QUANTITATION, PHENOTYPIC CHARACTERIZATION AND IN SITU LOCALIZATION OF LYMPHOID CELLS IN THE BRAIN PARENCHYMA OF RATS WITH DIFFERING SUSCEPTIBILITY TO CORONAVIRUS JHM-INDUCED ENCEPHALOMYELITIS

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INTRODUCTION

Intracerebral infection of rats with Coronavirus JHM may cause multiple neurological syndromes ranging from acute lethal encephalitis to subclinical demyelination[1,2]. The outcome of the infection is determined by both the type of virus used for inoculation as well as the genetic background of the host animal[1,2,3,4]. Data accumulated from different inbred strains of rats suggest that important host factors which influence the course of the disease include the maturation of viral target cells[5] and the state of immunocompetence of the animal[6,7]. Although virus-specific immunity in the cerebrospinal fluid of affected animals has been examined repeatedly in the past[8,9,10], little information has been collected about the dynamics of immune reactions taking place in the brain parenchyma. However, these local immunological events are almost certainly decisive for the clinical course of the infection. Therefore, we attempted to analyse the kinetics of the lymphoid cell infiltration following intracerebral inoculation of two rat strains with coronvirus JHM. In order to detect relationships between neurological symptomatology and local immune reactions in the brain tissue we selected two inbred strains which are known to behave differently after intracerebral infection. Whereas Lewis rats often develop a subacute demyelinating encephalomyelitis accompanied by severe paralytic signs of disease, Brown Norway (BN) rats reveal no signs whatsoever, although small foci of nodular demyelination can be detected in periventricular areas of the brain[2].

MATERIAL AND METHODS

Preparation of virus

The virus we have used throughout all experiments is a derivative of that we obtained from H. Wege[11]. In our laboratory it was passaged once through mouse brain followed by a passage through sac(-) cells. This virus was designated JHM (HS).

Inoculation of animals

Rats of the inbred strains, Lewis and BN (3 weeks old), were inocu-

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lated intracerebrally with 1000 plaque forming units (PFU) of JHM (HS) per animal in 40 µl of cell free tissue culture supernatant. For control purposes rats were inoculated with tissue culture medium from non-infected sac(-) cells. Inoculated animals were checked daily for signs of neurological disease.

Preparation of lymphocytes from rat brain

Animals were killed and perfused with PBS. Before removing the brain, cerebrospinal fluid was sampled from the cisterna magna. The brain and the spinal cord were minced through a steel sieve. The dissociated material was collected by low speed centrifugation and subjected to enzymatic digestion with collagenase and DNAse. Subsequently leukocytes were separated according to their density by a Percoll-step gradient.

Identification of lymphocyte subsets

Leukocytes isolated from the brain were stained by direct and indirect immunofluorescence and analysed by flow cyto-fluorgraphy (FACS). Monoclonal antibodies (mabs) used for staining were OX1 (specific for rat leukocyte common antigen [L-CA]), OX33 (specific for a high molecular weight L-CA present on B-cells), OX8 (specific for the CD8 molecule on cytotoxic T-cells), W3/25 (specific for the CD4 molecule on helper T-cells) and R73 (specific for the alpha/beta chains of the T-cell antigen receptor [TCR]). OX1, OX33, OX8 and W3/25 are commercially available antibodies, R73 was a gift from T. Hünig (Max-Planck Institut, Martinsried, FRG).

Determination of antibody secreting cells

JHM-specific antibody secreting cells were determined by enzyme-linked immunospot (ELISPOT) assay as described by Sedgwick and Holt[12]. Leukocytes isolated from brain and resuspended in RPMI medium were plated on recangular plastic wells which had been coated with JHM virus. During an overnight incubation, antibodies secreted by JHM-specific plasma cells attached to the viral antigen coated to the bottom of the plate. These antibodies were detected by enzyme-labeled secondary antibodies and incubation in a substrate which is converted by the enzyme to a visible coloured spot. Each spot was counted as a single antibody secreting cell.

Titration of JHM specific antibodies

Heat-inactivated CSF samples were diluted in MEM and aliquots of 50 µl were incubated with 100 $TCID_{50}$ of JHM virus for one hour in a microtiter plate. Subsequently DBT cells were added to the wells and after 4 days the cultures were examined for cytopathogenic effects (CPE) after staining the cell monolayer with May-Grünwald stain. The neutralization titer was considered as the highest dilution of CSF protecting more than 50% of the cell monolayer from CPE.

In situ localization of lymphocyte subsets and viral antigens

Serial frozen sections (7-8 µm thick) were cut from the cerebellum of an infected Lewis rat 23 days post infection (dpi). Each section was stained with a different mab to detect viral antigens as well as lymphocyte subsets in situ. The following antigens were stained in subsequent sections: (1) viral nucleocapsid (mab #556 [gift of H. Wege from this institute]), (2) cytotoxic (CD8+) T-cells (mab OX8), (3) helper (CD4+) T-cells (mab W3/25), (4) B-cells (mab HIS 14 [gift of F. G. M.

Kroese, University of Groningen, The Netherlands]) and (5) macrophages (mab ED1 [gift of Ch. D. Dijkstra, Vrije Universiteit Amsterdam, The Netherlands]). Alkaline phosphatase labeled secondary antibody was used to localize primary mabs and incubation of the sections with fast red substrate resulted in a bright red precipitate. Digital pictures of the stained sections were taken by a video camera mounted on a microscope. With the aid of a computer program the red color of the precipitates could be changed in the picture to any desired color. Subsequently all pictures were merged into a single picture on the computer screen, resulting in a multi-colored topographical map of the examined area in the brain.

RESULTS

Clinical course of the infection

Intracerebral inoculation of Lewis rats with JHM (HS) caused, in over 90% of the animals, a subacute demyelinating encephalomyelitis. Clinical signs of paralytic disease began at day 6 and were most severe 12 dpi. Thereafter animals started to recover and after 28 dpi no signs of an apparent neurological disease remained. In contrast, none of the BN rats inoculated with the same amount of the identical virus developed visible neurological symptoms.

Kinetics of lymphocyte infiltration into the CNS

The dynamics of leukocyte populations in the brain parenchyma was analysed by killing one animal each day after infection up to 4 weeks pi and staining of the isolated leukocyte fraction with different mabs. FACS analysis of the cells confirmed that, in average more than 50 % carried the rat L-CA. Examination of B-lymphocytes within this leukocyte population (staining with either mab OX33 or polyclonal anti-rat IgG) revealed the following pattern: In both rat strains there was a sharp increase of B cells shortly before the onset of clinical symptoms in Lewis rats, followed by a drop in the B-cell count and a second smaller peak at the time when Lewis rats recovered from disease. CD4+ T-cells detected by the mab W3/25 were abundant in the brain of both rat strains. In Lewis rats their numbers peaked two times, first, just prior to the onset of overt disease at the time when B-cells reached their first maximum and a second time when these rats recovered. The course of the CD4+ T-cell infiltration in BN rats paralleled the influx of these cells into the brain of Lewis rats, but their number was slightly lower than in the latter strain. Striking differences between the rat strains were detectable in the numbers of CD8+ T-cells isolated from the CNS. FACS analysis after double labeling the cells with mabs OX8 and R73 revealed much higher numbers of cytotoxic T cells (CD8+, TCR α/β+) in the Lewis rats compared to BN rats. It is interesting to note that, in both rat strains an initial increase of these cells in the brain was noticeable. However, in BN rats, numbers dropped rapidly in the following weeks whereas in Lewis rats a second peak appeared at the same time that B-cells and CD4+ T-cells peaked and animals were reconvalescent.

Characterization of the humoral immune response in the CNS

Using the ELISPOT assay we determined the number of JHM-specific antibody secreting plasma cells present in the CNS at different times after infection. Almost no antibody secreting plasma cells could be detected in the Lewis rats at the time of the initial peak of B-lymphocytes. However, during recovery from disease the number of JHM-specific antibody secretors reached its maximum. This was seen exactly at the

time when numbers of B- and helper T-cells reached their second peak. BN rats showed more and earlier antibody secreting cells than the Lewis rat, although during the first increase of B-lymphocytes in the brain their numbers were almost as low as in Lewis rats. Since the amount (or possibly the affinity) of antibody which was secreted by the individual plasma cell was higher in BN rats than in Lewis, it was very likely that this would be reflected in the titer of virus-specific antibodies in the cerebrospinal fluid of individual animals. Using a microneutralization assay we determined the JHM-specific neutralization titers in CSF specimens. Animals from both rat strains revealed very low neutralizing titers at 6 dpi. In Lewis rats the titers increased slowly up to 3 weeks pi and dropped thereafter to almost non-detectable levels. The highest titers were detected shortly after the number of JHM-specific antibody secreting plasma cells was at a maximum in the CNS. At this time the animals had recovered almost completely from neurological disease signs. In contrast, neutralizing titers in BN rats increased quickly within the second week and reached much higher levels than in the Lewis rats. Maximal titers were seen roughly 2 days after JHM-specific plasma cells reached their maximum in the parenchyma. Subsequently a drop was noticeable but the titers remained significantly higher than in Lewis rats.

Arrangement of infiltrating cells in a demyelinated area of the CNS.

To establish a picture of the interaction of infiltrating cells with virus-infected cells at the site of demyelination we stained viral antigens and infiltrating leukocytes by immunohistochemistry in serial sections of a frozen cerebellum taken from a Lewis rat 23 dpi. Computer-aided image analysis of individual sections enabled the development of a multi-colored, single picture showing the topographical distribution of leukocytes and viral antigens within a demyelinated plaque close to the brain stem. CD8+ T-cells were detected in close proximity to viral antigen at the border of the demyelinated area. Macrophages occupied the virus-free center and CD4+ T-cells as well as a few B-cells were scattered in the suroundings of this plaque in the presence of viral antigen. All leukocyte populations could be identified in the perivascular cuff of an adjacent blood vessel. Double immunofluorescence studies in the same part of the brain revealed that oligodendrocytes were the major target of the virus and infected cells expressed major histocompatibility (MHC) class I antigens at high density.

DISCUSSION

In this paper we have attempted to summarize our present knowledge about the immunological events taking place in the CNS of Lewis- and BN rats after intracerebral infection with coronavirus JHM. In agreement with earlier reports this infection caused a subacute encephalomyelitis accompanied by transient paralysis in Lewis rats, whereas BN rats remained clinically healthy[2]. However, we also noticed distinct differences to data published previously. The number of Lewis rats which recovered from paralytic disease increased from 30%[2] to over 90% in our rats. Consequently the incidence of fatal encephalitides was lower than 10% in our animals, compared to 60%[2] as has been reported earlier. Moreover, no variation in the incubation time of the virus was evident. All of the paralytic Lewis rats revealed first signs of neurological symptoms between 4 to 6 dpi, whereas the onset of the classical subacute demyelinating encephalomyelitis has been reported to vary between 14 days and 8 months[13,14]. These differences most likely reflect the high degree of genetic variance of JHM virus, which makes comparison of data between different laboratories difficult, unless the same batch of virus is used. In recent years it has become clear, that multiple passages of

JHM virus through tissue culture results in a considerable loss of neuropathogenicity. Therefore, we prepared large amounts of a virus batch which was passaged only once through tissue culture after replication in the brain of suckling mice and used this virus throughout all experiments. This might explain the high reproducibilty of disease induction in our Lewis rats, which was a prerequisite for the kinetic analysis of the immunological events in the brain of these animals.

In the CNS of Lewis- as well as BN rats the numbers of B-lymphocytes revealed 2 maxima. The first peak was observed 1 or 2 days before onset of neurological symptoms in Lewis rats and the second peak shortly before Lewis rats started to recover from their disease. Previous examinations in this laboratory have shown that the permeability of the the blood brain barrier (BBB) is increased in diseased Lewis rats[9]. This could facilitate the transfer of leukocytes from blood vessels into the brain parenchyma and B-Lymphocytes would contribute to this infiltration. After sensitization of those cells which carry virus-specific immunoglobulin on their surface, differentiation into antibody secreting cells (ASC) could occur in the presence of helper T-cells. Our finding, that the numbers of helper T-cells in the brain of both rat strains followed exactly the alterations seen in the B-lymphocyte subset, is supportive of this hypothesis. As a result of these events the majority of ASC should be detectable in the second peak of lymphocytes and indeed in both rat strains, the number of ASC increased from almost non detectable levels in the first peak up to several thousand in the second peak.

A few days later, titers of neutralizing antibodies reached their maxiumum in cerebrospinal fluid specimens. Although in Lewis rats, titers remained significantly lower than in BN rats, they may have contributed to the recovery from neurological symptoms, because improvement of the Lewis rats started immediately after neutralizing antibodies had reached a certain level. Consistent with this is our observation that animals which died from acute encephalitis were antibody negative. Low titers of these antibodies as seen early in infection of Lewis rats and a slow rise in the subsequent days allows distribution of infectious virus in the CNS for a considerable period of time. Therefore, Lewis rats suffer frequently from wide spread infection of the CNS including the grey matter of the spinal cord, which undoubtly contributes to the severe paralytic disease. In contrast, the early rise of high neutralizing titers as seen in BN rats, may limit the spread of infectious virus into the grey matter and thus will prevent severe neurological symptomatology. As shown by Watanabe and coworkers[2], histopathological changes in BN rats indeed are confined to very small areas in the white matter close to the ventricles. However, these animals seemed to be infected persistently, because viral antigens were demonstrated in the brain up to 60 dpi[2]. As we have described earlier, the continous expression of viral antigens in these animals allows the maintenance of a vigorous synthesis of JHM-specific antibody response with restricted heterogeneity[10]. These data are in good agreement with our present observation, that even 4 weeks pi JHM-specific antibody titers were significantly higher in BN rats than in Lewis rats.

The persistent infection of BN rats probably is caused by the low numbers of cytotoxic T-lymphocytes recruited into the brain in the course of the infection. This is in sharp contrast to the Lewis rats. After a first increase of cytotoxic T-cells their numbers reached a much higher second peak at the time when these rats recovered from their clinical disease. At this time we cannot present functional data on these cells, however, we have good reason to believe that they act by killing JHM-infected oligodendrocytes. Computer-aided image analysis of serial sections of Lewis brain during convalescence, detected cytotoxic T-cells

in close proximity to virus-infected cells and double immunofluorescence studies in the same area of the brain identified oligodendrocytes as the major target cell for the virus. In addition infected cells did express high levels of MHC class I antigens which is necessary for sucessful recognition of infected cells by CD8+ cytotoxic T-lymphocytes. Although these data do not directly prove a cytotoxic activity of T-cells in these animals, we believe that in convalescent Lewis rats, killing of JHM infected oligodendroglia cells is an important immunological effector mechanism. As a consequence, virus would be eliminated from the brain finally leading to recovery from the infection. However, it remains to be determined if, and to what extent, killing of infected oligodendrocytes contributes to the neurological symptomatology in these animals.

In summary, intracerebral infection of Lewis- and BN rats is a useful model to study the role of the local immune response during coronavirus-induced demyelinating encephalomyelitides. Our data suggest that both compartments of the immune system, the antibody response as well as the action of cytotoxic T cells, are required for a sucessful recovery from neurological disease. Lack of virus-specific antibodies will cause wide spread infection of the brain which may result in a fatal outcome and low numbers of cytotoxic T-cells will prevent efficient elimination of the virus from the brain leading to a persistent infection. At present, experiments are under way to functionallycharacterize cytotoxic T-cells from the brain of Lewis and BN rats.

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