Abstract

Two patients with clinical and radiological evidence of limbic encephalitis associated with an invasive lymphoepithelial thymoma who improved after thymectomy and radiotherapy are reported. The serum of both patients and the CSF of one of them contained different types of antibodies that immunoreacted with human and rat brain and newborn rat thymus. After treatment of the tumour, the antibody titres decreased. Similar antibodies were not found in various controls. Two out of 16 patients with thymoma, myasthenia gravis, and no CNS involvement had low titres of antibodies reacting with the brain. It is suggested that in some patients with thymoma, an autoimmune reaction involving antigens common to the brain and thymus is possibly misdirected against the CNS.

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Lymphoepithelial thymomas are tumours originating in the epithelial component of the thymus that are accompanied by a variable proportion of normal lymphocytes. A characteristic of thymomas is their frequent association with probable or definite autoimmune neuromuscular disorders. The pathophysiology of myasthenia gravis, the most common of them, has been extensively studied. The disease depends on an immunological reaction between acetylcholine receptors (AChRs) of the neuromuscular junctions and similar epitopes of normal or tumoral thymic cells. By contrast, only a few patients have been reported who had CNS involvement. Most of them had limbic encephalitis. This disorder usually belongs to a well known group of carcinoma associated paraneoplastic neurological syndromes that are ascribed, from recent data, to an autoimmune response primarily directed against tumour antigens that are also shared with the CNS. In this study, we report clinical and immunological investigations of two patients with thymoma and limbic encephalitis who had antibodies reacting with brain and thymus.

Patients and methods

PATIENT HISTORY

Patient 1

A 58 year old right handed woman was referred in July 1990 because of agitation, confusion, and loss of memory for recent events. She had almost permanent visual and auditory hallucinations. On examination, she was confused and anxious. Neurological examination was otherwise normal. She had no fever. Electroencephalography showed epileptic discharges in the right temporal lobe. Her CSF contained 0·30 g/l protein and 36 lymphocytes/mm³. Brain MRI was normal on T1 weighted sequences and showed mild increased signals in the right hippocampus on T2 weighted sequences. There were no antinuclear antibodies in the blood and CSF. A chest radiograph showed a large thoracic mass in the anterior mediastinum. The patient was treated with carbamazepine. Confusion disappeared within a few days; she was well oriented and could retain three words after five minutes. She still complained of memory loss. Thoracic surgery was performed in August 1990, 28 days after the onset of neurological symptoms. A macroscopically invasive predominantly epithelial thymoma (stage III of the classification of Masaoka et al) was removed. Treatment was completed with mediastinal radiotherapy. In April 1991, the patient’s family stated that her memory had greatly improved since her operation and that she performed her household management almost normally. For the first time in June 1991, she underwent memory tests. According to her cultural level, the Wechsler memory scale-revised (WAIS-R) intelligence quotient (IQ) was normal (82) for verbal material and slightly impaired for visual material (71). On memory tests, she had mild difficulties for immediate and delayed recall of the Rey-Osterrieth complex figure. There was no aplastic anaemia, no anti-AChR antibodies and no antistreptolysin antibodies. A second brain MRI was normal. In November 1993, there was no recurrence of the thymoma. She did not complain of any residual disability. Blood samples were obtained in July 1990 and November 1993.

Patient 2

A 67 year old right handed woman became suddenly confused with agitation, hallucinations, and subacute memory loss in April 1993. When she was referred to us in June 1993, she was alert and cooperative but seemed depressive. Her recent memory was...
severely altered. She could not recall any item after three minutes. Memory for remote events was less impaired. She had no confabulation. On the WAIS-R scale, her IQ was 76 and the memory quotient (MQ) was 65 according to the Wechsler memory scale-revised. Neurological examination was otherwise normal. Electroencephalography showed asymptomatic epileptic discharges in the left temporal lobe. She was treated with carbamazepine and the electrical abnormalities disappeared. Her neurological status, however, did not improve. Her CSF was normal. On T1 weighted sequences, brain MRI showed enlargement of the left hippocampus with pronounced hypersignals on T2 weighted sequences. Slight hypersignals were present on the right side. A test for herpes simplex virus with the polymerase chain reaction was negative in the CSF. Electromyography showed no myasthenic decrement. She had no anaesthetic anaemia. The antistriated muscle antibody titre was 1/10 and there were no anti-AChR and antinuclear antibodies. Chest CT showed a tumour in the anterior mediastinum. Thoracic surgery was performed on July 1993 and a mixed lymphoepithelial thymoma with microscopic invasion of the capsule (stage II of the classification of Masaoka et al) was removed. Treatment was completed with mediastinal radiotherapy. In September 1993, her neurological state had dramatically improved. She was well oriented, her IQ was 84, and her MQ 83. In November 1993, her IQ was 103 and her MQ 89. In April 1994, her IQ was 98 and her MQ 96, she performed her household management normally except for the necessity of noting her shopping requirements. Brain MRI was normal. Blood samples were obtained in June and July 1993, and after surgery, in December 1993 and in April 1994.

Serum samples
The following serum samples were used as controls: serum from a patient with high titre of anti-Hu antibodies (confirmed by Dr F Graus, Barcelona), serum samples from 30 normal subjects, 16 patients with lung or gynaecological cancer, 130 patients with various neurological diseases, and 16 patients with lymphoepithelial thymoma, myasthenia gravis, and no CNS disorders (kindly provided by Dr Bady, Lyon).

Methods
Details of our methods have been reported elsewhere.13 They are reported here in brief.

Tissue preparation—For immunofluorescence, anaesthetised OFA rats were perfused with 4% paraformaldehyde and 0.2% picric acid. Tissues (adult rat brain, newborn rat brain, E18 rat embryo, newborn rat thymus, and adult rat liver) were postfixed in the same solution and frozen. Human tissues (brain samples of a 71 year old woman without neurological disease and of a 24 week human embryo and the thymus of a 25 year old woman with myasthenia gravis and thymic hyperplasia) were fixed by immersion in the same solution and frozen. For western blots, fresh samples of rat and human tissues were homogenised with potassium phosphate buffer containing Triton X 100 and phenylmethylsulphon fluoride. To precipitate non-specific immunoglobulins, the human thymus was incubated with protein A sepharose and centrifuged. The supernatants were diluted in tris HCl buffer with sodium dodecylsulphate, bromophenol blue, diethioctetol, and glycerol.

Immunofluorescence—This was performed on frozen sections treated with bovine serum albumin and Triton X 100 and incubated for 12 hours with the serum samples diluted at room temperature. In some experiments, the serum of a normal control patient was added as blocking serum. After several washes, the sections were incubated with fluorescein conjugated rabbit antihuman antiserum (Dakopatts, Denmark). Control tests were also performed with the fluorescein conjugated rabbit antihuman antiserum alone. With this method, serum samples of patients with various neurological diseases and normal subjects were always negative when diluted over 1/500 and 1/1000 was fixed as being the lower limit of positivity.

Western blots—Proteins (6 μg per lane) were separated by electrophoresis in a 12% polyacrylamide minigel and electrically transferred to PVDF membranes (immobilin membranes, Millipore). Blots were incubated with bovine serum albumin to saturate additional protein binding sites, and then for 12 hours with the patient’s serum diluted 1/100. Bound antibodies were visualised by incubating the blots with biotinylated rabbit antihuman IgG antiserum (Jackson, Baltimore) and then with streptavidin peroxidase (Jackson, Baltimore). The colour reaction was developed with diaminobenzidine.

Results
Patient 1
Before thymectomy, the serum and the CSF tested by immunofluorescence contained a high titre of IgG antibodies (serum end point dilution 1/15 000, CSF 1/100). In adult rat brain, IgGs labelled the cytoplasm and processes of cells situated in the white matter of the brainstem and cerebellum. Their morphology and distribution in bead rows suggested that they were oligodendrocytes. In addition, cells having the morphology and distribution of neurons were labelled in the granular layers of the dentate gyrus (fig 1), olfactory bulb, and retina. In newborn rat and E18 rat embryo, there was a diffuse labelling of postmitotic cells throughout the central and peripheral nervous system. This labelling corresponded to a specific pattern that we have previously reported with the serum of a patient with paraneoplastic encephalomyelitis and undifferentiated carcinoma.14 In addition, the serum of patient 1 labelled some neurons in the rat brainstem and substantia nigra. There was no labelling in newborn rat thymus and human brain. Because of endogenous
IgGs, the human thymus gave no specific labelling. On western blots, the patient’s IgGs labelled a band of apparent molecular weight 66 kDa in human brain, rat brain and newborn rat thymus (fig 2). There was no labelling in rat liver. With adult human thymus, despite preincubation with protein A sepharose, the positive bands corresponded to non-specific endogenous IgGs. In November 1993, three years after thymectomy, the antibody titre was 1/100.

Patient 2
Before thymectomy, the serum tested by immunofluorescence in adult rat brain contained IgG antibodies (titre: 1/3000) that diffusely labelled the nucleus of cells having the morphology and distribution of neurons (fig 3). Six and nine months after operation, the antibody titre was 1/1000. Immunocytochemistry was negative in human brain, E18 rat embryo, and newborn rat thymus. With human thymus, as with patient 1, labelling corresponded to non-specific endogenous IgGs. Western blots (fig 4), showed immunoreactivity with bands of apparent molecular weight 36 kDa in the human and rat brain, and in the newborn rat thymus. In addition, a band of circa 70 kDa was labelled in the brain alone. None of these bands was labelled in the liver. Western blots with human thymus gave non-specific results as with patient 1. Antibodies were not detected in the CSF. On western blots of adult rat brain, a control serum containing anti-Hu antibodies gave a different pattern (fig 4).

Control serum samples
Serum samples from normal subjects, patients with neurological diseases, and patients with lung or gynaecological cancers gave no specific staining. Among the 16 patients with thyromma, myasthenia gravis, and no CNS disorders, five had antinuclear antibodies and seven had antistriated muscle antibodies. One serum gave a cytoplasmic labelling of neurons but no positivity was seen on western blots of rat brain. Another serum with antinuclear antibodies labelled the cytoplasms of glial cells at 1/100 dilution and labelled a band of 70 kDa on western blot of adult rat brain.

Discussion
The pathological features of limbic encephalitis consist of neuron loss, inflammatory cell reaction, and gliosis restricted to the limbic system or associated with disseminated encephalomyelitis.17 18 Recent memory impairment, confusion, hallucinations, temporal lobe epilepsy (sometimes prominent9), depression, and anxiety are the main symptoms.17 18 Some patients have regressive hippocampal high signals on T2 weighted sequences of brain MRI.20 21 Although a histological examination was not available in our patients, their clinical and radiological features similar to those mentioned above strongly support the diagnosis of limbic encephalitis. Despite significant differences in the delay in diagnosis, the lateralisation of temporal lobe injury on brain MRI, and the severity of symptoms, both patients had a good outcome after treatment of the thyromma. Patient 2 recovered rapidly and patient 1, who predominantly had confusion and epilepsy, showed only slight residual memory impairment. Improvement after

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**Figure 1**  Patient 1: immunofluorescence on adult rat brain. (1) Cytoplasmic labelling of cells and processes in the inner granular layer of the dentate gyrus (DG). (2) Cytoplasmic labelling in the cerebellar white matter (GL = granular layer). The serum is diluted at 1/100 and visualised with an antihuman IgG fluorescein conjugated antiseraum.

**Figure 2**  Patient 1: western blot with the serum diluted at 1/100. A = Adult human thymus; B = newborn rat thymus; C = rat liver; D = adult rat brain; E = human brain; F = newborn rat brain. A band of 66 kDa is labelled in newborn rat thymus and in rat and human brain. There is no labelling in rat liver. With human thymus, the labelling is non-specific.
treatment of the underlying tumour is not uncommon in patients with limbic encephalitis.\textsuperscript{11,12,22} This suggests that before irreversible neuron damage, functional disturbances possibly induced by epileptic discharges or other mechanisms under the dependence of the inflammatory reaction can be corrected by early treatment of the tumour.

Most patients with limbic encephalitis have oat cell carcinoma of the lung and anti-Hu antibodies.\textsuperscript{23} These antibodies recognise neuronal nuclear proteins of 35–45 kDa molecular weight, possibly involved in the development of the CNS.\textsuperscript{24,25} Tests for anti-neuronal antibodies in the two patients with limbic encephalitis and thymoma showed that they had two different types of specific antibodies. In the serum and CSF, patient 1 had a high titre of antibodies that displayed the same characteristics as the antibodies of a patient with uveitis, paraneoplastic encephalomyelitis, and carcinoma previously tested in our laboratory.\textsuperscript{16} In the white matter of rat cerebellum, these antibodies react with the cytoplasm of glial cells that by their morphology and distribution are possibly oligodendrocytes.\textsuperscript{16} Also, cells are labelled in various neuronal structures of rat brain. By western blot, these antibodies recognise a 66 kDa protein mainly expressed in the developing CNS.\textsuperscript{14} The antibodies of patient 2 gave a different pattern. The titres were low in the serum and absent in the CSF and they diffusely labelled the nuclei of cells having the morphology of neurons. The molecular weight of the labelled proteins showed that they were not anti-Hu antibodies.\textsuperscript{24} In both patients, immunofluorescence with human brain was negative probably because the fixation did not preserve the antigens in the post-mortem time. The reactivity with similar proteins in western blots of rat and human brain indicates that the antigens are present in both species.

The decrease in antibody titres after treatment of the thymomas strongly supports the hypothesis that the immunological perturbations were triggered by the tumours. By analogy with myasthenia gravis, one can speculate that in the particular immunological environment of thymoma, an antigenic similarity between brain and thymus may lead to the production of antineuronal antibodies and in some cases to an autoimmune attack misdirected against the CNS.\textsuperscript{20,26,27} Thus the antibodies of the two patients with limbic encephalitis reacted with proteins of the same apparent molecular weight in human and rat brain, and in newborn rat thymus. The absence of these proteins in an adult human thymus suggests that they are mainly expressed during thymus maturation. As we could not obtain tumour fragments, we do not know whether these antigens were also present in the thymomas.

In summary, our studies provide additional support for the association of thymoma and CNS disorders. They also support the hypothesis that brain lesions depend on immunological perturbations involving neural and thymic antigens and are triggered by the tumour. As different types of antibodies were detected, however, and as only one patient had CSF antibodies, these immunological perturbations may have different relevances.

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The well known tetrad of supranuclear ophthalmoplegia, axial rigidity, pseudobulbar palsy, and subcortical dementia is the hallmark of progressive supranuclear palsy (PSP), a disease of late middle life, terminating in five to 10 years. Dr Steele's recent observations may be of interest.

"Progressive supranuclear palsy is the name Dr J Clifford Richardson chose to designate an unusual clinical syndrome he first identified in the 1950s. Neurofibrillary degeneration is the hallmark of this fatal brain disease, and during our study of Richardson's patients, Professor Jerzy Olszewski and I also observed granulovacuolar degeneration, and widespread nerve cell loss and gliosis in subcortical and brain stem nuclei. The histopathological features bear a striking resemblance to those seen in postencephalitic parkinsonism after von Economo's epidemic encephalitis, and in the parkinsonism-dementia complex of Guam (PDC).

During the past 30 years, neurologists confirm that progressive supranuclear palsy is a universal, sporadic and not uncommon neurodegeneration of middle and late life. Many fine studies...have advanced our understanding of PSP but its cause, and thereby its cure, is still to be revealed. These historical notes tell of our observations from 1955 to 1975. We are pleased that colleagues remember these early descriptions and honor us by calling this disease, the Steele-Richardson-Olszewski (SRO) syndrome."
Limbic encephalitis and immunological perturbations in two patients with thymoma.

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