(1)各種ホルモンの測定：急性期に異常を検出しなかった。(2)ウイルス学的検討：単純ヘルペスウイルス，水痘・帯状疱疹ウイルス，サイトメガロウイルス，ウイルス分離など検索し得た範囲で全て陰性。(3)脱髄のマーカー：経時的な髄液にて全検体で陰性。(4)髄液中サイトカインの動態：IL-6は全検体で軽度高値。IL-1・・IL-2・IFN-・は全検体陰性。IL-10は軽度高値の1例以外陰性。TNF-・は軽度高値の2例以外陰性。(5)髄液中抗GluR抗体の検出：測定した7例中6例にて抗GluR・2抗体(IgM 3例，IgG 5例)を、7例中5例で抗GluR・2抗体（IgM 4例，IgG 4例)を検出した。(6) 抗カリウムチャネル抗体の測定：1例の血清・髄液にて陰性。(7) 血中・髄液中のカテコールアミン・セロトニン・GABA濃度の測定：2例の血清・髄液にて正常範囲であった。

D.考察

今回の研究結果と従来の報告例を考え合わせると、本症の病態は、不特定なウイルス感染を契機に惹起された宿主免疫の賦活化による抗原提示により、抗GluR抗体などの自己抗体が産生され、これがGluRなどを活性化させ、その結果として高度の痙攣や遷延経過を呈し、2次性に広範な機能的脳障害を呈していると推定した。さらに軽度ながら、T細胞の活性化からIL-6などの炎症性サイトカインが誘導され、細胞障害性に機能障害をさらに助長していると推察された。

E. 結論

本症の病因・病態の検討から、髄液中IL-6の高値を認め、抗グルタメート受容体抗体を高頻度に検出した。本症の位置づけは、ウイルス感染とそれを契機とした宿主側の自己免疫的な機序が関与し、さらに炎症性サイトカインによる機能障害も加わった、非ヘルペス性広範性脳炎・脳症であると考えた。

F.健康危険情報

なし。

G.研究発表

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H.知的財産権の出願・登録状況
該当事項無し。

Ⅲ　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Ⅳ　研究成果の刊行物・別刷

Cytotoxic T cells in paraneoplastic limbic encephalitis

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Abstract

Paraneoplastic neurologic syndrome (PNS) is one of cause of limbic encephalitis. Patients with PNS show antibodies in their sera and cerebrospinal fluid reacting with malignant tumor and neurons, such as anti-Hu, anti-Ma (Ta), anti-CRMP-5 (CV2), anti-VGKC, or anti-amphiphysin antibody, however, there is no evidence showing that antibodies in PNS can cause neuronal loss even ex vivo. We found the same HLA B7 supertype among seven Japanese patients with PNS and anti-Hu antibodies. We examined cytotoxic T cell (CTL) activity against five peptides derived from Hu protein using reversed immunogenetics in three patients with anti-Hu antibodies and PNS, as reported in patients with anti-Yo antibodies and paraneoplastic cerebellar degeneration previously. Two peptides reacted and one peptide weakly reacted with CTL in patients with sensory neuronopathy. A patient with limbic encephalitis ahowed marked CTL activity reactong with the fourth peptide. The other (5th) peptide reacted with CTL in both patients with different phenotypes. The results suggest that peptides reacting with CTL were defferent among clinical phenotypes such as limbic encephalitis and sensory neuronopathy. We also present here a trial of induction of CTL by an immunixation with peptides of Yo or Hu protein.

1. Introduction

Patients with carcinoma rarely show some neurologic symptoms and signs not caused by a metastasis, compression to nerves, infection, cerebrovascular disorder, complications of chemotherapy or radiation, nor nutritional deficits. Most of these patients show antibodies against autologous tumor and neurons of central nervous system (CNS) or peripheral nervous systme (PNS) in their sera and cerebrospinal fluids (CSF). These condition is called as

paraneoplastic neurologic syndrome (PNS), includes encephalomyelitis, paraneoplastic cerebellar degeneration (PCD), subacute sensory neuronopathy, retinal paraneoplastic syndrome, opsoclonus-myoclonus syndrome and stiff-person syndrome [1- 7]. Antibodies are examined by immunohistochemistry and immunoblotting [8]. It is believed that these antibodies are induced by the stimulation of tumor cells and then cause neuronal loss. However, most antigens reacting these antibodies are cytosolic or nuclear and the mechanism causing neuronal loss by these antibodies has not been shown except for a few exceptions. In the case of anti-receptor antibodies reacted with cell surface antigens such as anti-N-type voltage-gated calcium channels in Lambert-eaton mysthenic syndrome [9] & anti-metabotropic glutamate receptor1 in Hpdgkin's disease and cerebellar ataxia [10], small animals received antibodies of patients showed the same neurologic signs or neurophysiological findings as those in patients. Paraneoplastic neurologic syndrome in the narrowest sence is diagnosed by the presence of antibodies reacting with neurons and also autologous tumor cells, so antibodies not reacting with autologous tumor cells is excluded. Neurologic symptoms are usually occur before the detection of carcinoma. The antibody-neurologic symptoms or syndrome-malignant tumor complexes are characteristic clinical features (Table 1) [6, 11], so the antibody suggesting the origin of malignant tumor is convinient as a cancer marker. Caicinoma is found in most patients within four yeras after the onset of neurologic symptoms, but paraneoplastic neurologic syndrome is not excluded even in patients whose carcinoma is not detected four years after the onset of neurologic symptoms because carcinoma often disappear.

2. Limbic encephalitis

Patients with limbic encephalitis show short-term memory loss, seizures, or psychiatric symptoms. Several causes of limbic encephalitis were reported including; herpes simplex virus [12], human herpesvirus 6 [13], varicella-zoster virus [14],Japanese encephalitis [15], non-herpetic acute limbic encephalitis in Japan [16], paraneoplastic, associated with autoimmune disorders such as Sjogren syndrome [17] or systemic lupus erythyematosus [18] have been shown. More than 30 patients with non-herpetic acute limbic encephalitis have been reported in Japan. The clinical feature differ from other type of limbic encephalitis in the bilateral hippocampus and amygdala MRI abnormalities, no viral genomes nor antibodies for herpesviruses, the absence of tumors and antibodies in PNS, and lower levels of IL-6 and IFN-γ in the CSF than those of patients with herpes simplex encephalitis [16]. Although pathognomonic antibodies have not been shown, memory loss with Hodgkin's disease called as Ophelia syndrome, have been reported [19-21].

3. Paraneoplastic limbic encephalitis

Patients with paraneoplastic limbic encephalitis show short-term memory loss (47%), seizures (53%), acute confucional state (46%), and psychiatric abnormalities (42%) and develop over days or weeks. More than half of patients show neurological symptoms before tumor diagnosis (Table 2). Diagnostic criteria for paraneoplastic limbic encephalitis is recommended (Table 3)[22].

In paraneoplastic limbic encephalitis, several antibodies including, anti-Hu [23], anti-CV2 (CRMP-5) [24], anti-Ma2 (Ta) [25], anti-VGKC [26], or anti-amphiphysin antibody [27] is reported.

4. Paraneoplastic encephalomyelitis/sensory neuronopathy with anti-Hu antibodies (Anti-Hu syndrome)-the pathogenesis-

Animals immunized with purified recombinant HuD fusion protein showed high-titer anti-HuD antibodies but did not show any neurologic symptoms nor neuronal loss [28].

The mechanism by which antibodies cause neuronal loss, is not yet understood. Intrathecal synthesis of anti-Hu antibodies [29, 30] and antibody deposition in the nervous system [31] Neuronal destruction was shown by anti-Hu antibodies in vitro [32], that was not confirmed by another investigator [33].

The idea that antibodies reacting with cytosol (Yo protein) or nuclear protein (Hu protein) cause neuronal damage, is difficult to be acceptable because antibodies can not enter neuronal cell body except for a special conditions [34, 35]. We and others showed that neurons take up antibodies after intracerebral injection in guinea pig [36] or mouse [37]. So, we examined the role of T cells reacting with peptides derived from cytosol or nuclear antigens. In anti-Hu syndrome, CD8 positive T cells may be seen in close contact with sensory neurons [38, 39], marked Human leukocyte Antigen (HLA) class I molecules expression by sensory neurons [39], and detection of similar oligoclonal T cell populations in both the nervous and the neoplastic tissues [39, 40].

5. HLA analysis and reverse immunogenetics

CD4-positive T cells usually recognize a peptide that binds to HLA or major histocompatibility complex (MHC) class II molecules, and CD8-positive T cells recognize a peptide that binds to HLA or MHC class I molecules. Peptides of 8-12 amino acids (nonamers are predominant) bind to the peptide-binding groove of HLA or MHC class I molecules. The bottom of the groove of HLA or MHC class I molecules has a β–sheet structure [41]. Peptides that were bound to HLA or MHC molecules were removed and analyzed. Many kinds of peptide can bind to a HLA or MHC class I molecule but as a rule, peptides with a limited number of amino acids can bind to the specific pockets. In many HLA or MHC class I molecules, the second and ninth positions of 9mer-peptides are the dominant anchor residues. Allele specificity has been shown in the interaction between peptide and HLA, or the MHC molecules [42]. Peptides reacting with CD4- or CD8-positive T cells may be due to the allele specificity of HLA or MHC class II or I molecule, respectively. The method called reverse immunogenetics [43] was used in the development of vaccines.

We found a peptide from the Yo protein with the HLA-A24-specific peptide-binding motifs (xYxxxxxxL) (Yo-1: AYRARALEL; amino acid positions 231-239 of the Yo protein) reacting CTL in patients with anti-Yo antibodies and PCD [44-46]. Next, we found peptides reacting CTL in patients with polymyositis and HLA A24 by the same strategy [47]. We examined HLA in patients with anti-Hu syndrome. The same HLA class I molecule was not detected but another same molecule was found. Nine HLA class I supertypes, namely A1, A2, A3, A24, B7 (B*0702-05, B*1508, B*3501-03, B*51, B*5301, B*5401, B*5501-02, B*5601-02, B*6701, B*7801), B27, B4, B58, B62 supertype, were classified according their peptide motifs [48, 49]. All of the seven Japanese patients with anti-Hu syndrome showed B7 supertype (B51, B54 or B56) [50].

6. Cytotoxic T cells in anti-Hu syndrome

We showed CD8-positive CTL activity against autologous fibroblast injected recombinant HuD fusion protein [51]. We examined CTL activity against five peptides derived from Hu protein with the HLA-B7 supertype-specific peptide-binding motifs [52] (Table 4). No CTL activity was detected in three patients with small cell lung carcinoma and HLA B7 supertype but neither anti-Hu antibodies nor neurological symptoms. CTL activity was examined by 51Cr release method for 4 hours after 4 days stimulation with 100 μg/ml peptide. Target antigens were synthetic Hu peptides such as Hu-1 (GPFGAVNNV; HuD protein amino acid positions 318-326), Hu-2 (PPSACSPRF; 265-273), Hu-3 (DPKDAEKAI: 96-104), Hu-4 (KPSGATEPI; 198-206) and Hu-5 (SPRFSPITI; 270-278). CD8 positive T cells as an effector cells were purified by two cycles of a Magnetic Cell Sorting (MACS) system that used CD8

microbeads and colums (Miltenyi Biotec, Germany) as reported previously [46]. After two cycles, CD8 rich fraction increased to 98.8%. Patient 1 and 2 had sensory neuronopathy, and patient 3 had limbic encephalitis. Although the numer of patients is small, we would like to emphasized that peptides reacting with CTL may be different among patients with clinical phenotype such as sensory neuronopathy and limbic encephalitis. Hu-1 may be the target in paraneoplastic limbic encephalitis and Hu-3 and 4 may be in sensory neuronopathy, respectively (unpublished data).

Recent study showed that no CTL activity was detected in patients with anti-Yo antibodies and PCD by interferon-γ (IFN-γ) release method [53], however, each assay to examine CTL is not the same value of CTL activity. Some responding CD8 positive T cells degranulate and cause cytolysis but do not produce IFN-γ [54] and many MHC class I tetramer positive cells do not produce detectable levels of IFN-γ after direct ex vovi stimulation with cognate peptide [55]. To examine CTL activity, cytolytic activity should be important. We used ^{51}Cr release from target cells to examine CTL activity. We would like to indicate another important subject whether HLA-A*0201 molecule and its cognate peptides are good for CTL assay in caucasian patients with PNS. HLA-A*0201 is expressed in about 50% of the caucasian popoulation, however, caucasian investigators did not show which HLA molecule is common in patients with PNS to examine CTL activity. We examined HLA class I in Japanese patients and found the same HLA supertype (B27 supertype including A24 in anti-Yo antibodies in patients with PCD [46, 56], and B7 supertype in anti-Hu syndrome [49, 50]) and could use each cognate peptides for an examination of CTL activity.

6. Immunization

We tried to induce CD8 positive CTL in mice bearing the same peptide motifs as humans with HLA A24 but adequet CTL activity was not elicited in mice by Yo-1 and bone marrow derived dendritic cells (data not shown). Lipopeptide (XSKKKKAYRARALEL) was used as antigen because a lipid-tailed peptide induce high levels of the major histocompatibility complex (MHC) class I-restricted CTL with one or three subcutaneous immunizations [57]. Briefly, peptide was dissolved in sterile PBS before use. 10μg/100μl lipopeptide was immunized female BALB/c mice subcutaneously without adjuvant twice at interval of 10days. The spleen cells (SPC) was obatained 10days after the second injection. $4x10^6$ SPC and $1x10^6$irradiated SPC are incubated with 100μg/ml Yo-1 in 2 ml α-modification of Eagle's medium (α−MEM) including 10% fetal calf serum (FCS), 2mM glutamine, 50μM 2-mercaptoethanol, 10mM Hepes (pH 7.2), and streptomycin and penicilline in 24 well multiplate. Two day later, 10u/ml IL-2 added to wells and CTL was estimated from the amount of ^{51}C release for 4 hours incubation by P815 as a target cell pulsed with 100μg/ml Yo-1 . CD8 positive T cells were purified by two cycles of a MACS system (Miltenyi Biotec, Germany) [52]. CTL activity was 4.4% at 5:1 as E/T ratio (unpublished data).

For Hu specific CTL induction, bone marrow derived dendritic cells pulsed with Hu-3 or Hu-4 was given in BALB/c mice by subcutaneous injection twice. The lymph node cells were collected 10days after the second immunization and incubated with 100μg/ml Hu-3 or Hu-4 in α-MEM including 10% FCS and added IL-2 at the 2nd day and 4th day after the incubation. CTL was examined by ^{51}C release method using CD8 positive T cells purified by MACS system twice as an effector cells and fibroblasts as a target [45]. CTL activity of CD8 positive T cells was 2.9% against Hu-3 at 17:1 as E/T ratio, and 4.4% against Hu-4 at 13:1 (unpublished data). CD8 positive T cells of non-immunized mice showed 0% as CTL activity against Hu-3 or Hu-4 peptide. Immunized mice did noy show any neurologic signs nor pathological findings. New methods to induce more potent CTL activity are needed.

After mice immunized by DNA vaccination with HuD were challenged by s.c. implantation of a neuroblastoma cell line, tumor growth inhibition and CD3 positive lymphocytic infiltrates

with a higher CD8+:CD4+ ratio is shown, however, none of the animals developed neurological deficits or neuropathological evidence [58] CD8 positive T cells can enter the CNS via blood brain barrier [59] and CTL may play some roles in PNS. The immunological background of petients with PNS may be different from that of naive mice immunized to induce CTL, as discussed below.

7. Conclusion

We detected CTL precursor in the peripheral blood of patients with paraneoplastic neurologic syndrome and anti-Yo or anti-Hu antibodies even after a short stimulation (4 or 5 days incubation) in vitro. CTL can be induced even in healthy donors for 3 weeks incubation by twicely stimulation with a peptide [60]. Although all SCLCs express HuD, anti-Hu antibodies are identified in only 16% of patients with SCLC, usually at low titers, and are associated with indolent tumor growth [61]. Therefore, the immunological background should be specific in patients with PNS. We found activated macrophages in patients with anti-Yo antibodies and paraneoplastic cerebellar degenaration [62]. We should examine what is different of immunoregularion between patients with SCLC alone and those with SCLC and PNS. We suggest that peptides reacting with CTL were defferent among clinical phenotypes of anti-Hu syndrome. Recently we found several peptides of Yo protein reacting with CTL (data not shown), although there was not association between peptide and clinical phenotype in patients with anti-Yo antibodies and PCD.

We found that the anti-Yo or anti-Hu antibody on mouse-brain-derived neurons in a primary culture system and found that these antibodies did not kill neurons, but induced the expression of cell adhesion molecules and accelerated neuronal differentiation [63]. More studies are needed to clear the role of anti-neuronal antibodies in PNS.

We do not produce animal models by immunization with recombinant Yo nor HuD protein or peptides reacting with CTL. We do not know which peptides can cause neuronal loss. We do not know whether peptides showing high CTL activity can cause neuronal loss. Yo and HuD are potent immunostimulator and antibodies can be produced easily after nuclear or cytosol protein exposed by cell apoptosis. The mechanism of CTL induction is different from that of antibody production and it is not necessary that CTL react with the peptide from the same protein reacting with autoantinodies. However, peptides presented here at least react with CTL in patients' blood and important candidates for target of CTL. We do not know the role of antibodies in the neuronal loss but we have to establish a therapy to suppress CTL activity.

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