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Borna Disease Virus Nucleoprotein (p40) Is a Major Target for CD8$^+$-T-Cell-Mediated Immune Response

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Experimental infection of rats with Borna disease virus (BDV) and natural BDV infection of horses and sheep leads to a virus-induced T-cell-mediated immunopathology in the central nervous system. Earlier work revealed the importance of the BDV-specific T-cell response and of CD8$^+$ effector cells in particular in the destruction of virus-infected cells. Evidence was also presented that this major histocompatibility complex class I-restricted lysis detected in vitro might play a functional role in the immunopathogenesis of Borna disease. The present study employed different vaccinia virus recombinants expressing single BDV-specific proteins to investigate the specificity of the cytolytic CD8$^+$-T-cell response, revealing a major epitope on the BDV nucleoprotein p40. In contrast, no direct evidence in favor of the presence of in vivo relevant cytotoxic T-lymphocyte epitopes on other BDV-specific proteins was found.

Borna disease (BD) is a persistent viral infection of the central nervous system caused by the single-negative-strand, nonsegmented RNA Borna disease virus (BDV) (17). BDV has a wide host range in naturally and experimentally infected animals and, most probably, also infects humans (11, 13, 22), in whom it might even be detectable for long periods in blood (20a). BDV exists of six open reading frames encoding the nucleoprotein (p40), the phosphoprotein (p24), the glycosylated matrix protein (p16→gp18), and the glycoprotein (p57→gp84 or gp94) (4, 9, 10, 15, 24); furthermore, the BDV antigenome encodes a protein with a molecular mass of approximately 180 kDa, which represents the putative L-polymerase (6, 16, 23) and the most recently identified unglycosylated p10 (26, 30).

For experimental investigations, the rat is the most widely used animal model. After intracerebral infection, the animals develop an encephalomyelitis in which the infiltrating cells could be characterized as CD4$^+$ and CD8$^+$ T cells and macrophages (1, 7). BDV-specific CD8$^+$ T cells represent the effector cell population in the acute phase of the disease and significantly contribute to the destruction of virus-infected brain cells in vivo. Moreover, evidence was presented that this T-cell population also participates in the degenerative encephalopathy that results in severe cortical brain atrophy in the chronic phase of the disease (2, 19, 20, 27, 28). In addition to the immunopathological reaction caused by BDV-specific CD8$^+$ T cells, the same T-cell population was shown to have beneficial effects in BD. BDV-specific CD4$^+$ T cells given prior to infection induce CD8$^+$ T cells which eliminate the virus without causing significant cell damage (18).

The p40 and p24 proteins appear to be the most abundant proteins produced during BDV infection. As early as 6 to 10 days after intracerebral infection of rats, p40 is detectable in the brain by immunohistochemistry (5, 28a). Around day 15 postinfection (p.i.), p24 can be detected. Thus, the p40 protein appears to be the protein expressed at the highest levels at early times p.i. in the brain, followed by p24. Likewise, experimentally and naturally infected animals first develop detectable antibodies directed against p40 and p24 at 2 to 3 weeks p.i., whereas antibodies directed against the two glycoproteins are not detectable before 10 to 14 weeks after infection (12). In contrast to the humoral immune response to BDV, little is known about the specificity of the cellular immune response against different BDV-specific proteins. Whereas CD4$^+$ T cells have been induced for the p24 and p40 proteins after immunization with purified protein (18, 20, 21), the antigen specificity of cytolytic CD8$^+$ T cells has never been investigated.

To determine which virus-specific antigens are recognized by cytotoxic T lymphocytes (CTL), we made use of the following different vaccinia virus (VV)-BDV recombinants, each expressing only one BDV-specific protein: VV-BDV p40 (VV-p40), VV-BDV p24 (VV-p24), VV-BDV gp18 (VV-gp18), and VV-BDV gp94 (VV-gp94). Wild-type VV, strain WR, was used as a control. The recombinant viruses were generated by J. C. de la Torre, Scripps Research Institute, San Diego, Calif., and virus stocks were propagated by infection of BHK-21 cells at a low multiplicity of infection and virus titers were determined on BHK-21 cells in our laboratory. Trypsin virus stocks contained about 1×10^9 to 5×10^9 PFU/ml. The VV-BDV recombinants were used for intravenous (i.v.) infection of Lewis rats to generate a cellular immune response. The distinct VV-BDV recombinant viruses were also used for infection of syngeneic astrocyte target cells (F10 cells). In addition to spleen cells from rats infected with VV-BDV recombinants, as a second source of effector cells, lymphocytes were isolated from the brain of acutely BDV-infected rats at 19 days p.i. to represent the in vivo situation (19, 27). In all experiments, persistently BDV-infected F10 cells were used as target cells in cytotoxicity assays as described previously (19).

Earlier experiments by Zinkernagel et al. (31) had indicated that a potent cellular immune response can be induced and found in the local lymph nodes after intra-footpad infection of Lewis rats with wild-type VV. The immune response was dominated by major histocompatibility complex (MHC) class I-restricted CD8$^+$ T cells starting 3 days after infection, peaking at 5 to 7 days, and disappearing after 10 to 12 days (31).

To generate large amounts of virus-specific T cells, female Lewis rats were infected i.v. and immune cells were isolated

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TABLE 1. Induction of cytotoxic T-cell responses to BDV-specific proteins by VV-BDV recombinants

Expt[d]	Recombinant virus used for infection[a]	% Specific lysis[b] of F10 target cells infected with:		
		VV	BDV[e]	NL[c]
I	VV-p24	65, 60, 33, 14	11, 16, 10, 5	5, 1, 1, 2
	VV-p40	84, 74, 30, 13	**41, 21, 13, 9**	0, 1, 2, 1
	VV-gp18	50, 44, 24, 7	14, 13, 10, 1	2, 1, 0, 0
	VV-gp94	46, 48, 28, 8	**29, 13, 11, 3**	0, 1, 0, 0
II	VV-p24	49, 32, 19, 8	3, 7, 0, 0	3, 8, 4, 2
	VV-p40	57, 35, 20, 9	**26, 19, 9, 3**	0, 2, 1, 0
	VV-gp18	53, 34, 16, 10	5, 12, 7, 11	1, 9, 5, 1
	VV-gp94	51, 63, 41, 16	**32, 26, 16, 6**	0, 1, 0, 0
III	VV-p24	45, 24, 9, 5	18, 9, 3, 0	12, 8, 3, 0
	VV-p40	62, 29, 14, 3	**35, 21, 9, 3**	7, 9, 2, 2
	VV-gp18	47, 20, 5, 2	0, 3, 2, 0	1, 2, 1, 2
	VV-gp94	40, 25, 7, 0	**24, 14, 4, 4**	11, 7, 3, 1

[a] Six- to eight-week-old female Lewis rats were infected i.v. with 10^7 PFU of VV-BDV recombinants and killed 6 days later. Values of specific lysis are mean values.
[b] Results shown for effector/target cell ratios of 30:1, 10:1, 3:1, and 1:1, respectively.
[c] NL, noninfected F10 cells.
[d] Experiments were performed independently. Two animals were infected with the respective VV-BDV recombinant in all experiments.
[e] Boldface values indicate VV-BDV recombinant-specific lysis of BDV-infected F10 target cells.

from the spleen. To evaluate the peak of the cellular immune response after i.v. VV infection, different infectious doses of VV-BDV recombinant virus or VV trypsin stocks ranging from 10^4 to 10^7 PFU were injected. Six days after infection, the animals were killed, spleens were extracted, and a single spleen cell suspension was prepared. To determine the in vitro cytotoxicity of $CD8^+$ T cells, spleen cells were coincubated with ^{51}chromium-labeled (100 μCi of $^{51}Cr/5 \times 10^6$ cells) persistently BDV-infected syngeneic F10 cells, VV wild-type-infected F10 cells (multiplicity of infection, 5), and uninfected F10 cells. At the end of the incubation period, 50-μl aliquots of supernatant were taken and counted in a gamma counter. Percent specific lysis was calculated by the formula [(test release − minimal release)/(maximal release − minimal release)] × 100. Rats receiving an infectious virus dose of 10^7 or 10^6 PFU were able to generate a cellular immune response against VV, whereas lower infectious doses were less efficient. Furthermore, we tested the peak activity of the T-cell-mediated immune response after i.v. infection with VV at infectious doses of 10^7 or 10^6 PFU. Lewis rats were killed 6 or 8 days after infection, and spleen cells were tested for their cytotoxic activity. The $CD8^+$-T-cell response to VV or BDV infection was drastically decreased on day 8 p.i. in comparison to that on day 6 after infection, independent of the infectious dose (data not shown).

Based on these findings, in all of the succeeding experiments, described below, effector spleen cells were harvested on day 6 from rats infected with 10^7 PFU of VV or VV-BDV recombinants. Antibodies directed against the BDV-specific proteins were detectable after day 15 by Western blot analysis (data not shown).

We then investigated whether VV-BDV recombinants were able to induce cytotoxic T-cell responses directed against BDV-specific proteins. Therefore, Lewis rats were infected with either VV-p24, VV-p40, VV-gp18, VV-gp84/94, or wild-type VV strain WR. As shown in Table 1, effector cells generated by VV-p40 regularly caused lysis of BDV-infected target cells, whereas VV-p24 and VV-gp18 did not trigger cytotoxicity; however, effector cells induced in rats after VV-gp94 infection always had cytotoxic capacity, in one experiment (Table 1, experiment II) causing even higher specific lysis than that of VV-p40 effectors. VV wild-type-infected target cells were always lysed by spleen cells from all infected rats, while no lysis was found when non-infected F10 cells were used as targets.

Since brain lymphocytes represent the relevant effector cell population (19, 27) in the acute phase of the disease (day 19 p.i.), these cells were isolated and tested for their lytic activity on VV-BDV recombinant virus-infected F10 cells. As shown in Table 2, this effector cell population from BDV-infected rats was able to lyse BDV-infected F10 cells and VV-p40-infected F10 cells but not VV-p24-, VV-gp18-, or VV-gp94-infected F10 cells, at an effector/target cell ratio of 10:1 (Table 2, experiments I and II). To further investigate whether higher numbers of effector cells would cause lysis of VV-p24-, VV-gp18-, or VV-gp94-infected F10 cells, we increased the effector/target cell ratio to 20:1 (Table 2, experiment III). To show that all target cells were infected with the different VV recombinants, spleen cells from VV wild type strain WR-infected rats were used as controls. These effectors lysed target

TABLE 2. Lytic activity of effector cells on VV-BDV recombinant virus-infected F10 cells

Effector cells[a]	% Specific lysis of F10 target cells infected with:						
	BDV	VV	NL[b]	VV-p24	VV-p40[e]	VV-gp18	VV-gp84/94
Expt I[c]							
BDV	80, 66, 25	0, 1, 0	0, 2, 0	6, 0, 0	**76, 42, 9**	4, 1, 0	5, 0, 0
VV	3, 2, 0	58, 38, 24	4, 1, 0	62, 33, 12	68, 31, 28	66, 41, 17	57, 37, 21
Expt II[c]							
BDV	69, 22, 0	0, 0, 0	3, 1, 0	3, 1, 0	**100, 68, 32**	0, 1, 0	0, 0, 0
VV	7, 6, 5	79, 43, 18	5, 9, 0	59, 36, 17	63, 37, 28	56, 31, 17	54, 30, 14
Expt III[d]							
BDV	100, 70, 32, 17	0, 2, 1, 0	2, 2, 1, 0	3, 2, 3, 0	**81, 60, 25, 8**	2, 0, 0, 0	13, 4, 0, 0
VV	2, 7, 5, 2	52, 34, 13, 3	5, 5, 4, 0	46, 38, 12, 4	53, 44, 16, 7	56, 50, 23, 9	49, 42, 17, 0

[a] Effector cells were isolated from the brain of BDV-infected Lewis rats 19 days p.i. and cultivated overnight (BDV), or female Lewis rats were infected i.v. with 10^7 PFU of VV and spleen cells were taken 6 days p.i. (VV).
[b] NL, noninfected F10 cells.
[c] Results are shown for effector/target cell ratios of 10:1, 3:1, and 1:1, respectively, for BDV and 100:1, 30:1, and 10:1, respectively, for VV.
[d] Results are shown for effector/target cell ratios of 20:1, 7:1, 2:1, and 0.7:1, respectively, for BDV and 100:1, 30:1, 10:1, and 3:1, respectively, for VV.
[e] Boldface values indicate BDV-specific lysis of F10 target cells infected with VV-BDV recombinants.

cells infected with wild-type strain WR, VV-p24, VV-p40, VV-gp18, and VV-gp94 (Table 2). This proves that all VV-BDV recombinant viruses were infectious and in addition to VV-specific antigen should also express the particular BDV-specific antigen. The latter was verified by reverse transcription-PCR using p24-, p40-, gp18-, or gp84/94-specific primer and by Western blot analyses of VV-BDV-infected target cells, which revealed the presence of BDV-specific mRNA and BDV-specific proteins (data not shown).

The results demonstrate that the nucleoprotein p40 is a major target for the MHC class I-restricted cytotoxic T-cell response in Lewis rats after BDV infection. Therefore, at least one epitope recognized by BDV-specific $CD8^+$ T cells must be located on this virus-specific protein. However, we cannot formally exclude the possibility that subdominant epitopes exist on p24, gp18, and gp84/94 of BDV. Particularly, the gp84/94 would be a candidate (Table 1); however, regarding the relevant in vivo situation as represented by brain lymphocytes, this viral protein does not appear to be a major target (Table 2). This problem of additional epitopes cannot be solved at present, since irrespective of the system used, $CD8^+$ T cells from rats have never been successfully cultured, which would be necessary to address this question. Epitopes which are subdominant in the acute immune response may play a role in the chronic viral infection as it has been demonstrated for lymphocytic choriomeningitis virus in mice (8, 25, 29). In this respect, the moderate T-cell activity found on BDV-infected F10 cells from spleen cells of rats infected with VV-p24 or VV-gp18 (Table 1) might be unspecific or might be exerted by a few antigen-specific T cells generated against these proteins. In contrast, when we used T cells isolated from the brain of BDV-infected rats, no lysis was found on target cells expressing p24 or gp18 of BDV and only marginal lysis was found on F10 cells expressing BDV gp84/94 (Table 2, experiment 3). We did not address the question of whether fragments of p24, gp18, or gp84/94 can be processed and presented to induce $CD8^+$ T effector cells or if these proteins are available in the appropriate amount, time frame, and place after infection, as described for other infectious systems (3, 14, 32). The BDV p40 protein is the first virus-specific antigen expressed early after infection and in considerable amounts. The fact that anti-p40 antibodies are the first virus-specific antibodies synthesized, around day 15 p.i. (17), and the fact that the p40-specific T-cell activity is also detectable early after infection make this virus-specific protein a major target antigen in both the humoral and cellular immune reaction in BD. No functional role for p40-specific antibodies has been recognized in BD so far, but as shown here, the cytotoxic T-cell immune reaction specific for the p40 protein might result in immunopathology or immunoprotection (2, 18–20, 27).

As reported earlier, the time point of the MHC class I-restricted cytotoxic T-cell response determines whether virus-infected cells are eliminated very early or whether the immune reaction proceeds to an immunopathological disease (18). Therefore, we propose that the MHC class I-restricted recognition of the BDV p40 protein represents the key event in BD development. Furthermore, the anti-p40 $CD8^+$-T-cell response presents itself as the prime candidate for protective cellular immunity in BD.

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REFERENCES

1. **Bilzer, T., and L. Stitz.** 1993. Brain cell lesions in Borna disease are mediated by T cells. Arch. Virol. Suppl. **7:**153–158.
2. **Bilzer, T., and L. Stitz.** 1994. Immune-mediated brain atrophy. $CD8^+$ T cells contribute to tissue destruction during borna disease. J. Immunol. **153:**818–823.
3. **Blum, J. S., C. Ma, and S. Kovats.** 1997. Antigen-presenting cells and the selection of immunodominant epitopes. Crit. Rev. Immunol. **17:**411–417.
4. **Briese, T., W. I. Lipkin, and J. C. de la Torre.** 1995. Molecular biology of Borna disease virus. Curr. Top. Microbiol. Immunol. **190:**1–16.
5. **Carbone, K. M., S. A. Rubin, A. M. Sierra-Honigmann, and H. M. Lederman.** 1993. Characterization of a glial cell line persistently infected with borna disease virus (BDV): influence of neurotrophic factors on BDV protein and RNA expression. J. Virol. **67:**1453–1460.
6. **de la Torre, J. C., L. Bode, R. Durrwald, B. Cubitt, and H. Ludwig.** 1996. Sequence characterization of human Borna disease virus. Virus Res. **44:**33–44.
7. **Deschl, U., L. Stitz, S. Herzog, K. Frese, and R. Rott.** 1990. Determination of immune cells and expression of major histocompatibility complex class II antigen in encephalitic lesions of experimental Borna disease. Acta Neuropathol. (Berlin) **81:**41–50.
8. **Gallimore, A., T. Dumrese, H. Hengartner, R. M. Zinkernagel, and H. G. Rammensee.** 1998. Protective immunity does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides. J. Exp. Med. **187:**1647–1657.
9. **Gonzalez-Dunia, D., B. Cubitt, and J. C. de la Torre.** 1998. Mechanism of Borna disease virus entry into cells. J. Virol. **72:**783–788.
10. **Gonzalez-Dunia, D., B. Cubitt, F. A. Grasser, and J. C. de la Torre.** 1997. Characterization of Borna disease virus p56 protein, a surface glycoprotein involved in virus entry. J. Virol. **71:**3208–3218.
11. **Gonzalez-Dunia, D., C. Sauder, and J. C. de la Torre.** 1997. Borna disease virus and the brain. Brain Res. Bull. **44:**647–664.
12. **Hatalski, C. G., S. Kliche, L. Stitz, and W. I. Lipkin.** 1995. Neutralizing antibodies in Borna disease virus-infected rats. J. Virol. **69:**741–747.
13. **Hatalski, C. G., A. J. Lewis, and W. I. Lipkin.** 1997. Borna disease. Emerg. Infect. Dis. **3:**129–135.
14. **Karrer, U., A. Althage, B. Odermatt, C. W. Roberts, S. J. Korsmeyer, S. Miyawaki, H. Hengartner, and R. M. Zinkernagel.** 1997. On the key role of secondary lymphoid organs in antiviral immune responses studied in alymphoplastic (aly/aly) and spleenless (Hox11(−)/−) mutant mice. J. Exp. Med. **185:**2157–2170.
15. **Kliche, S., T. Briese, A. H. Henschen, L. Stitz, and W. I. Lipkin.** 1994. Characterization of a Borna disease virus glycoprotein, gp18. J. Virol. **68:**6918–6923.
16. **McClure, M. A., K. J. Thibault, C. G. Hatalski, and W. I. Lipkin.** 1992. Sequence similarity between Borna disease virus p40 and a duplicated domain within the paramyxovirus and rhabdovirus polymerase proteins. J. Virol. **66:**6572–6577. (Erratum, **67:**1746, 1993.)
17. **Narayan, O., S. Herzog, K. Frese, H. Scheefers, and R. Rott.** 1983. Behavioral disease in rats caused by immunopathological responses to persistent borna virus in the brain. Science **220:**1401–1403.
18. **Nöske, K., T. Bilzer, O. Planz, and L. Stitz.** 1998. Virus-specific $CD4^+$ T cells eliminate Borna disease virus from the brain via induction of cytotoxic $CD8^+$ T cells. J. Virol. **72:**4387–4395.
19. **Planz, O., T. Bilzer, M. Sobbe, and L. Stitz.** 1993. Lysis of major histocompatibility complex class I-bearing cells in Borna disease virus-induced degenerative encephalopathy. J. Exp. Med. **178:**163–174.
20. **Planz, O., T. Bilzer, and L. Stitz.** 1995. Immunopathogenic role of T-cell subsets in Borna disease virus-induced progressive encephalitis. J. Virol. **69:**896–903.

20a. **Planz, O., C. Rentzsch, A. Batra, H.-J. Rziha, and L. Stitz.** 1998. Persistence of Borna disease virus-specifc nucleic acid in blood of psychiatric patient. Lancet **352:**623.

21. **Richt, J. A., L. Stitz, H. Wekerle, and R. Rott.** 1989. Borna disease, a progressive meningoencephalomyelitis as a model for $CD4^+$ T cell-mediated immunopathology in the brain. J. Exp. Med. **170:**1045–1050.
22. **Rott, R., and H. Becht.** 1995. Natural and experimental Borna disease in animals. Curr. Top. Microbiol. Immunol. **190:**17–30.
23. **Schneemann, A., P. A. Schneider, S. Kim, and W. I. Lipkin.** 1994. Identification of signal sequences that control transcription of Borna disease virus, a nonsegmented, negative-strand RNA virus. J. Virol. **68:**6514–6522.
24. **Schneider, P. A., C. G. Hatalski, A. J. Lewis, and W. I. Lipkin.** 1997. Biochemical and functional analysis of the Borna disease virus G protein. J. Virol. **71:**331–336.
25. **Schulz, M., P. Aichele, M. Vollenweider, F. W. Bobe, F. Cardinaux, H. Hengartner, and R. M. Zinkernagel.** 1989. Major histocompatibility complex-dependent T cell epitopes of lymphocytic choriomeningitis virus nucleoprotein and their protective capacity against viral disease. Eur. J. Immunol. **19:**1657–1667.
26. **Schwemmle, M., M. Salvatore, L. Shi, J. Richt, C. H. Lee, and W. I. Lipkin.** 1998. Interactions of the borna disease virus P, N, and X proteins and their functional implications. J. Biol. Chem. **273:**9007–9012.

27. **Sobbe, M., T. Bilzer, S. Gommel, K. Noske, O. Planz, and L. Stitz.** 1997. Induction of degenerative brain lesions after adoptive transfer of brain lymphocytes from Borna disease virus-infected rats: presence of $CD8^+$ T cells and perforin mRNA. J. Virol. **71:**2400–2407.
28. **Stitz, L., O. Planz, T. Bilzer, K. Frei, and A. Fontana.** 1991. Transforming growth factor-beta modulates T cell-mediated encephalitis caused by Borna disease virus. Pathogenic importance of $CD8^+$ cells and suppression of antibody formation. J. Immunol. **147:**3581–3586.
28a. Stitz, L., K. Nöske, O. Planz, E. Furrer, W. I. Lipkin, and T. Bilzer. 1998. A functional role of neutralizing antibodies in Borna disease: influence on virus tropism outside the central nervous system. J. Virol. **72:**8884–8892.
29. **van der Most, R. G., A. Sette, C. Oseroff, J. Alexander, K. Murali-Krishna, L. L. Lau, S. Southwood, J. Sidney, R. W. Chesnut, M. Matloubian, and R. Ahmed.** 1996. Analysis of cytotoxic T cell responses to dominant and subdominant epitopes during acute and chronic lymphocytic choriomeningitis virus infection. J. Immunol. **157:**5543–5554.
30. **Wehner, T., A. Ruppert, C. Herden, K. Frese, H. Becht, and J. A. Richt.** 1997. Detection of a novel Borna disease virus-encoded 10 kDa protein in infected cells and tissues. J. Gen. Virol. **78:**2459–2466.
31. **Zinkernagel, R. M., A. Althage, and F. C. Jensen.** 1977. Cell-mediated immune response to lymphocytic choriomeningitis and vaccinia virus in rats. J. Immunol. **119:**1242–1247.
32. **Zinkernagel, R. M., S. Ehl, P. Aichele, S. Oehen, T. Kundig, and H. Hengartner.** 1997. Antigen localisation regulates immune responses in a dose- and time-dependent fashion: a geographical view of immune reactivity. Immunol. Rev. **156:**199–209.