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Prevention of Virus Persistence and Protection against Immunopathology after Borna Disease Virus Infection of the Brain by a Novel Orf Virus Recombinant

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The *Parapoxvirus Orf virus* represents a promising candidate for novel vector vaccines due to its immune modulating properties even in nonpermissive hosts such as mouse or rat. The highly attenuated Orf virus strain D1701 was used to generate a recombinant virus (D1701-VrVp40) expressing nucleoprotein p40 of Borna disease virus, which represents a major antigen for the induction of a Borna disease virus-specific humoral and cellular immune response. Infection with Borna disease virus leads to distinct neurological symptoms mediated by the invasion of activated specific $CD8^+$ T cells into the infected brain. Usually, Borna disease virus is not cleared from the brain but rather persists in neural cells. In the present study we show for the first time that intramuscular application of the D1701-VrVp40 recombinant protected rats against Borna disease, and importantly, virus clearance from the infected brain was demonstrated in immunized animals. Even 4 and 8 months after the last immunization, all immunized animals were still protected against the disease. Initial characterization of the immune cells attracted to the infected brain areas suggested that D1701-VrVp40 mediated induction of B cells and antibody-producing plasma cells as well as T cells. These findings suggest the induction of various defense mechanisms against Borna disease virus. First studies on the role of antiviral cytokines indicated that D1701-VrVp40 immunization did not lead to an enhanced early response of gamma or alpha interferon or tumor necrosis factor alpha. Collectively, this study describes the potential of the Orf virus vector system in mediating long-lasting, protective antiviral immunity and eliminating this persistent virus infection without provoking massive neuronal damage.

The type species *Orf virus* of the genus *Parapoxvirus* of the poxvirus family has been proposed as a candidate for novel vector vaccines. Orf virus has several invaluable characteristics, including the induction of a strong immune response and the advantage of not being pathogenic in most hosts, including rats. Further important features of Orf virus are the absence of systemic virus spread, even in immunocompromised individuals or after intravenous injection of high virus doses, and the short-lived duration of Orf virus-specific immunity allowing frequent reinfections (for review, see references 3 and 21). Recently, we reported the successful use of Orf virus recombinants, derived from the highly attenuated, cell culture-adapted strain D1701-V, expressing *Pseudorabies virus* (suid herpes 1) glycoproteins for protection against lethal challenge infection of mice (15). After having demonstrated the protective capacity against a cytolytic herpesvirus infection, now we describe the utility of this vector virus for immunoprotection against an immunopathological disease and persistent infection with a noncytolytic neurotropic RNA virus, *Borna disease virus* (BDV).

BDV infection in rats represents a useful model of immunopathology in the brain. After infection of rats with this neurotropic and noncytolytic single-stranded RNA virus distinct neurological symptoms of deficiency can be observed (25, 31; for review, see reference 27). In parallel with the replication of BDV in the brain, a vigorous immune response is induced in immunocompetent animals that leads to a severe meningoencephalitis. The cellular immune response is induced in secondary lymphoid organs and the invasion of activated primed T cells into the brain causes severe local inflammation (1, 44, 49). Of unique and crucial importance are $CD8^+$ T cells, which are present at the site of degenerative brain alterations and, most importantly, have been shown to exert virus-specific cytotoxicity in vitro directed against the p40 nucleoprotein (3, 12, 33, 35–38, 42) and as recently demonstrated, also against the p10 protein (22). The nucleoprotein p40 also represents a major target antigen for the humoral immune response, and anti-p40 serum antibodies are found early after infection, whereas antiviral antibodies against the phosphoprotein p24 and glycoprotein gp94, the latter mediating protection against disease, are detected later in BDV infection (16, 48).

Interestingly, despite a strong immune response against BDV, the virus is usually not cleared from the brain but rather persists in cells of neural origin throughout the brain, namely neurons and astrocytes (7, 8, 26). However, the induction of an early potent cellular immune response, either by transferring virus-specific T cells or by infection with a high virus dose,

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leads to protection against disease, mainly due to the early initiation of virus elimination from all areas of the brain (16, 32, 34, 42, 44). To defeat BDV infection, this strategy, however, is impossible to pursue from a practical point of view, and therefore, we made use of the new Orf virus vector system for immunization against this disease of the central nervous system.

The present study demonstrates the generation of the Orf virus recombinant D1701-VrVp40 expressing the major BDV immunogen, nucleoprotein p40, and its successful use to protect rats against intracerebral BDV challenge infection. Most notably, intramuscular application of that Orf virus recombinant not only prevented the dissemination of BDV throughout the central nervous system, but moreover eliminated persistent BDV from the brain. Immunization with D1701-VrVp40 elicited a strong and long-lasting p40-specific humoral response, and animals were still protected 6 or 8 months after the last booster immunization. This is the first report demonstrating prevention of immunopathogenesis and termination of persistent BDV infection of the central nervous system by the use of a recombinant live virus in a host nonpermissive for the vector virus.

MATERIALS AND METHODS

Cells and viruses. The D1701-VrV recombinant, which is deleted in the virus-encoded VEGF-E gene and expresses the *Escherichia coli* β-galactosidase, was derived from the highly attenuated and Vero cell line-adapted Orf virus strain D1701-V as reported recently (15). All Orf viruses were propagated and plaque purified as described (10, 15). The fourth rat brain passage of BDV He/80 was used as the challenge virus (40), and focus-forming units (FFU) were determined on the guinea pig cell line CRL1405 as described (16). Brain homogenates (10% in phosphate-buffered saline) were prepared and tested for infectious BDV by titration in CRL cells with a detection limit of 1.2×10^2 FFU (32, 47).

Plasmid constructions. The construction of plasmids pdV550, pdV, pdV-Rec1, and pdV-ATG, which allow substitution of the Orf virus VEGF-E gene for foreign genes, has been reported recently (15). The BDV p40 gene sequence was amplified by reverse transcription (RT)-PCR (37) with primers p40-H (5′-CAA AAGCTTCACACGCA**ATG**CCAC-3′) and p40-E (5′-TCATGAATTCTTA GTTT AGACCAGT-3′). The resulting PCR product (1,140 bp) was cloned into plasmid pCRII-TOPO according to the manufacturer's instructions (Invitrogen Life Technol.) to obtain plasmid p40HE-1. The BstXI fragment of p40HE-1 containing the complete p40 gene was blunt-end ligated into EcoRV-cleaved pdV-ATG, which results in plasmid pTM-40 to be used for recombinant virus generation. Southern blot hybridization and DNA sequencing (10) proved the correct construction of each plasmid (data not shown).

Generation and selection of recombinant D1701-VrVp40. The strategy of generation and selection of the BDV p40-expressing Orf virus recombinant has been described recently (15). Briefly, Vero cells were infected with the VEGF-E negative, β-galactosidase-expressing virus D1701-VrV (multiplicity of infection of 0.1) and transfected 2 h later with 2 μg of pMT-40 and SuperFect transfection reagent according to the recommendations of the manufacturer (Qiagen). Plaque titration of cell lysates on Vero cells with a 1% SeaPlaque agarose overlay containing 300 μg of Bluo-Gal (Invitrogen Life Technol.) per ml allowed the isolation of white virus plaques indicative of successful exchange of the *lacZ* gene in D1701-VrV. Immunostaining of virus plaques with the BDV p40-specific monoclonal antibody 38/17C1 (50) confirmed expression of p40. After four consecutive rounds of plaque purification, virus stocks were prepared and titrated in Vero cells. Comparative restriction enzyme analysis and Southern blot hybridization of D1701-VrV and D1701-VrVp40 DNA demonstrated the correct substitution of the *lacZ* gene cassette by the p40 gene (data not shown). In addition, PCR with *lacZ* gene-specific primer pairs was applied to exclude any contamination of the different recombinant virus stocks with parental *lacZ*-positive D1701-VrV (data not shown).

Expression of p40 protein was detected by Western blotting 4 h after infection of Vero cells with D1701-VrVp40 with increasing amounts at later times postinfection (data not shown). These results demonstrated the correct expression and early synthesis of p40 due to its control by the early *vegf-e* promoter of D1701. Furthermore, after D1701-VrVp40 infection of a fibroblast cell line from Lewis rats, which is nonpermissive for Orf virus, early expression of p40 was immunohistochemically detectable in 20 to 60% of the cells depending on the multiplicity of infection used for infection (data not shown).

Western blot analysis. Total cell proteins of infected Vero cells or brain homogenates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electro-blotted onto a polyvinylidene difluoride Western blotting membrane (Roche Applied Sciences), incubated with antibodies as described (15, 47), and specific proteins were visualized by chemiluminescence (Lumi-light, Roche Applied Sciences).

Immunization of rats and BDV challenge infection. Lewis rats were immunized at an age of 4 to 5 weeks in 2-week intervals, and 2 weeks after the last immunization 5×10^3 to 10×10^3 FFU of BDV were used for intracerebral challenge infection (47). The recombinant virus stocks used for immunization were tested for correct insertion and expression of BDV p40 as described above. All animals were examined daily and weighed, and disease symptoms were scored by three independent observers. The appearance of BDV-specific neurological signs was assessed and scored for for slight incoordination and vigilance (score of 1), slight paresis and distinct ataxia (score of 2), and marked paralysis or paresis (score of 3).

Detection of serum antibodies. The BDV-specific humoral antibody response was determined by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis with brain lysates from infected and diseased rats as the antigen (16). The ELISA titers of the individual sera were determined in three or four independent assays.

Histology and immunohistochemistry. Tissues were fixed in 4% paraformaldehyde containing lysine-HCl and sodium-meta-periodate (9) and further processed as described (47). To increase the sensitivity of immunohistochemistry, avidin-biotin complex reagents (Vector Lab) were used in combination with tyramide signaling amplification according to the manufacturer's recommendations (NEN Perkin Elmer). In addition to the BDV p40-specific monoclonal antibody 38/17C1 the following rat-specific antibodies (from BD Biosciences or Serotec) were used: OX-8 ($CD8a^+$ cells), OX-38 ($CD4^+$ cells), OX-22 ($CD45RC^+$ cells), and anti-rat immunoglobulin G. To detect activated microglia or macrophages, monoclonal antibody ED1 (Camon) was used. Sections were hematoxylin stained, analyzed, and photographed with a Leica DMRBE/DC400 microscope.

In situ cytohybridization. Thin sections of deep-frozen or paraffin-embedded rat brains fixed in 4% paraformaldehyde containing lysine-HCl and sodium-meta-periodate were analyzed for the presence of BDV p40-specific mRNA by the use of digoxigenin-labeled probes (Roche Applied Sciences) as described (9, 32).

RT-PCR. Gamma interferon (IFN-γ), IFN-α, tumor necrosis factor alpha (TNF-α), and β-actin as a housekeeping gene control was detected in the different rat brains by RT-PCR (OneStep RT-PCR, Qiagen) with specific primers as reported (43). Trizol (Invitrogen Life Technol.) was used to isolate total RNA from brains stored in RNAlater (Qiagen) followed by DNase treatment (DNAfree, Ambion). PCR products were separated in ethidium bromide-stained NuSieve agarose (Biozym) composite gels.

RESULTS

Induction of BDV-specific serum antibody response after immunization with D1701-VrVp40. Nucleoprotein p40 represents one of the major antibody-inducing antigens of BDV, and anti-p40 antibodies are usually detected early after infection with BDV. Therefore, the anti-p40 serum antibody response has been evaluated by ELISA in all rats used in these experiments mainly as a read-out for the efficiency of the D1701-VrVp40-induced immune reaction. For immunization, different doses of D1701-VrVp40 were tested after either intramuscular or intravenous application. The results indicated that the immunization effect was dependent not only on the dose but also on the route of application. Rats immunized three times intramuscularly with only 10^5 PFU of the recombinant Orf virus displayed no detectable BDV-specific humoral immune response until 3 weeks after challenge infection (Fig. 1A). Only weak serum antibody titers (1:40) were found after

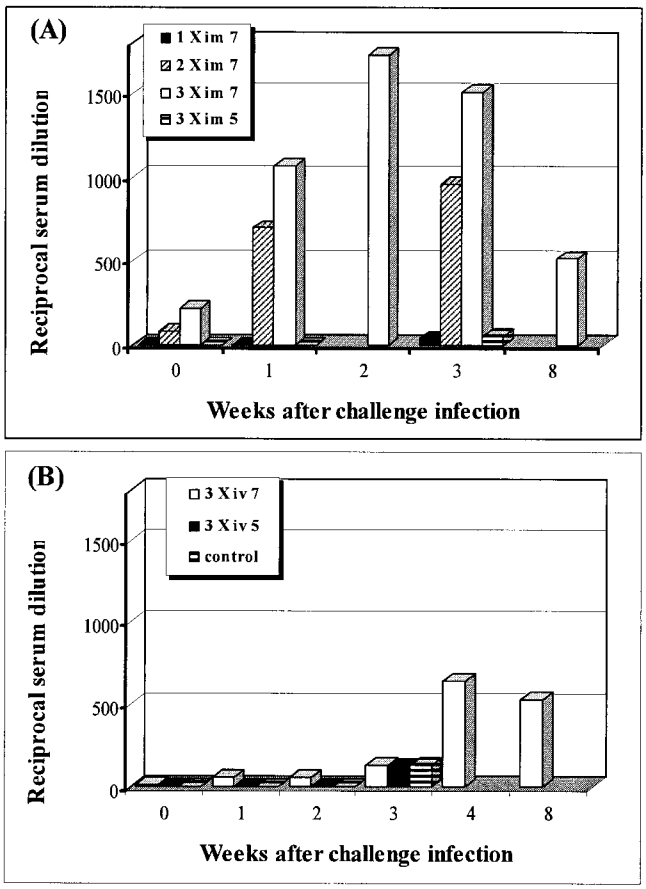

FIG. 1. BDV-specific serum antibody response in immunized rats. Sera were taken at the indicated week after challenge infection from rats that received one (1 X), two (2 X), or three (3 X) intramuscular doses of 10^7 PFU (im 7) or 10^5 PFU (im 5) of D1701-VrVp40 (A) or three intravenous doses of 10^7 PFU (3 X iv 7) or 10^5 PFU (3 X iv 5) of D1701-VrVp40 (B). Control rats were immunized three times with 10^7 PFU of the parental virus D1701-VrV (control). BDV-specific antibodies were determined by ELISA, and titers are shown as the mean reciprocal serum dilution (for details, see text).

immunization with a single dose of 10^7 PFU of D1701-VrVp40 given intramuscularly, which increased after challenge infection (Fig. 1A). Similarly, one to three intravenous immunizations were not very efficient, and low specific serum antibody titers (1:80 to 1:160) were finally detectable at 3 weeks after challenge infection (Fig. 1B).

The strongest BDV-specific humoral immune response was obtained after two or three intramuscular immunizations with 10^7 PFU of D1701-VrVp40. As can be seen in Fig. 1A, animals immunized twice (three rats tested) exhibited specific antibodies up to a serum dilution of 1:80 before challenge infection, which increased thereafter. Three consecutive intramuscular injections of 10^7 PFU of D1701-VrVp40 induced specific humoral antibodies with a mean titer of 1:220 before challenge infection (Fig. 1A, 3 X im7, 0 weeks), the titers of 26 serum samples from different immunized animals ranged between 1:40 and 1:1,280. One week after challenge infection the BDV-specific antibody response was boosted to mean serum titers of 1:700 (19 sera tested, 1:80 to 1:2,560), increasing to 1:1,500 (seven sera tested, 1:320 to 1:5,120) 3 weeks after challenge infection. Taken together, these results demonstrate that intramuscular injection of 10^7 PFU of D1701-VrVp40 led to a clearly higher specific humoral immune response before and after challenge infection compared to intravenous application.

Protection from disease after immunization with D1701-VrVp40. Rats were immunized as described above and challenge infected intracerebrally, and the protective effect was evaluated by daily monitoring the body weight and clinical score of the individual animals. Without challenge infection, the immunized rats did not show any disturbance in their health status. As controls, animals were left untreated or were control immunized with either the parental D1701-VrV (n = 3) or pseudorabies virus glycoprotein gC-expressing recombinant D1701-VrVgC (20) (n = 6). All control animals showed constant decrease of body weight starting at day 12 after BDV challenge and developed severe neurological symptoms and marked body weight loss (Fig. 2). All diseased animals became moribund within 21 days after BDV infection and had to be sacrificed.

The protective effect of immunization was dependent on the dose and the frequency of application of the recombinant vector virus. Rats immunized only once intramuscularly with 10^7 PFU or up to three times intravenously or intramuscularly with low doses (10^5 PFU) of D1701-VrVp40 suffered from Borna disease comparable to nonimmunized control rats as well as those animals, though the severity of Borna disease was clearly reduced in all animals receiving three low intramuscular doses (data not shown). A more pronounced protection was found after two intramuscular injections of 10^7 PFU of D1701-VrVp40 (n = 3). These rats showed almost normal increases in body weight and developed only moderate neurological symptoms such as incoordination and slight ataxia (data not shown).

For complete protection from Borna disease, however, three intramuscular immunizations with 10^7 PFU of D1701-VrVp40 were needed. All the rats, tested in three different independent experiments (n = 16), constantly gained weight (Fig. 2A) and developed only mild transient clinical signs (ruffled fur for approximately 5 days) lacking any neurological symptomatology after BDV challenge infection (Fig. 2B) and remained healthy over the entire observation period up to 140 days after challenge infection (data not shown). Similarly, animals receiving three intravenous injections of 10^7 PFU of D1701-VrVp40 (n = 3) showed a continuous increase in body weight after challenge infection (Fig. 2A), but in contrast to the intramuscularly immunized rats, the mild nonneurological symptoms occurred earlier and remained until 31 days postinfection, when all animals were free of any disturbances of general health status (Fig. 2B).

Next, we were interested to test for the duration of the protective immune response mediated by the threefold intramuscular application of 10^7 PFU of D1701-VrVp40. One group of immunized rats (A, n = 8) were reimmunized 4 months later and 2 weeks thereafter challenge infected intracerebrally. Rats of group B (n = 7) and group C (n = 9) were challenge infected 4 and 8 months, respectively, after the last immunization without being reimmunized. Before challenge infection, BDV-specific serum antibody titers ranged from 1:80 to 1:5,120 (mean titer, 1:647), whereby most of the sera (13 out of 24) were ELISA positive in a dilution of 1:320 to 1:640 (data not shown). The additional reimmunization of group A in-

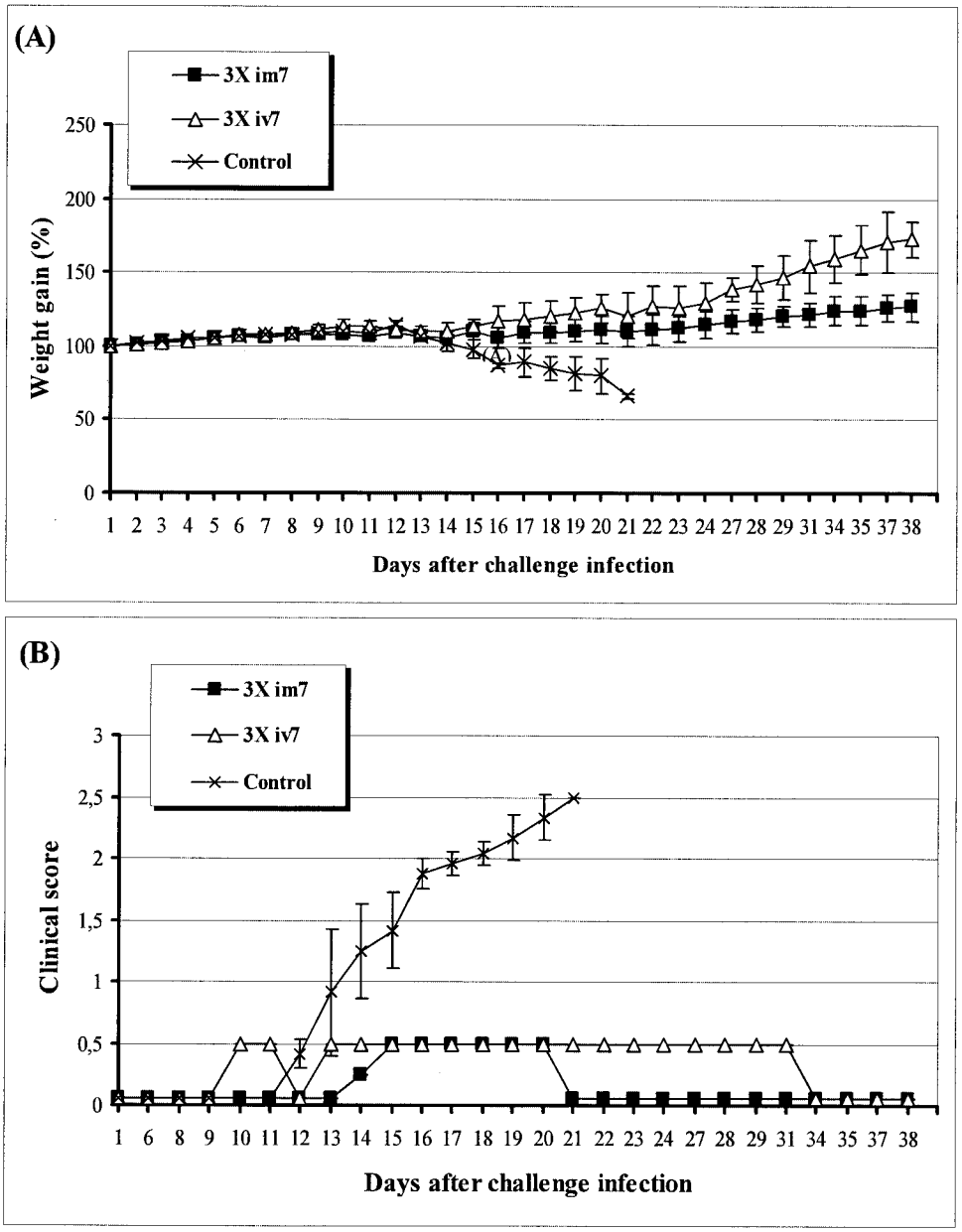

FIG. 2. Protection of rats against BDV challenge infection. Loss of body weight (A) and appearance of BDV-specific neurological symptoms (B) at the indicated days after intracerebral challenge infection of rats that received 10^5 PFU of D1701-VrVp40 intravenously three times (3 X iv 5) or intramuscularly (3 X im 5). Control animals were immunized with three intramuscular doses of parental D1701-VrV (control). Two or three intramuscular injections of 10^7 PFU of D1701-VrVp40 (2 X im 7 and 3 X im 7, respectively) protected the rats against loss of body weight and BDV-specific clinical symptoms after challenge infection. Similar protection was found after intravenous application of three doses of 10^7 PFU of D1701-VrVp40 (3 X iv 7), whereas a single intramuscular injection of 10^7 PFU of D1701-VrVp40 (1 X im 7) did not protect the animals against Borna disease. BDV-specific symptomatology was scored as 0.5 (mild transient clinical signs, ruffled fur), 1.0 (slight incoordination and vigilance), 2.0 (slight paresis and distinct ataxia), and 3.0 (marked paralysis or paresis). The standard error of mean is given by the bars.

creased the BDV-specific serum antibody titers only approximately twofold. The prechallenge sera taken either 4 months or 8 months after the last immunization displayed comparable titers. All immunized animals of the three groups were completely protected against the disease over an observation period of 18 months (groups A and B) or 14 months (group C), except for one rat of group A and one rat of group C. Both animals had to be sacrificed approximately 4 weeks after challenge infection due to loss of weight and BDV-specific symptomatology, and spread of BDV was found in their brains by immunohistochemistry (data not shown). The age-matched nonimmunized control animals ($n = 3$) displayed severe BDV-specific neurological symptoms between 22 and 25 days postinfection and had to be sacrificed (data not shown).

Detection of BDV in the central nervous system of immunized rats. To investigate whether immunization with D1701-

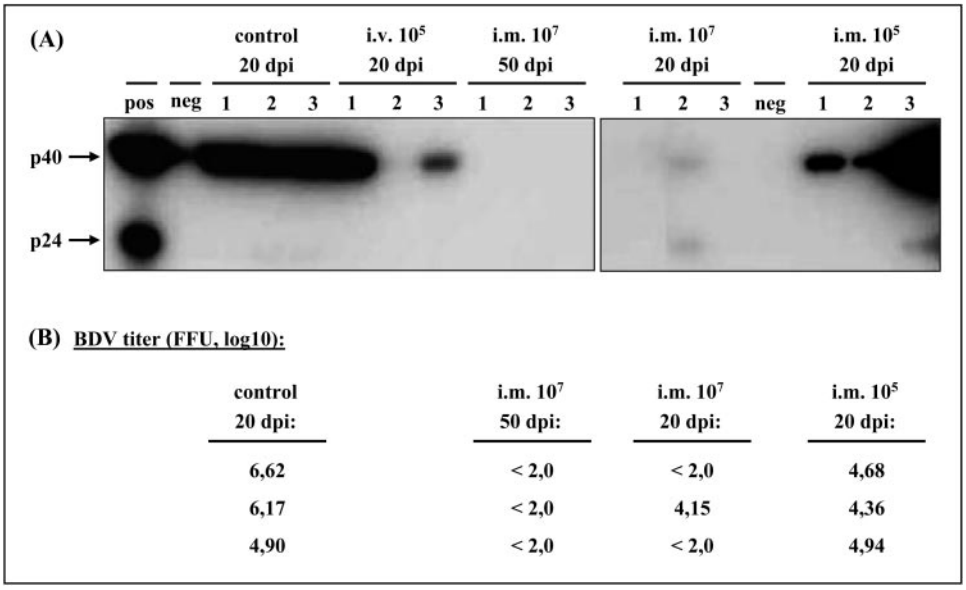

FIG. 3. Presence of BDV proteins in the brain of infected rats. (A) Brain lysates of three individual rats (1 to 3) were obtained from animals that were control immunized (control) or immunized three times intravenously or intramuscularly with 10^5 PFU or 10^7 PFU of D1701-VrVp40. In addition, BDV-positive (pos) and BDV-negative (neg) brain lysates were included, and Western blot analysis was performed with BDV p40- and p24-specific monoclonal antibodies. Detection of specific antigens was achieved by chemiluminescence. Note that the X-ray film in the right panel was exposed approximately 10 times longer than that in the left panel. (B) BDV titers were determined in duplicate 10% (wt/vol) brain homogenates and are indicated for the individual brains of the tested control- and D1701-VrVp40-immunized rats, which were also analyzed by Western blotting.

VrVp40 controlled BDV replication in the rat brain, we first tested brain homogenates for the presence of viral antigen by Western blotting. As shown in Fig. 3A, at day 20 after challenge infection (20 days postinfection) viral proteins could be detected in all individual brains of the diseased control animals. Clearly reduced amounts of BDV-specific antigens were found in the brains of rats after three intravenous or particularly after intramuscular applications of 10^5 PFU of D1701-VrVp40 (Fig. 3A, the right panel was exposed approximately 10-fold longer than the left panel). In contrast, after three intramuscular immunizations with 10^7 PFU of the Orf virus recombinant, viral antigens were undetectable with the exception of one brain lysate, which exhibited trace amounts of BDV p40 and p24 at 20 days postinfection (Fig. 3A, intramuscular 10^7 no. 2). After 50 days postinfection (three animals tested, Fig. 3A) and 140 days postinfection (six animals tested, data not shown), again no BDV proteins were found in brain lysates of any of the rats.

These findings were substantiated by titration experiments of individual brain lysates taken 20 days after challenge infection (Fig. 3B). Compared to the control animals, intramuscular immunization with 10^7 PFU led to approximately 10-fold lower titers of BDV after a single application of D1701-VrVp40, whereas two immunizations reduced the BDV load in the brain approximately 40-fold (Fig. 3B). After three immunizations, no infectious BDV could be detected, except from the brain of one rat that exhibited a 100-fold-reduced virus titer compared to that of controls (Fig. 3B). These results are in good agreement with the Western blotting results (Fig. 3A). Animals that received three low doses of D1701-VrVp40 (10^5 PFU intramuscularly) still harbored infectious virus in the brain, although approximately 40-fold reduced compared to the controls.

For a more sensitive identification of virus-infected cells, BDV-specific in situ cytohybridization was performed with brain sections. Figure 4 demonstrates representative results for rats that received three intramuscular injections of 10^7 PFU of the Orf virus recombinant before challenge infection. Virus spread into cortical areas and accumulated in the hippocampal region (Fig. 4A and B), very similar to the nonimmunized or control-immunized rats (data not shown). However, at 20 to 22 days postinfection, clearly fewer BDV-positive cells were detectable in the brains of all immunized animals (Fig. 4C) compared to the control-immunized animals (Fig. 4D). In all cases, we found that immunization reduced the presence of challenge virus in cortical regions, and BDV appeared to be restricted essentially to the hippocampus. However, even there, significantly fewer neurons were infected (Fig. 4C) compared to the hippocampus of control rats (Fig. 4D). Most notably, at later times after challenge infection either only trace amounts of or no BDV-infected cells could be found in the brain of immunized rats by in situ cytohybridization or immunohistochemistry. At 50 days postinfection, only very small numbers of BDV-positive cells were detectable in the hippocampus of all immunized rats (Fig. 4E). At 140 days postinfection, BDV was found in single neuronal cells only in the brains of two of the six immunized rats (Fig. 4G to H). Collectively, the results demonstrated that a threefold intramuscular immunization with the Orf virus recombinant D1701-VrVp40 not only mediated protection of rats against the central nervous system disease but also prevented the dissemination of BDV after

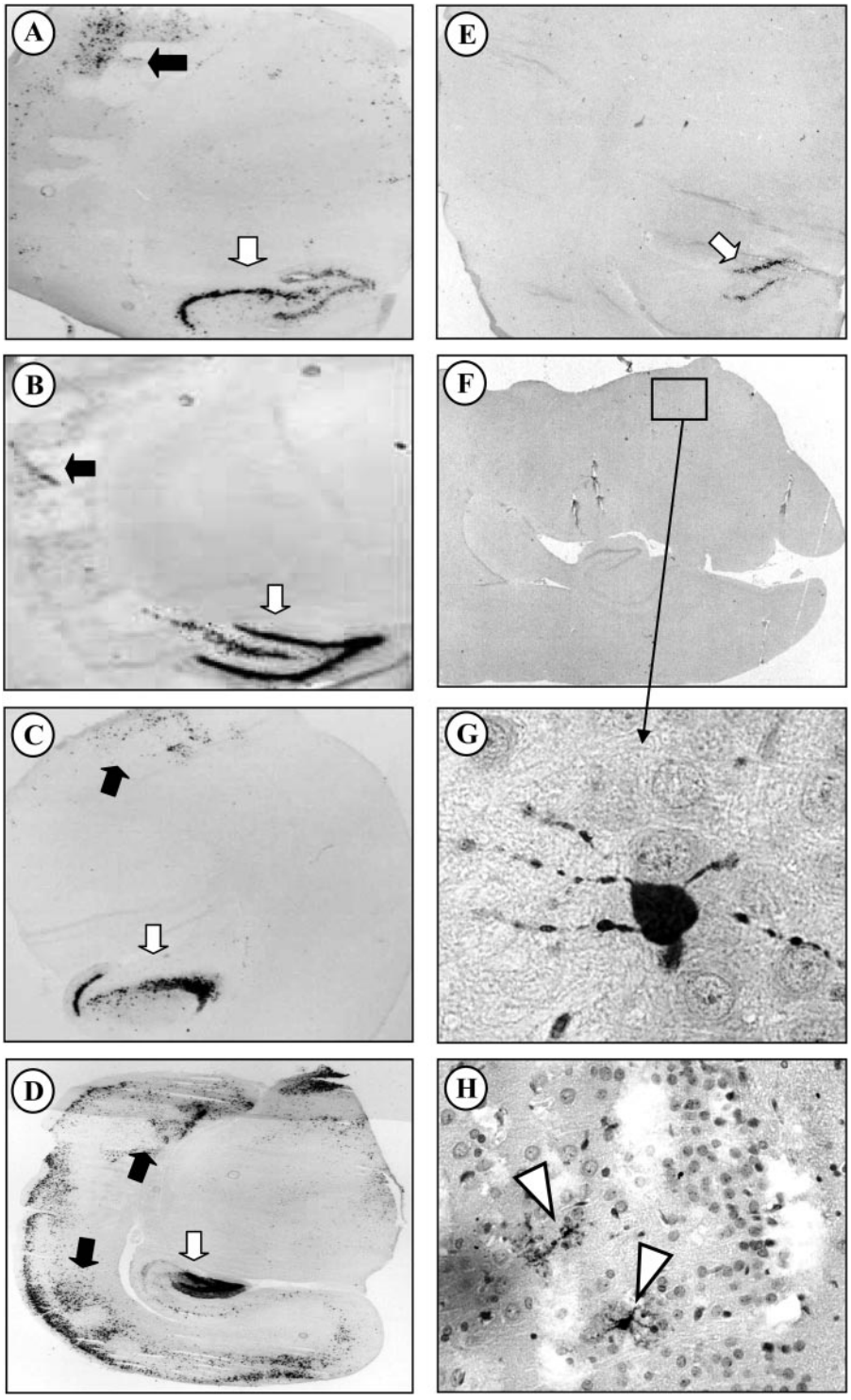

FIG. 4. Detection of BDV RNA and proteins by in situ cytohybridization and immunohistochemistry in the brain of immunized rats after challenge infection. Thin sections (4 μm) of paraffin-embedded brains were subjected to in situ cytohybridization with BDV p40-specific, digoxigenin-labeled probe (A to E) or to immunohistochemistry with the p40-specific monoclonal antibody 34/17C1 (F to H). After three intramuscular injections of 10^7 PFU of D1701-VrVp40, BDV-harboring neural cells were detected in the cortical (black arrows) and the hippocampal (white arrows) brain regions 10 days (A), 13 days (B), and 20 days (C) after challenge infection. However, compared to control-immunized rat 20 days postinfection (D), the number of BDV-positive cells was drastically reduced. Note that at 50 days postinfection, only trace amounts of BDV-positive cells were detectable in the hippocampus by in situ cytohybridization (E), and at 140 days postinfection, only in two of the immunized rats could single neurons positive for BDV antigen be detected (G and H).

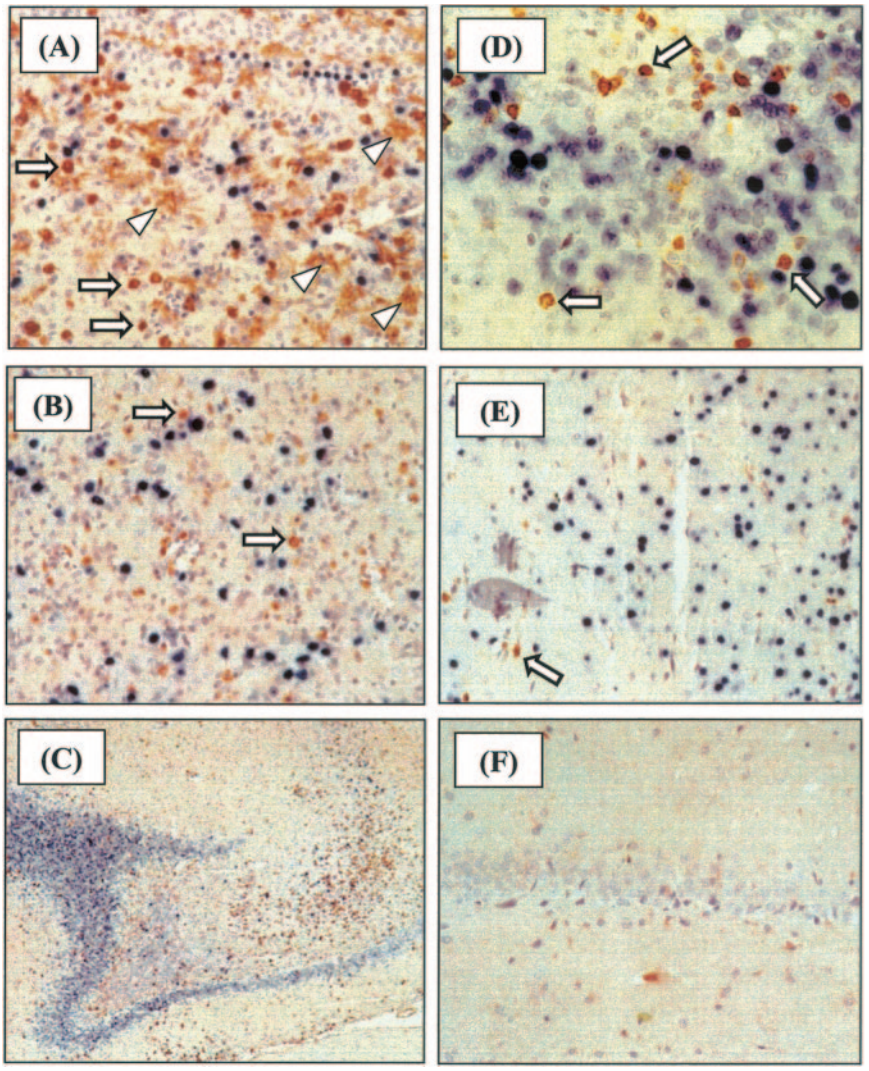

FIG. 5. Detection of lymphocytes and BDV RNA in brains 20 days postinfection by combined in situ cytohybridization and immunohistochemistry. Thin sections of brains from immunized (A to D) and control-immunized (E) rats were analyzed 20 days postinfection by in situ cytohybridization with BDV p40-specific, digoxigenin-labeled probe (dark blue-stained cells) followed by immunohistochemistry (brown-stained cells) with (A) monoclonal antibody OX-38, detecting CD4-positive lymphocytes (arrows) and cells with microglial morphology (arrowheads), and (B) monoclonal antibody OX-8, demonstrating CD8-positive lymphocytes invading BDV-infected areas. (C and D) Brain sections from immunized rats revealed massive cell infiltrations reacting with monoclonal antibody OX-22, specific for CD45RC-positive lymphocytes (arrows), in contrast to nonimmunized, challenge-infected animals (E). (F) Nonimmunized, noninfected rat brain negative for BDV RNA and virtually no CD45RC-positive cells.

intracerebral challenge infection and most importantly eliminated most if not all BDV persisting in the brain.

Cellular infiltrates in the brain of immunized rats challenged with BDV. In order to gain insight into the possible mechanisms of BDV elimination in the D1701-VrVp40-immunized rats, immunohistochemistry was performed to detect and to phenotype cellular infiltrates in the brain. Since earlier work had revealed the importance of T cells in protecting rats from encephalitis and immunopathological disease, brains were scrutinized for the presence of $CD4^+$ and $CD8^+$ T cells. Both cell types could be detected in the parenchyma of immunized animals in areas also positive for BDV nucleic acid as demonstrated by in situ cytohybridization. The cells stained either for CD4 (Fig. 5A) or for CD8a, a phenotypic marker for $CD8^+$ T cells (Fig. 5B), sharing morphological characteristics of lymphocytes with round nuclei (Fig. 5, arrows). In addition, numerous CD4-positive cells morphologically resembled microglia or macrophages (Fig. 5A, arrowheads) that could be confirmed by ED-1 staining (data not shown).

The most salient finding, however, was the demonstration of considerable numbers of perivascular and parenchymal lymphocytes reacting with monoclonal antibody OX-22 in the brains of immunized rats, which were present very near BDV-positive cells (Fig. 5C and D and Fig. 6). In contrast, in the brains of nonimmunized challenge-infected animals only very small numbers of those CD45RC-positive cells were seen (Fig. 5E). Due to this finding and the quite impressive specific antibody response in immunized rats, we also tried to detect

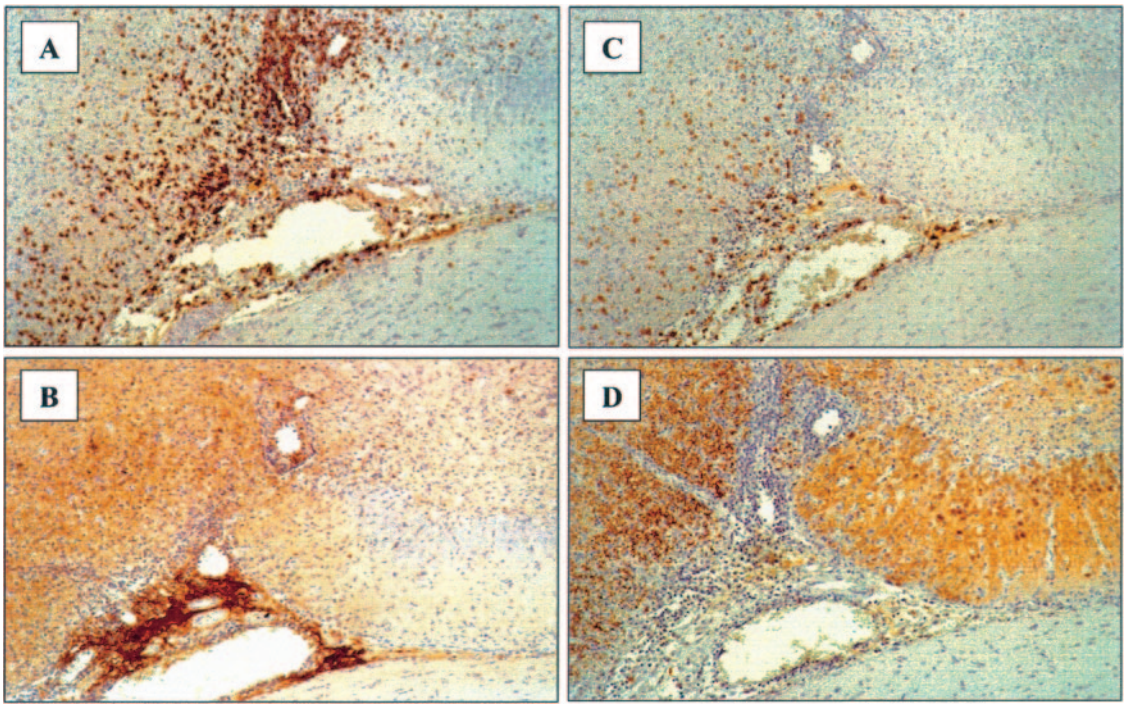

FIG. 6. Brain infiltration of CD45RC-positive lymphocytes is accompanied by a large number of immunoglobulin G-producing cells. Serial thin sections of the brain of an immunized rat 20 days after challenge infection were subjected to immunohistochemistry (brown-stained cells) to demonstrate the presence of cells positive for (A) CD45RC, (B) rat immunoglobulin G, (C) CD8, and (D) BDV p40 antigen. Note the attraction of a remarkably large number of perivascular and parenchymal CD45RC-positive lymphocytes and immunoglobulin G-producing cells to BDV-infected areas and t the presence of fewer CD8-positive lymphocytes.

immunoglobulin in the brain parenchyma. Indeed, pronounced immunoglobulin G staining could be demonstrated coinciding with the presence of numerous CD45RC-positive cells that accumulated perivascularly as well as in the adjacent parenchyma (Fig. 6A and B). Staining of serial sections also revealed the presence of $CD8^+$ T cells (Fig. 6C) and BDV p40 antigen (Fig. 6D) in these brain areas. Collectively, immunohistochemistry of the brains of immunized and nonimmunized rats did not reveal striking differences in infiltrating T cells but indicated an increased presence of CD45RC-positive, potential B cells in immunized rats (see Discussion).

IFN-γ, IFN-α, and TNF-α production. Since the antiviral immune response mediated by the Orf virus recombinant cleared BDV from the brain without gross neurological damage, we asked whether locally produced cytokines might be involved rather than cytolytic effector mechanisms. Key players in the noncytolytic control of viral infections can be IFN-γ, IFN-α, and TNF-α (for review, see reference 20). Therefore, we used total RNA isolated from individual brains of immunized and nonimmunized rats from day 5 to day 21 after challenge infection for RT-PCR. In both immunized and nonimmunized rats, IFN-γ mRNA was detectable later than 5 days postinfection, with maximal transcription around 15 days postinfection (Fig. 7A). A slightly increased transcription of IFN-γ mRNA was found in the brain of this immunized rat at day 11 after challenge infection in that particular experiment. However, additional RT-PCR assays with different RNA preparations from that brain resulted in a weaker PCR product (data not shown). A similar variability of signal strength can be seen from the RT-PCR results obtained with brain RNA isolated from three different animals at days 20 and 21 after infection (Fig. 7, lanes 20 and 21). Transcription of IFN-α (data not shown) and TNF-α (Fig. 7B) was already found 5 days postinfection in the brain of all animals, continuing in comparable amounts until 21 days postinfection. Again, there was no obvious difference between immunized and nonimmunized rats. BDV p24-specific mRNA was detected in all brains from the challenge-infected rats (Fig. 7C).

DISCUSSION

The present study describes the protection of rats from an immunopathological disease caused by the noncytolytic BDV with a novel parapoxvirus vector expressing nucleoprotein p40 of BDV. In rats and many other species, BDV causes a persistent virus infection in the brain in the presence of a functional immune system (for review, see reference 49). In rats, most brain areas usually remain productively infected throughout the lifetime of the infected animals due to the broad spectrum of cells infected with BDV, neurons, astrocytes, and microglia, in all cortical and brain stem areas (6–8).

The rat represents an excellent model for Borna disease, and thus, the protective immune response induced by the BDV p40-expressing Orf virus recombinant was investigated in these animals. The rationale to use the recently described Orf virus vector system for generating a BDV p40-expressing recombinant to immunize against BDV challenge infection is based on its powerful induction of a specific humoral and cell-mediated

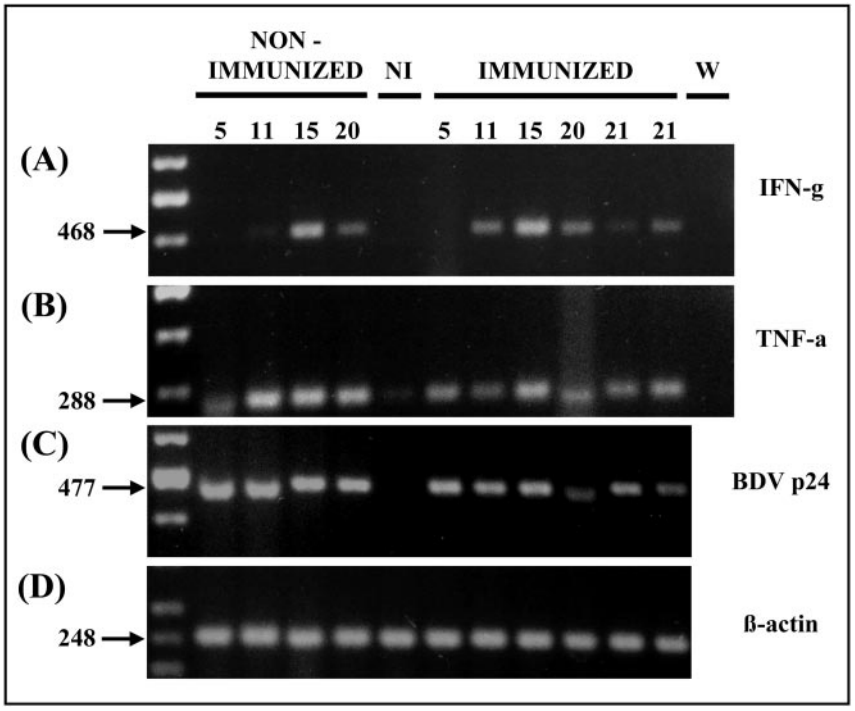

FIG. 7. RT-PCR analysis of brain RNA. Total brain RNA was isolated from a nonimmunized, noninfected animal (lane NI) and from individual nonimmunized or immunized rats 5, 11, 15, 20, and 21 days after challenge infection (the numbers above each lane indicate days postinfection); at day 21 postinfection, RNA was prepared from two different rats. RT-PCR was performed to detect mRNA specific for IFN-γ (A), TNF-α (B), BDV p24 (C), and β-actin (D). The size of each amplicon is indicated to the left in bases. Lane W shows the negative control that contained water instead of RNA.

immune protection, as demonstrated recently against a lethal herpesvirus infection in mice (15). The Orf virus type species of the genus *Parapoxvirus* has a very narrow host range (sheep and goats), a restricted skin tropism, lack of systemic infection, and an exceptionally strong stimulation of fast innate cellular immune mechanisms at the site of infection (for review, see references 5, 20, and 21). We have now made use of this vector system to further investigate its usefulness as a candidate vaccine and to further analyze the mechanisms mediating protection against persistent BDV infection in rats. Like mice (15), rats are also nonpermissive for Orf virus infection, and, therefore, it was important to monitor whether immunization with D1701-VrVp40 would induce an immune response against the p40 antigen.

A prerequisite for immunization studies was the demonstration of early p40 expression after in vitro infection of cells nonpermissive for Orf virus. Upon in vivo application, a direct correlation of the D1701-VrVp40 dose used for immunization, the frequency of application, the route of application, and specific serum antibody titers was observed. As shown before (15), the most effective dose for recombinant immunization was found to be 10^7 PFU given three times (immunization and two booster injections), and the most effective route was intramuscular. This immunization regimen resulted in strongest specific serum antibody titers and complete protection against Borna disease. The higher amounts of D1701-VrVp40 required for solid protection against BDV challenge infection might be explained by the lack of productive Orf virus recombinant infection at the site of injection. Thereby, the administration of larger amounts of recombinant virus might provide the early expression of sufficient foreign antigen to induce a potent immune response. A correlation between the amount and the duration of antigen expression and the strength of an efficient specific immune response has been reported (33). The superiority of the intramuscular route compared to intravenous administration might be due to the restricted tropism of Orf virus allowing more efficient antigen processing as well as presentation to and recognition by attracted immune cells.

The functional importance of the induced anti-p40 response was then tested in challenge experiments. Interestingly, rats were protected over a wide time range after the last booster injection of D1701-VrVp40. Challenge virus infections both early (2 weeks) after the end of the immunization regimen and as late as 8 months after the last booster resulted in complete protection from the disease. It should be stressed in this context that the p40-specific T-cell response so far has been regarded as a major mechanism both for protection from immunopathological disease and for virus elimination from the host. In cases of experimental disease and after transfer of brain lymphocytes, numerous T cells were always found throughout the brain, usually accompanied by degenerative brain lesions in the vicinity of these cells (3, 12, 25, 36, 44, 46), but in D1701-VrVp40 immunized rats T-cell numbers in the brain were not spectacular. Remarkably, hardly any destructive lesions were seen, either in the cortex or even in the hippocampus. This finding suggests that the local immune response in the brain after BDV infection differs from that in the immunized, BDV-infected rats.

Changes in the pathological and clinical picture after immunization with a vaccinia virus p40-expressing recombinant (VV-N) prior to BDV challenge infection have been described (30). These authors reported better control of virus replication in VV-N-immunized rats as represented by reduction of BDV nucleic acid and a 10- to 100-fold reduction in virus titer in the brain. This limitation of virus replication, however, was achieved only at the expense of severe inflammatory response in the brain and consequently disease exacerbation. This is in fundamental contrast to the effects of the Orf virus D1701-VrVp40-induced protection shown here. At 3 weeks postinfection in the brain of two of the tested immunized animals, BDV titers were below detection levels, and one rat exhibited at least 100-fold less BDV than the control rats. At approximately 2 months postinfection, infectious BDV was no longer recovered from brain homogenates of all immunized animals tested.

These results were consistent with the demonstration of viral nucleic acid by in situ hybridization and the detection of viral antigen by immunohistochemistry. Both techniques revealed a steady decrease of BDV-positive cells in the brains of immunized rats compared to control animals. At later time points, 2 and 5 months postinfection, the brains of immunized rats were almost free of BDV. None of the infected, immunized rats developed aggravated disease or late onset of disease. As for the use of vaccinia virus p40, it was shown by Lewis et al. (30) that viral replication could only be suppressed for a limited period of time (21 days). Therefore, we conclude that the parapoxvirus vector might have induced a more efficient anti-p40 response than the vaccinia virus vector, which allowed a rapid limitation of BDV replication and spread without causing adverse effects. A direct comparison of vaccinia virus p40 and D1701-VrVp40 with identically immunized and challenge-infected animals will clarify those questions.

Interestingly, in all hippocampi of the immunized healthy rats, viral antigen and nucleic acid were detectable for a rather prolonged period, albeit at clearly lower levels compared to nonimmunized rats, whereas virus had already been eliminated from nearly all other parts of the brain. The hippocampus is regarded as the prime target structure for BDV infection of the brain, and diseased rats exhibit dramatic losses of hippocampal neurons (7, 17, 26, 44); however, that was never observed in the immunized rats. Since the noncytolytic BDV persists without causing major damage to infected cells, the control of a local antiviral response guarantees the survival of this central brain structure. In general, virus restriction in the brain of D1701-VrVp40-immunized rats was clearly visible around 20 days postinfection, and virus clearance lasted until approximately 50 days postinfection This type of kinetic of BDV clearance seems to differ from successful virus elimination from the brain with high-dose BDV infection. In the latter case, more rapid prevention of BDV replication was achieved, as exemplified by the absence of viral RNA between days 7 and 68 after infection (34).

This effect might be explained by induction of the entire spectrum of the antiviral immune response due to the presence of all viral antigens in high-dose virus preparations (16). In addition to the p40-specific $CD8^+$ T-cell response, other effector mechanisms might be of importance. Cytotoxic T cells specific for gp94 cannot be excluded (37) as well as the recently described p10-specific cytotoxic T lymphocytes (22). Thus, it is conceivable that the p40-specific immunity obtained by priming with the Orf virus recombinant is capable of limiting BDV replication early after challenge infection and facilitates virus clearance by the subsequently induced immune response against complete BDV.

The time needed to clear challenge virus from the brain resembled that reported after transfer of primed $CD4^+$ T cells (32). Therefore, we speculated that immunization with the Orf virus recombinant might provide an early and fast $CD4^+$ T-cell response after challenge infection that provides efficient help for specific $CD8^+$ T cells. Since immunization with the Orf virus recombinant was initiated in the periphery, this might not be entirely reflected by the situation in the brain, where between 5 and 20 days postinfection no significantly enhanced $CD4^+$ T-cell numbers were detectable compared to that of nonimmunized infected rats. However, considerable numbers of CD45RC-expressing lymphocytes accumulated in close association to p40 antigen, where CD8-positive cells were also found in the parenchyma. High expression of rat CD45RC has been reported on B cells (51) and on T lymphocytes (for review, see reference 2). This phenotype either represents naïve rat $CD4^+$ T cells providing help to B cells during primary immune response or is found on the majority of resting, long-lived memory $CD4^+$ T cells (2). This subpopulation of antigen-experienced $CD4^+$ T cells is also found in cervical lymph nodes (42), can easily migrate across endothelial venules (45), and can also exert an immunoprotective function (2).

The role of cervical lymphocytes in Borna disease has recently been demonstrated (1). From the experiments presented, we cannot conclude whether immunization with D1701-VrVp40 mediated the generation of an extraordinarily potent pool of those memory T cells or led to rapid induction of specific B cells and antibody-producing plasma cells or both. Interestingly, immunohistochemistry demonstrated the coincident deposition of large amounts of immunoglobulin, but the specificity of these local antibodies needs to be determined in future work. The presence of B cells and deposits of immunoglobulins in cortical areas as well as in the hippocampus of BDV-infected rats have been demonstrated earlier (12, 23, 42), however, without proof of specificity. In mice, antigen-experienced memory B cells can be restimulated to differentiate to plasma cells outside of lymphoid organs (33), requiring $CD4^+$ T cells for generating long-term humoral immunity (11). Work by Hatalski et al. (24) indicated a switch from an initial Th1-type cellular immune response to a Th2-like humoral immune response in the chronic phase in BDV-infected rats, which apparently correlated with a decrease of the inflammatory reaction. Further studies are required to determine a Th1- versus Th2-type response after immunization with D1701-VrVp40.

The participation of nonneutralizing antiviral antibodies in protection against Borna disease has not yet been addressed. With regard to the findings presented it becomes necessary to investigate whether antibodies directed against defined viral antigens (other than inducing neutralizing antibodies) might play a role in the course of BDV infection or in the control of virus replication and virus elimination. For other neurotropic RNA viruses, such as Sindbis virus, flavivirus (tick-borne encephalitis virus) or murine coronavirus JHM, a prominent role of antiviral antibodies, also with nonneutralizing functions, has been demonstrated for nondestructive virus clearance from the

brain by restriction or prevention of viral gene expression (14, 18, 19, 28). There is growing evidence for the requirement of B cells in virus clearance of primary central nervous system infection, but the role of antibodies as primary mediators of noncytolytic virus elimination remains to be resolved.

As discussed recently (13, 18), antibody binding to the surface of or entering the infected cell might initiate intracellular signaling cascades. As a consequence, attracted T cells and activated microglia can produce cytokines such as IFN-γ or TNF-α, resulting in cytokine-mediated virus elimination by noncytolytic T cells (4, 14, 18, 29), including noncytolytic CD8-positive T cells (19). However, the impact of IFN-α, IFN-γ, or TNF-α in D1701-VrVp40-mediated elimination of BDV does not appear very likely, since we could not detect significant differences in transcription of these mRNAs compared to that of nonimmunized rats. Therefore, more detailed analyses have to elucidate what other protective and regenerative factors are induced after immunization with D1701-VrVp40. With the parapoxvirus vector system, we now have a model at hand that would allow us to reveal the immune effector mechanisms responsible for the nondestructive elimination of BDV from the brain.

In conclusion, for the first time, protection against immune-mediated neuropathology after BDV infection was achieved by the application of a new recombinant vector virus expressing only BDV p40 antigen. This should be an important step towards an ideal vaccine, which is capable of inducing an efficient and long-lasting specific immune response leading to restriction and elimination of this persistent virus infection without provoking massive neuronal destruction. Studies are now in progress to scrutinize cytokines and additional potential immune modulators that are induced by this Orf virus recombinant and might not only be responsible for an optimized interplay with the well-known anti-BDV T-cell-mediated component, but might also influence early stages of virus infection (39). Moreover, the use of this vector system can now enable more intense studies on the role of the local antibody response in elimination of persistent viruses. Finally, this study provides another example of the exceptional capacity of the Orf virus vector system in mediating protective antiviral immunity.

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