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The effect of B-cell depletion in the Theiler's model of multiple sclerosis

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ABSTRACT

B cell depletion (BCD) is being considered as a treatment for multiple sclerosis (MS), but there are many uncertainties surrounding the use of this therapy, such as its potential effect in individuals with concurrent viral infections. We sought to discover what effect BCD, induced by an anti-CD20 monoclonal antibody, would have on Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD). Mice were injected with the anti-CD20 monoclonal antibody 5D2, 14 days before or 14 days after infection with TMEV. Efficacy of depletion of B cells was assessed by flow cytometry of $CD19^+$ cells. Mouse disability was measured by Rotarod, viral load was measured by real time PCR for TMEV RNA. Binding and neutralizing antibody levels were determined in sera and CSF by ELISA, and in CNS by real time PCR for IgG RNA. Inflammation, microglial activation, axonal damage and demyelination were assessed using immunohistochemistry.

5D2-induced BCD was confirmed by demonstration of nearly absent $CD19^+$ cells in the blood and lymphoid tissue. Systemic and CNS antibody responses were suppressed during 5D2 treatment. Higher viral loads were detected in 5D2-treated mice than in controls, and the viral levels correlated negatively with IgG production in the brain. Overall, 5D2 caused worsening of the early encephalitis and faster progression of disability, as well as exacerbation of the pathology of TMEV-IDD at the end stage of the disease. These data indicate that BCD in humans might worsen CNS viral infections and might not improve disability accrual in MS.

1. Introduction

Multiple sclerosis (MS) is an idiopathic neuroinflammatory disease of the central nervous system (CNS) thought to be due to a viral or autoimmune etiology. Based to a great extent on experience gained in the EAE (experimental autoimmune encephalomyelitis) model [1], emphasis on the immunology of the disease has been predominantly on the role of T cells rather than B cells. Over the past few years, there have been two major changes in basic research into MS. First, the importance of viral models of demyelination has been increasingly recognized [2,3]. Second, B cells have increasingly moved into the spotlight in MS research [4–6]. In both MS and its animal models, B cells and plasma cells are commonly found in active lesions [7,8] and antibodies have been identified in areas of demyelination [9,10]. In addition, B cell depletion (BCD) using anti-CD20 monoclonal antibodies [11] has resulted in decreased attacks in MS patients [12].

The purpose of this investigation was to elucidate the consequences of anti-CD20 therapy in a mouse viral model of MS. The Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease (TMEV-IDD) model is characterized by progressive weakness and robust systemic and CNS antiviral antibody and cellular responses [13,14], as well as a pathology that closely mimics that of MS [15,16]. We used an anti-mouse CD20 monoclonal antibody, 5D2, to evaluate the effect of BCD in TMEV-IDD. These data represent the first study of BCD in viral models of MS.

2. Methods

2.1. Mice, virus growth, viral quantitation, intracerebral inoculation

All animal work utilized protocols reviewed and approved by the University Animal Care and Use Committee at UMDNJ-New Jersey Medical School. SJL mice purchased from Harlan Laboratories (Indianapolis,

Abbreviations: BCD, B cell depletion; MS, multiple sclerosis; TMEV, Theiler's murine encephalomyelitis virus; TMEV-IDD, Theiler's murine encephalomyelitis virus-induced demyelinating disease; CD, cluster of differentiation; RT-PCR, reverse transcription polymerase chain reaction; RNA, ribonucleic acid; CSF, cerebrospinal fluid; ELISA, Enzyme-Linked ImmunoSorbent Assay; CNS, central nervous system; IgG, immunoglobulin G; EAE, experimental autoimmune encephalomyelitis; HHV-6A, human herpes virus 6A; EBV, Epstein Barr virus; VZV, Varicella Zoster Virus; HERVs, human endogenous retroviruses; p.i, post infection; MNCs, mononuclear cells; BAbs, binding antibodies; Nab, neutralizing antibodies; mRNA, messenger ribonucleic acid; CPE, cytopathic effect; FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyanate; SCH, spinal cord homogenate; BCA, bicinchoninic acid assay; PBS, Phosphate buffered saline; IBA-1, Ionized calcium binding adaptor molecule 1; APP, amyloid precursor protein; SEM, standard error of mean.

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IN) were 4–8-week old females, and were housed in isolator cages in the Research Resource Facility at New Jersey Medical School. Mice were inoculated intracerebrally (i.c.) with 3×10^8 plaque forming units (PFU) of TMEV, BeAn strain in a final volume of 30 μL of PBS. The virus used was the BeAn strain of TMEV, obtained originally from Steven Miller (Northwestern University), and passaged in a hamster fibroblast line, BHK, as previously described [17]. PFU were determined by a cytopathic effect (CPE) assay.

Mice were necropsied at every 10 days post infection (p.i.) for Expt. (−14) until day 90 p.i. For Expt. (+14), biweekly necropsy was performed until day 150 p.i. Techniques used in were performed as previously described [18,19], including anesthesia, i.c. injections, perfusion with PBS, CSF collection by cisternal tap, and the collection of blood and other tissues.

2.2. Anti-CD20 treatment

The monoclonal anti-mouse CD20 antibody 5D2 was obtained through a Material Transfer Agreement between Genentech and UMDNJ. Pilot experiments were performed to determine optimal dosing for prolonged depletion of peripheral blood B cells, which was found to be 10 mg/kg intraperitoneally (i.p.) twice a month. In Expt. (−14), treatment was initiated with 5D2, 15 mice, or control IgG, 15 mice, 14 days prior to infection with TMEV. Expt. (+14) was identical except that treatment was begun at 14 days p.i.

2.3. Enumeration of mononuclear cells (MNCs) in the CNS, total IgG and anti-TMEV binding antibody, and neutralizing antibody (NAb) assay

MNCs from CNS tissues were harvested by Percoll density gradient centrifugation (70%/30%) as described previously [18,19]. Total viable cells were then enumerated.

Capture ELISA was used to quantitate total IgG in both CSF and serum. In brief, donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as coating antibody, and horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson) was used as detecting antibody. A purified murine IgG (Jackson ImmunoResearch Laboratories) was serially diluted as a standard positive control on each plate.

TMEV-specific BAb level was determined as previously described [20]. Each sample was assigned an arbitrary unit of anti-TMEV binding activity according to a standard curve derived from a known positive control in the anti-TMEV ELISA.

TMEV-specific neutralizing antibody (TMEV-NAb) was measured using the modified cytopathic effect assay (CPE) [13]. In brief, 1500 PFU of TMEV was incubated with serial 3-fold dilutions of mouse serum and CSF for 2 h, followed by incubation with BHK cells for 48 h in a 96-well microplate format. Live BHK cells were then exposed to naphthol blue-black dye, and absorbance read on an ELISA reader at 620 nm. Neutralizing titers were expressed as the inverse of that dilution of serum able to block 90% of the cytopathic effect of the virus; i.e. to lower the cytopathic effect of 1500 PFU of virus to 150 PFU of virus under conditions in which the change from 1500 PFU to 150 PFU is in the linear part of the curve of the cytopathic effect dilution curve.

2.4. Real time reverse transcription polymerase chain reaction (RT-PCR) for in situ IgG production and TMEV viral load

Expression of IgG and TMEV RNA was tested as previously described with specific primers and probes for murine IgG1 and TMEV [19,21]. Total RNA was isolated from fresh, homogenized tissue samples using TRIzol® RNA Isolation Reagents (Life Technologies, Grand Island, NY); total RNA (50 ng/uL) was then reverse transcribed using random hexamer primers with the qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). cDNA was finally used as a template for the real time RT-PCR analysis based on the 5′ nuclease assay.

Transcriptional expression was normalized using the housekeeping gene glyceraldehyde-phosphate-dehydrogenase (GAPDH) as reference. The IgG mRNA level was expressed as the relative expression index of two to the power of $-\Delta Ct$, where $-\Delta Ct = -(Ct_{IgG} - Ct_{GAPDH})$. Differently, to determine the viral load in spinal cords, a standard curve was first obtained by plotting the Ct values of the samples against known TMEV concentration; copy number was determined by regression analysis from the standard curve.

2.5. Rotarod testing for progressive disability

Progressive disability in mice was assessed as previously described [18]. Rotarod data were expressed as a neurological function index (NFI). The NFI value at any time point was the mean of the last 3 time indices divided by the mean time indices from day 15 to day 45 after infection. Time indices were the time on the Rotarod for that day divided by the mean of the 2 maximum times for that mouse.

2.6. B-cell enumeration

FACS analysis for CD19-positive B cells was used to determine adequacy of depletion of B cells, since CD19 and CD20 expression are similar [22]. MNCs from the various tissues or blood underwent FACS after labeling with a FITC-conjugated donkey anti-mouse CD19 antibody (Jackson Immunoresearch, West Grove, PA).

2.7. Histology, immunohistochemistry and demyelination (Luxol fast blue)-method of Klüver–Barrera staining

After perfusion of mice with PBS, spinal cords were removed by dissection, and immediately placed in 4% paraformaldehyde at 4 °C. Tissue was processed to paraffin wax and cut in a microtome, 10 μm in the axial plane. The spatial extent of the entire spinal cord was assayed to assess for local effects.

For immunohistochemical analysis, the tissue was de-waxed in xylene and rehydrated through alcohols. Primary antibodies against markers for microglial activation (IBA-1, Ab5076), T cell (CD3, Ab5076) and axonal damage (amyloid precursor protein—APP) were obtained from Abcam (Cambridge, MA, USA). Biotinylated secondary antibodies and avidin–biotin complex were from Vector Laboratories (Peterborough, UK). Avidin–horseradish peroxidase was obtained from DAKO (Cambridge, UK). Immunocytochemistry was carried out by the avidin–biotin-complex method with minor modifications depending on the antibody used [23].

For Luxol fast blue staining (Klüver and Barrera method), tissue sections were incubated with 0.1% Luxol fast blue (Sigma, St. Louis, MO, USA) at 56 °C overnight. Then, the slides were soaked in 0.05% lithium carbonate solution, distilled water, and 70% ethanol. Finally, the slides were dehydrated in absolute ethanol, cleared in xylene, and mounted. Lesion severity was evaluated by an examiner who was blinded to the experimental conditions [24].

2.8. Statistical analysis

All data are shown as mean ± SEM. The nonparametric Mann–Whitney U-test and Kruskal–Wallis tests were used for the statistical analysis, which was performed using GraphPad Prism version 6.00 for Mac (GraphPad Software, San Diego, California, USA). All reported p values are based on two-tailed statistical tests, with a significance level of 0.05.