FUNCTIONAL CHARACTERIZATION OF CD8⁺ LYMPHOCYTES DURING CORONAVIRUS MHV IV INDUCED ENCEPHALITIDES IN RATS

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INTRODUCTION

Intracerebral infection of rodents with the murine coronavirus JHM is a well established animal model to study the pathology of virus-induced primary demyelination of the central nervous system (CNS). Although it was assumed by Weiner¹ that cytopathogenic effects of the virus play the dominant role in this axonal loss of myelin sheaths, a growing body of evidence suggests now a significant contribution of the virus-specific immune response to the histopathological changes in the central nervous system as well as to the clinical course of the infection. In this context, in mice action of CD8⁺ T-lymphocytes appears to be a two-edged sword. On the one hand, they are necessary to clear JHM virus from infected brain tissue,^{2,3} on the other hand, in vivo depletion of this lymphoid subset reduces drastically the appearance of white matter destruction^{4,5} and adoptive transfer of either viral-specific⁶ or naïve syngeneic CD8⁺ splenocytes⁷ in immunosuppressed animals fully reconstitutes neurological disease. This suggests that cytotoxic T-lymphocytes may cause demyelination by killing of virus-infected oligodendrocytes and thereby contribute to the clinical symptomatology of the infection.

In the rat, previously published data from our laboratory⁸ support the idea that CD8⁺ T-lymphocytes might determine the clinical course of intracerebral infection by JHM virus. With respect to the inflammatory CD8⁺ response striking differences have been noticed between the disease-resistant inbred strain Brown Norway (BN) and the susceptible Lewis (LEW) strain. Whereas in LEW rats the virus challenge results in a strong biphasic infiltration of CD8⁺ T-cells into the CNS accompanied by extensive lesions of primary demyelination, in BN rats the amount of infiltrating cytotoxic T-lymphocytes is much less and

demyelination is limited to few and small foci around the ventricles^{8,9}. Pathogenic properties of CD8⁺ cells in JHM virus-infected rats is seen in a series of experiments reported by Schwender et al.⁷. Reconstitution of immunosuppressed LEW rats by purified naive CD8⁺ lymphocytes or a combination of CD4⁺ and CD8⁺ lymphocytes prior to infection results in an earlier onset and enhancement of neurological disease compared to completely reconstituted animals.

On the basis of these findings it was likely that CD8⁺ T-lymphocytes could cause neurological disease by MHC class I restricted killing of JHM virus-infected targets in the CNS of LEW rats. However, at this stage of the investigations it was not clear why infiltration of CD8⁺ T-lymphocytes in the few virus-affected areas of BN rats remained subclinical.⁸ Thus, we attempted to compare CD8⁺ T-lymphocytes from the CNS of both rat strains for their capability to kill JHM virus-infected targets *in vitro*.

MATERIALS AND METHODS

Viruses. For intracerebral inoculation of rats strain JHM of the mouse hepatitis virus (MHV) was propagated and purified according to Wege et al.¹⁰. Target cells used in cytotoxic assays were infected with JHM virus that had been passaged multiple times through the appropriate target cell line.

Animals. Three-week-old rats of the inbred strains Lewis (LEW, RT-1^b) and Brown Norway (BN, RT-1^a) were purchased from the "Zentralinstitut für Versuchstierzucht" (Hannover, FRG). After intracerebral inoculation with 100 µl of virus containing 10³ plaque forming units the neurological signs of each animal were scored daily on an arbitrary scale as described elsewhere.⁸

Isolation of lymphocytes. At the time points indicated animals were killed and extensively perfused with PBS. Brain and spinal cord were removed and lymphocytes isolated as previously described¹¹.

Lymphocytes from the cervical lymph nodes (CLN) were prepared using standard methods. To remove CD4⁺ T-cells, B-lymphocytes and natural killer- (NK-) cells immunomagnetic procedures were applied according to the manufacturer's recommendations. Briefly, lymphocytes were incubated with primary monoclonal antibodies (mabs) with specificity for the lymphocyte subset in question (mab W3/25 specific for CD4⁺ T-cells, mab OX 33 specific for B-lymphocytes and mab 3.2.3¹² specific for NK-cells). The mabs W3/25 and OX 33 are commercially available (Serotec, Wiesbaden, FRG) and the mab 3.2.3 was a kind gift from T. Hünig, University of Würzburg, FRG. After incubation cells were washed and resuspended in Hank's solution containing goat-anti-mouse antibodies linked to magnetic beads (DRG Instruments, Marburg, FRG). Subsequently, the cell

suspension containing tubes were placed onto a magnetic plate (Du Pont, Dreieich, FRG). Non-labeled CD8⁺ lymphocytes did not adhere to the magnetic plate and thus could be collected from the supernatant by centrifugation. Subsequently, they were resuspended in RPMI supplemented with 10% FCS and T-cell growth factors (TCGF) and maintained in culture for 5 to 6 days before assaying their cytotoxic capacity.

Cell lines. The cell lines LE 1B4 (RT-1A^l) and BN 3B2 (RT-1Aⁿ) were used as targets in cytotoxic assays. They were obtained after fusing of primary rat splenocytes with the mouse myeloma cell line SP2/0-Ag14 ¹³. Hybridomas were grown in RPMI supplemented with 10% FCS.

Cytotoxicity assay. After infection of the hybridoma cell lines LE 1B4 and BN 3B2 with JHM virus at a multiplicity of infection (moi) of 10-15 for 2h at 37°C cells were washed once with RPMI. Subsequently, infected as well as uninfected cells were irradiated, labeled with ⁵¹Cr (Du Pont, Dreieich, FRG), washed twice with RPMI/10% FCS and resuspended with effector cells at various proportions in RPMI/10% FCS. After 5h incubation at 37°C, 100 µl of cell free supernatant was removed and released radioactivity was determined in a gamma counter (LKB Wallac, Turku, Finland). Percent specific lysis was calculated according to the formula:
$$\frac{(\text{cpm in experiment} - \text{cpm spontaneous release}) \times 100}{(\text{cpm in 2\% Triton X 100} - \text{cpm spontaneous release})}$$

Spontaneous release never exceeded 25% of the maximum release.

RESULTS AND DISCUSSION

In a first attempt to unravel possible differences between LEW and BN rats with respect to the killing capacity of their CD8⁺ lymphocytes we isolated leukocytes from the CNS of infected animals 14 days past infection (dpi). At this time, LEW rats usually reveal a strong paralytic disease whereas BN rats show no overt signs of the infection. As demonstrated in figure 1 the *ex vivo* killing capacity was higher in BN rats compared to LEW rats. Of note is the fact that due to the higher proportion of CD8⁺ cells in leukocytes from LEW rats, a higher CD8⁺/target ratio is effective in this rat strain compared to BN rats. This suggests, that CD8⁺ cells from the CNS of LEW rats are less active or exhibit lower virus-specificity compared to BN rats. However, the overall specific killing of 10% as seen in CD8⁺ cells from BN rats was surprisingly low compared to other *ex vivo* killing assays in mice ¹⁴ Therefore we assumed that either the proportion of JHM virus-specific MHC class I restricted cells in the CD8⁺ population was low or that only few of them were in a state of activation that allowed killing. Our attempts to expand activated CD8⁺ cells from the brain by cultivation in TCGF failed completely. Possibly, this can be explained by data from Imrich et al.,¹⁵ who showed that T-lymphocytes isolated from the CNS at any time past infection neither did proliferate *ex vivo* nor could be stimulated to proliferation by mitogens or viral antigens.

Since CLN were identified in our laboratory as a site of T-cell sensitization to JHM virus and strong virus-specific proliferation¹⁵, we isolated CLN lymphocytes from intracerebrally infected rats at day 7 past infection. CD8⁺ T-lymphocytes were purified from the isolated lymphocyte fraction up to 98% (data not shown) and cultured in TCGF containing medium. Six days later these cells were assayed for their cytotoxic capacity. As seen in figure 2 specificity of CD8⁺ cell mediated killing is comparable in both rat strains. However, the overall cytolytic activity is considerable higher in CD8⁺ cells from BN rats. To date, we cannot unequivocally explain the strong unspecific killing of uninfected target cells in our assays. It is

Figure 1. Cytotoxic capacity of brain-derived CD8⁺ lymphocytes 14 dpi. White (LEW) and black (BN) bars represent cytotoxicity against syngeneic, virus-infected targets, hatched bars against uninfected, MHC I-matched cells. Lymphocytes from brain and spinal cord of 3 LEW and BN rats, respectively, were pooled and measured for their cytolytic potential *ex vivo*.

clear that this activity is not due to NK-cells, because they were removed prior to the *in vitro* culture of isolated lymphocytes and flow cytometric analysis of cultered CD8⁺ cells excluded the outgrow of NK-cells during in vitro expansion. To minimize the problem of non-specific killing experiments are in progress to expand exclusively JHM-specific CD8⁺ cells in isolated leukocytes.

In summary, these preliminary results indicate that after inoculation of rats with the neurotropic murine coronavirus JHM the recruited CD8⁺ T-lymphocytes are able to kill virus-infected targets. BN rats show not only a greater virus-specific cytotoxicity but also a higher overall cytolytic capacity compared to LEW rats. This suggests that generation and

Figure 2. Cytolytic activity of purified CD8⁺ T-lymphocytes isolated from the cervical lymph nodes of JHM-infected rats 7 dpi. Cytotoxicity was assayed against syngeneic, JHM-infected (white and black bars) or uninfected (hatched bars) targets. Results represent the mean value of 8 LEW and BN rats, respectively.

activation of cytotoxic T-lymphocytes is more effective in BN rats. These findings are in line with observations that BN rats recruit more rapidly and effectively virus-specific antibody secreting cells into the brain parenchyma¹¹ as well as the fact that sensitization of CLN T-lymphocytes to JHM virus occurs earlier past infection and is more vigorous in BN rats compared to LEW rats¹⁵. Therefore, we tend to believe that BN rats are able to limit viral spread in the CNS very early past infection due to a very rapid afferent limb of the JHM virus-specific immune response. Under these circumstances cytotoxic action of CD8⁺ cells in the few and small periventricular sites of virus infection remains subclinical.

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