (*6*) with purified neurofilaments prepared as described by Schlaepfer (*7*) and with normal brain suspension made by homogenization of 0.5 g of brain tissue from a 7-week-old white Lewis rat in 100 ml of PBS.

Neuroblastoma (N_{2a}) and glioblastoma (C6) cell lines were from the American Type Culture Collection, Rockville, Maryland. Sheep brain (0A1B) and rabbit cornea (SIRC) cell cultures were purchased from Flow Laboratories, Rockville, Maryland. Rabbit antiserum to human globulins, goat antiserum to human globulins, rabbit antiserum to human IgG, IgM, or IgA (immunoglobulins G, M, or A), goat antiserum to human IgG specific for the Fab fragment, and goat antiserum to rabbit IgG rhodamine and fluorescein conjugates were from Cappel Laboratories, Cochranville, Pennsylvania. Rabbit antiserum to human globulins and rabbit antiserum to human IgG peroxidase conjugates were obtained from Miles Laboratory, Elkhart, Indiana. Rabbit antiserums to neurofilament and rabbit antiserums to intermediate (10 n*M*) filaments were obtained from B. H. Toh, Monash University, Australia; rabbit antiserum to tubulin was obtained from G. Rutter, H. Pette Institute, West Germany; rabbit antiserum to glial fibrillar acidic protein was obtained from A. Bignami, Boston, Massachusetts; and rabbit antiserum to actin was made in our laboratory as described by Owen (*8*). Cells were viewed with a Zeiss photomicroscope equipped with epifluorescence.

Serum samples from 13 of the 22 CJD patients (59 percent) and from 8 of the 28 kuru patients (27 percent), when examined by the immunofluorescence test, contained high titers of specific antibodies against the perikarya and the axonal neurofilaments of central neurons; these antibodies were observed along the full axonal length and thus revealed its tract throughout the cultures (Figs. 1 and 2). These same serum samples were also positive in the immunoperoxidase test (Fig. 3). None of the serum samples from 25 normal subjects showed similar staining. Antibody was not detected in the undiluted cerebrospinal fluid from the 13 CJD patients with positive serum.

We also examined, in a later fully blind study, serum samples from 38 patients with CJD, 63 patients with kuru, 71 patients with other chronic neurological and autoimmune diseases (eight with Parkinson's disease with dementia; seven with myasthenia gravis; six with neurosyphilis; eight with multiple sclerosis; nine with Alzheimer's; nine with Guamanian amyotropic lateral sclerosis and parkinsonism-dementia; and 24 with other miscellaneous diseases), and 50 normal subjects. The serum from 45 percent of the patients with CJD, 22 percent with kuru, 13 percent with the other neurological diseases (two with Parkinson's disease with dementia; two with Alzheimer's; one with Guamanian parkinsonism-dementia; two with Pick's disease; one with subacute sclerosing panencephalitis; and one with brain lymphoma), and 10 percent of the normal subjects, showed a similar autoantibody reaction (*9*). These positively reacting serums have not yet been characterized.

All of the positively reacting serum samples from the 13 patients with CJD and eight with kuru were titrated by means of the immunofluorescence test. Samples from four of the CJD patients were positive in dilutions up to 1:320; samples from one of the patients with kuru were positive in dilutions up to 1:1280 and, from another, up to 1:640. All of these samples with high titers of

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autoantibodies were from patients in the late stages of their disease; the four CJD patients were those whose brain tissue had been used to transmit the disease experimentally to monkeys. The rest of the positive serum samples had antibody titers that ranged from 1:16 to 1:128.

The serum samples with the highest titers (four from CJD and two from kuru patients) were used for further characterization of the autoantibody. Antibodies against neurofibrils in the axonal skeleton were consistently detected with rabbit antiserum to human globulins or goat antiserum to human globulins conjugated with either rhodamine or fluorescein dyes (Fig. 1 and 2) or by immunoperoxidase staining (Fig. 3). The reaction was also consistently positive when we used mouse, rat, or hamster central neurons in culture. This neurofilament autoantibody proved to be IgG when tested with rabbit antiserum to IgG conjugated with fluorescein isothiocyanate (FITC). Fab fragments of IgG prepared from the positive serums from two CJD patients and from the negative serum of one control subject were reacted with the neuron cultures and then stained with fluorescein-conjugated IgG fraction of goat antiserum specific for human IgG Fab fragments. These antiserums gave the same pattern of positive staining as did whole CJD serum with fluorescein-conjugated antiserum to IgG, thus demonstrating the antigen-antibody binding as the basis of the observed reaction.

Positive serum from CJD patients did not react by the same immunostaining techniques with any of the following cell lines: glioblastoma C6, sheep fetal brain cells, rabbit cornea, neuroblastoma N_{2a}, or rat fetal lung fibroblasts; it also did not react with frozen sections of composite blocks of rat liver, kidney, and stomach. Serum tested with frozen sections of normal rat brain gave no conclusive results because of nonspecific staining that obscured any positive binding that may have been present.

The positive staining of cultivated neurons disappeared when the serum samples were immunoabsorbed with normal brain suspension or with a suspension of purified neurofilaments.

The autoantibody in serums from patients with CJD or kuru showed a pattern of staining similar to that obtained when neuron cultures were stained by an immunofluorescence technique with rabbit antibody to neurofilament, though the human autoantibody produced more neurofilament-specific and less background staining. In contrast, when the same preparation was tested by the double immunofluorescence technique, the human autoantibody produced a pattern of staining that was different from the one obtained when the same neurons were reacted with rabbit antibody to tubulin: this antibody to tubulin reacted with the microtubules of the dendritic network and with the cytoplasmic microtubules of the supporting cells in the culture (astrocytes, oligodendrocytes, and fibroblasts), whereas the autoantibody in the serum of CJD and kuru patients showed a clear specificity for the cell body and axon of neuron (Fig. 4). The picture was also different when the cultures were stained by the double immunofluorescence technique with rabbit antiserum specific for actin (Fig. 5), showing that the human autoantibody was not directed against microfilaments, another cytoskeletal structure. Finally, when the neuron cultures were stained by the double immunofluorescence technique with rabbit antiserum to glial fibrillary acidic protein and the human autoantibody, the latter showed a clear specificity for neurites whereas the former stained only astroglial cytoplasmic filaments (Fig. 6).

These comparisons of the patterns of immunofluorescence staining obtained

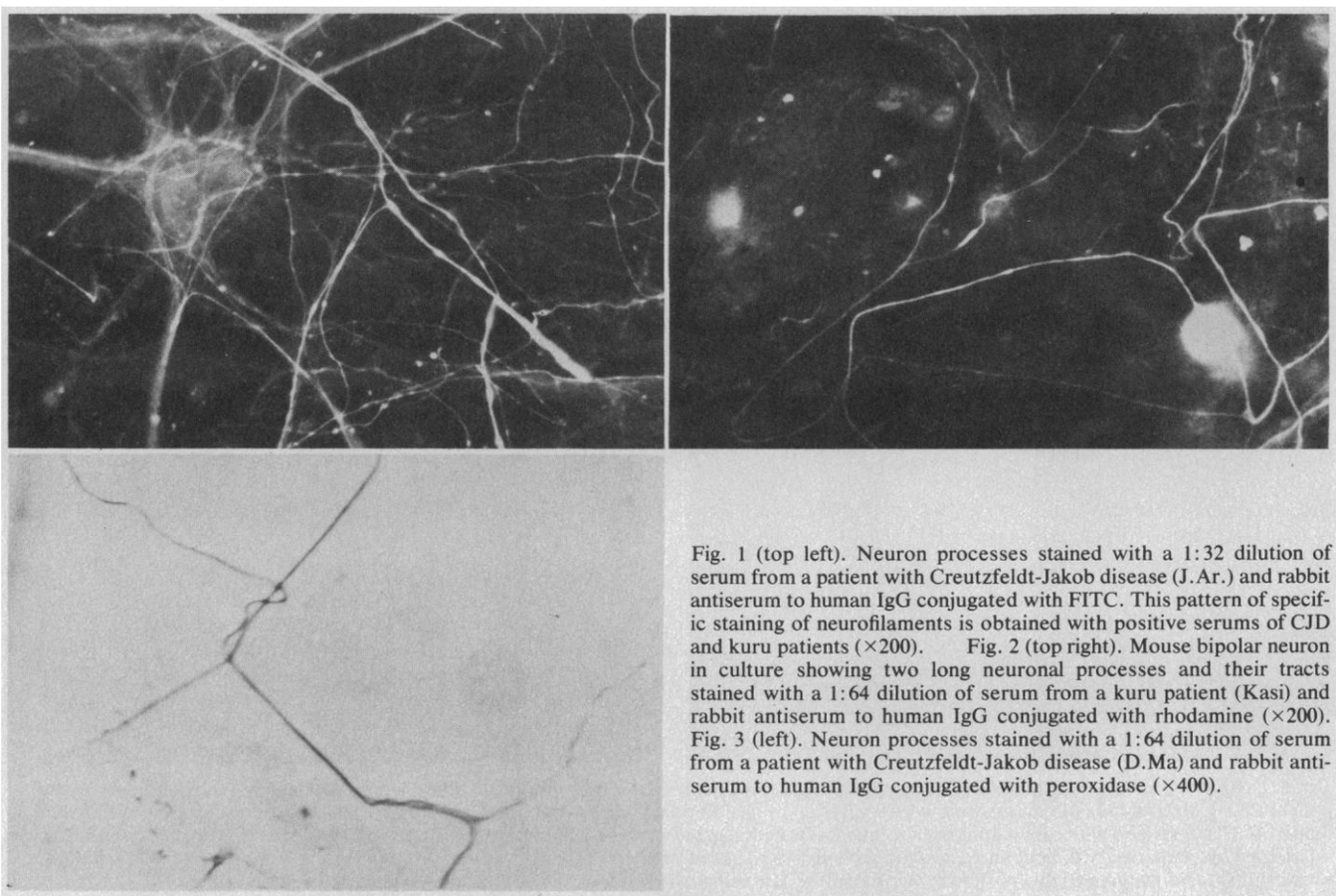

Fig. 1 (top left). Neuron processes stained with a 1:32 dilution of serum from a patient with Creutzfeldt-Jakob disease (J.Ar.) and rabbit antiserum to human IgG conjugated with FITC. This pattern of specific staining of neurofilaments is obtained with positive serums of CJD and kuru patients (×200). Fig. 2 (top right). Mouse bipolar neuron in culture showing two long neuronal processes and their tracts stained with a 1:64 dilution of serum from a kuru patient (Kasi) and rabbit antiserum to human IgG conjugated with rhodamine (×200). Fig. 3 (left). Neuron processes stained with a 1:64 dilution of serum from a patient with Creutzfeldt-Jakob disease (D.Ma) and rabbit antiserum to human IgG conjugated with peroxidase (×400).

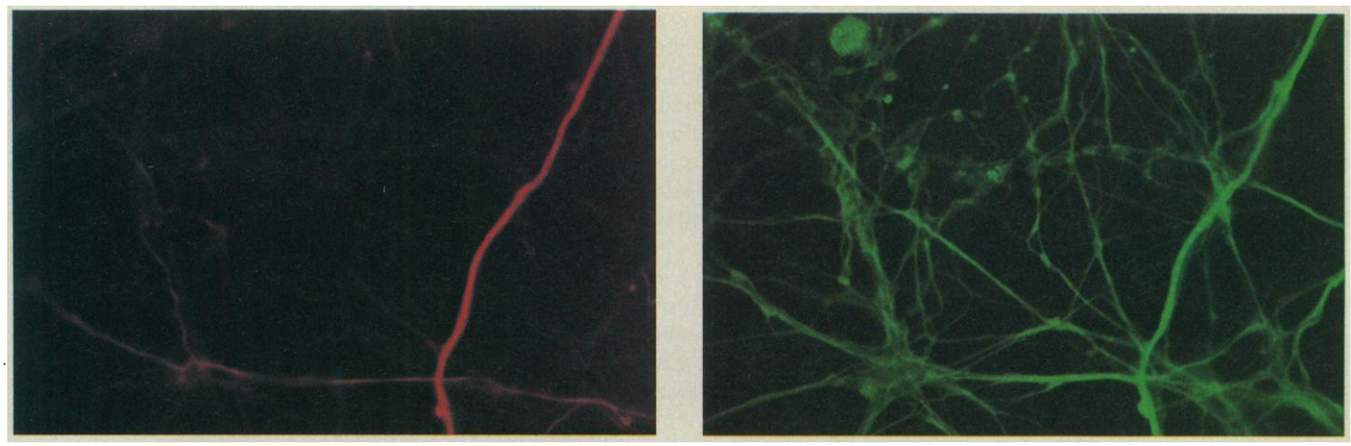

Fig. 4. Double immunofluorescence staining of mouse neurons in the same microscopic field contrasting the staining obtained with a positive serum from a patient with Creutzfeldt-Jakob disease and with antibody to tubulin. (Left) Positive serum from a patient (D.Ma.) with CJD diluted 1:32 and stained with rabbit antiserum to human IgG conjugated with rhodamine. (Right) Rabbit antiserum to tubulin diluted 1:32 and stained with goat antiserum to rabbit IgG fluorescein conjugate. Antibody to tubulin antibody stains the whole dendritic network of neurons while serum from a patient with CJD stains only selective neural processes, apparently axons (×200).

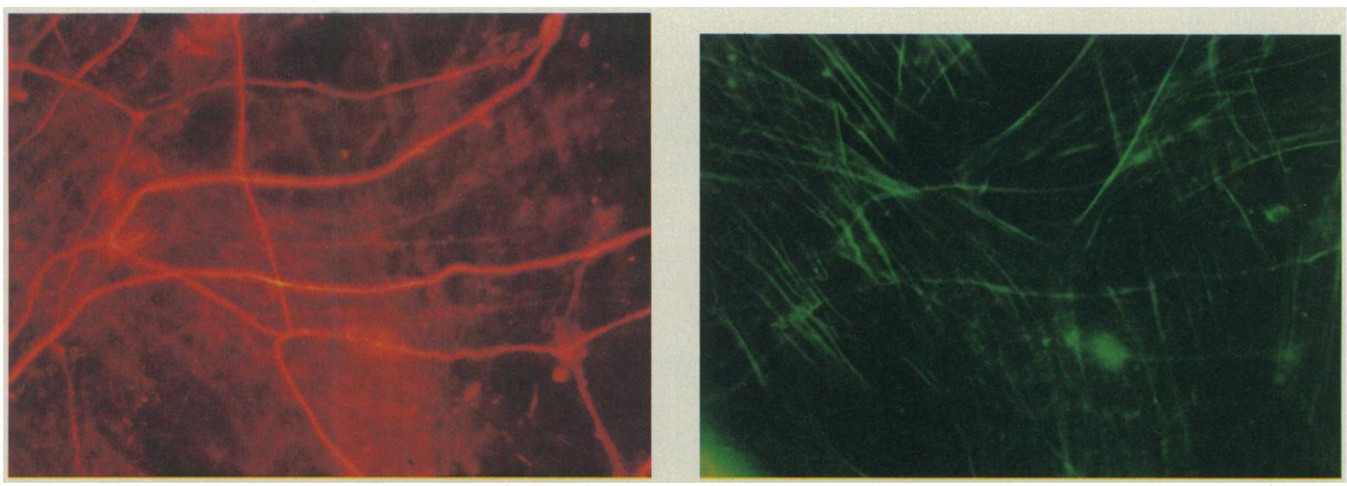

Fig. 5. Double fluorescent staining of mouse neurons in the same microscopic field contrasting the staining obtained with a positive serum from a patient with Creutzfeldt-Jakob disease and with antibody to actin. (Left) Positive serum from a patient (D.Ma.) with CJD diluted 1:32 and stained with rabbit antiserum to human IgG conjugated with rhodamine. (Right) Rabbit antiserum to actin antibody diluted 1:32 and stained with goat antiserum to rabbit IgG conjugated with fluorescein. The difference in the distribution of neurofilaments (left) and the cytoplasmic microfilaments of neuron-supporting fibroblasts is shown (×200).

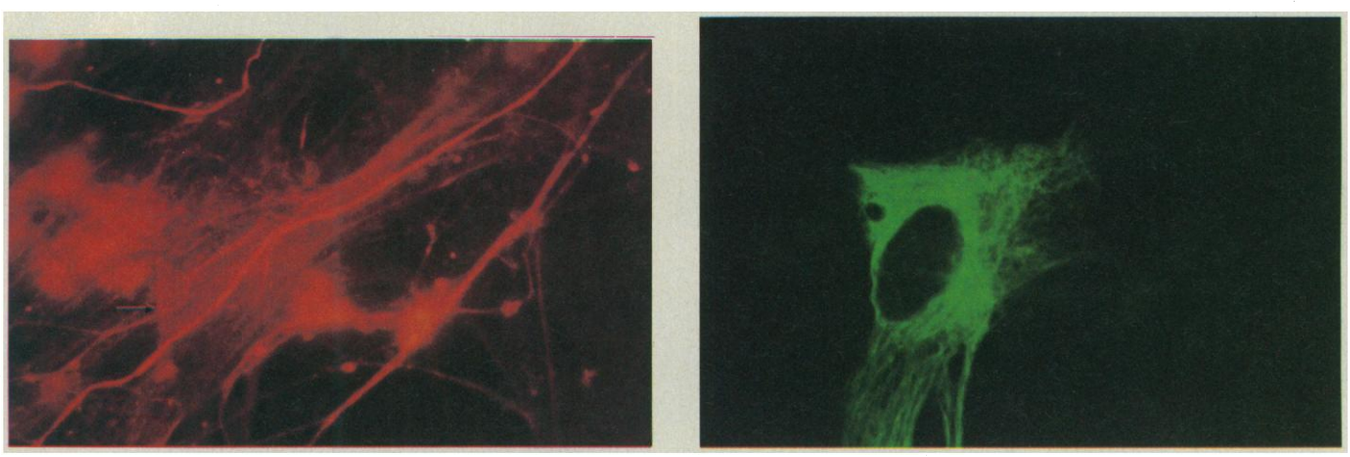

Fig. 6. Double fluorescence staining of mouse neurons in the same microscopic field contrasting the staining obtained with a positive serum from a patient with Creutzfeldt-Jakob disease and with antiserum to glial fibrillary acidic protein. (Left) Positive serum from patient (D.Ma.) with CJD diluted 1:32 and stained with rabbit antiserum to human IgG conjugated with rhodamine. (Right) Rabbit antiserum to glial fibrillary acidic protein diluted 1:32 and stained with goat antiserum to rabbit IgG conjugated with fluorescein. The difference between neurofilament staining (left) and astroglial filament staining (right) is shown. Arrow on the left shows the shadow of the astrocyte stained on right (×200).

with antiserums to different known skeletal filaments of cells support the idea that the autoantibody found in some CJD and kuru patients is directed against the neurofilaments. Furthermore, the absence of a reaction of the positive serums with the skeletal structure of dendrites in our neuron culture strongly suggests that the autoantibody is specific for a form of filament protein restricted to axons. That this human autoantibody reacts with normal rat, mouse, or hamster neural proteins shows that it is not species-specific.

Johnson *et al.* (*10*) in 1965 showed that patients with active chronic hepatitis developed an autoantibody against cytoskeletal proteins (*11, 12*); these autoantibodies (smooth muscle autoantibodies) were later found in patients with other diseases (*13–15*). Toh *et al.* (*16*) demonstrated the transient presence of autoantibodies to 10 n*M* filaments in the serum of children in the acute stage of common viral diseases; these autoantibodies decreased during convalescence. Linder *et al* (*17*) showed that complement can be activated in the absence of antibody and bound specifically to 10 n*M* filaments in a wide range of cells and noted the possible role of this mechanism in virus-infected cells. Dales and Chardonnet (*18*) reported evidence that another cytoskeletal structure, microtubules, may serve as the pathway used by viruses on their vectorial travel from the cellular surface toward the nucleus. Hiller (*19*) has demonstrated an intimate association of viral particles with the cytoskeleton of virus-infected cells, and their interaction with microfilaments. Allison (*20*) postulated that virus infection can lead to T-cell sensitization, and through a carrier effect can stimulate B cells to produce autoantibodies against cellular components.

The failure of the viruses of the transmissible spongiform encephalopathies to induce an immune response, either during the evolution of the natural or experimental disease or in animals hyperimmunized with high concentrations of virus, has been one of their most "unconventional" characteristics (*21*). Although previous serological tests to detect immune reactions in patients and animals with kuru, CJD, and scrapie were consistently negative, it was early postulated by us that the procedures employed, that is, complement fixation and Ouchterlony double-diffusion and precipitation tests, were not sensitive enough to detect a response (*22*). The autoantibody described in this report, although not specific for kuru and CJD, is the first evidence of immune system involvement in these diseases. However, it is too early in our investigations to speculate about the possible role of this autoantibody in the pathogenesis of CJD and kuru or the clinical value of the estimation of this antibody in the course of these diseases. Its occasional presence in patients with other neurological disorders and in healthy subjects must be evaluated.

J. SOTELO*
C. J. GIBBS, JR.
D. C. GAJDUSEK
Laboratory of Central Nervous System Studies, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205

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* Visiting scientist from Instituto Nacional de Neurologia, Insurgentes Sur 3877, Mexico 22 D.F.

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Endorphin-Mediated Increases in Pain Threshold During Pregnancy

Abstract. *Maternal pain thresholds in rats were determined during various stages of pregnancy and parturition by measuring the intensity of electric shock that elicited reflexive jumping. There was a gradual rise in the pain threshold between 16 and 4 days prior to parturition and a more abrupt rise 1 to 2 days before that event. This increase was abolished by long-term administration of the narcotic antagonist naltrexone. The endorphin system is thus an important component of intrinsic mechanisms that modulate responsiveness to aversive stimuli. The data also demonstrate the activation during pregnancy of an endorphin system that is apparently quiescent in nonpregnant female rats treated the same way.*

The biochemical demonstration of specific opiate receptors (*1*) and the discovery of endogenous ligands (endorphins) (*2*) for these receptors indicate that in addition to mediating the analgesic effects of alkaloids, the receptors are involved in mediating physiological processes. Since pregnancy, labor, and parturition are states that require adaptation to stress, considerable attention has been focused on the possible involvement of endorphins in the physiological sequelae of pregnancy. Although there is much circumstantial evidence that endorphin systems are activated during pregnancy (*3*), the specific behavioral and physiological consequences of this activation have not yet been elucidated. This report describes experiments showing that normal modulation of responses to painful stimuli during pregnancy can be blocked by administration of naltrexone, a pure, potent narcotic antagonist. The results indicate that endorphin systems are involved in raising maternal thresholds to pain.

Two groups of seven pregnant rats and two groups of seven nonpregnant rats were housed individually and given free access to food and water. Pain thresholds were determined for one group of pregnant animals beginning 16 days before parturition and continuing until 41 days afterward. One group of nonpregnant rats was tested in parallel with the pregnant group an equal number of times with the same interval between tests. To determine whether endorphins are involved in modulating responsiveness to aversive stimuli during preg-

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