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B-Lymphocyte Requirement for Vaccine-Mediated Protection from Theiler's Murine Encephalomyelitis Virus-Induced Central Nervous System Disease

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The role of humoral immunity in the protection of vaccinated SJL/J mice from central nervous system disease induced by the DA strain (DAV) of Theiler's murine encephalomyelitis virus was investigated in B-cell-deficient mice. Mice were depleted of B cells by treatment with a mouse monoclonal antibody specific for immunoglobulin M. DAV-vaccinated, B-cell-deficient mice failed to clear viral infection and were no longer protected from Theiler's murine encephalomyelitis virus-mediated central nervous system disease. $CD4^+$ T cells are required in this model of protection to provide help for the development of an antiviral antibody response in the central nervous system.

Intracerebral (i.c.) infection of SJL/J mice with the DA strain (DAV) of Theiler's murine encephalomyelitis virus (TMEV) causes a biphasic disease in the central nervous system (CNS). This disease is characterized by an early gray matter encephalitis followed by a persistent infection and chronic demyelinating disease in the white matter of the CNS (15, 17, 18, 29–31).

It has previously been shown that susceptible SJL/J mice can be protected from TMEV persistence and CNS disease by peripheral inoculation with a live or inactivated virus vaccine (5, 13). Intraperitoneal (i.p.) vaccination with either live DAV or the H7A6-2 mutant strain of TMEV (32, 33) results in rapid clearance of virus from the brain within the first week following intracerebral (i.c.) challenge with DAV (13). The protective immune response has been shown to require $CD4^+$ but not $CD8^+$ T cells (13). In this study, we investigated the role of B cells in this vaccine-mediated protection by generating B-cell-deficient mice. Mice were depleted of B cells by using anti-immunoglobulin M (IgM) (μ) antibodies, as has been described for other models of viral and bacterial pathogenesis (1, 2, 11, 26). We also investigate the role of $CD4^+$ T cells in mediating protection.

Mice were treated from birth with monoclonal antibodies to deplete IgM^+ B cells. Newborn mice (six to eight animals per group) were treated with ascites fluid IgG containing either AF6.78.25.4 (anti-μ antibody) or P3X638Ag (control antibody) (10, 12, 27). The mice were given i.p. injections of 0.05 ml of a 1-mg/ml solution of antibody on days 1, 2, 3, 4, 5, 6, and 7 and then were treated twice weekly with 0.1 ml of the antibody solutions. To monitor the B-cell depletion, peripheral blood was collected from four mice from each treatment group and analyzed by FACS for IgG^+ cells at 4 weeks of age. Erythrocytes were removed by treatment with Tris-buffered 0.17 M ammonium chloride and three washes with phosphate-buffered saline (PBS) (19). The lymphocytes were stained with fluorescein-conjugated goat anti-mouse IgG, IgA, or IgM (Sigma Chemical Co., St. Louis, Mo.). Ig-positive cells were 85.4 ± 4.2% depleted from peripheral blood. FACS analysis was repeated on splenocytes from four animals from each group at the time of perfusion (10 weeks of age) as well. Splenocytes at the time of perfusion were 92.3 ± 8.9% depleted of Ig-positive cells compared with control antibody-treated mice. A Student *t* test indicated that there was a significant difference in the number of Ig-positive cells between anti-μ- and control antibody-treated mice ($P < 0.0001$). These studies indicate that the B-cell depletions were maintained throughout the study. The presence of $CD3^+$ cells was also monitored in control and anti-μ-treated mice. A Student *t* test indicated that there was no significant difference in $CD3^+$ T-cell numbers between control and anti-μ-treated mice ($P = 0.213$). FACS profiles of IgG^+ and $CD3^+$ spleen cells from representative control and anti-μ-treated mice at the time of perfusion (10 weeks) are shown (Fig. 1).

To assess the functional effect of the anti-μ treatment on the proliferation of B- and T-cell subsets, the response of splenocytes to the mitogens lipopolysaccharide (LPS) and concanavalin A (ConA) were measured at 4 and 10 weeks of age. Spleen cells were removed from the representative mice in each group, and single-cell suspensions free of erythrocytes were prepared. The cells were resuspended at a concentration of 2×10^6 cells per ml, and 0.1 ml of this suspension was aliquoted in triplicate into 96-well plates (Corning Glass Works, Corning, N.Y.). A 0.1-ml volume of medium, LPS (10 μg/ml), or ConA (1 μg/ml) was then added to each well, and the plates were incubated at 37°C for 48 h. The cells were then pulsed with 0.5 μCi of [^{3}H]thymidine and harvested at 24 h. [^{3}H]thymidine uptake was determined by liquid scintillation counting. At both 4 and 10 weeks, there was no significant proliferation of the splenocytes from the anti-μ-treated mice in the presence of LPS (stimulation index, 1.1 ± 0.5), while the splenocytes from control antibody-treated mice proliferated in the presence of LPS (stimulation index, 6.3 ± 0.95) (Fig. 2). Splenocytes from both control antibody- and anti-μ-treated mice proliferated in the presence of ConA (Fig. 2).

Mice were vaccinated i.p. at 4 weeks of age with 10^7 PFU of the purified attenuated variant of DAV, H7A6-2 (13, 32, 33). The mice were challenged i.c. with 5×10^5 PFU of DAV 5 weeks following vaccination. Anti-DAV antibody responses in serum and cerebrospinal fluid (CSF) were measured in the control antibody- or anti-μ antibody-treated mice at the time of perfusion by enzyme-linked immunosorbent assay (ELISA),

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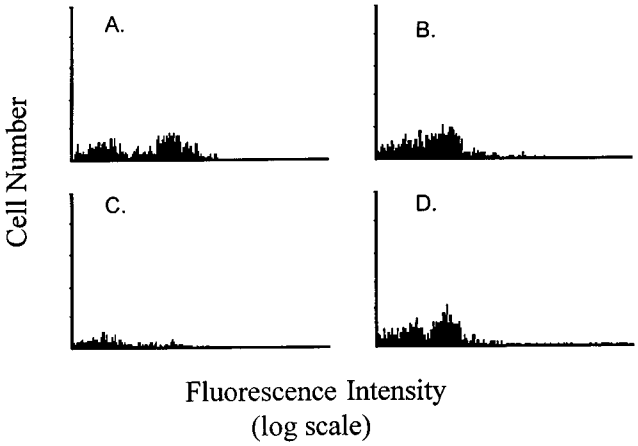

FIG. 1. FACS analysis of splenocytes from control antibody- or anti-μ-antibody treated 10-week-old mice at the time of perfusion. Splenocytes were prepared from mice treated from birth with either a control antibody, P3X.638Ag (A and B) or anti-μ antibody, AF6.78 (C and D). The cells were stained with fluorescein-conjugated antibodies to IgG,A,M (A and C) or CD3 (B and D) and were analyzed by FACS. Relative cell number is plotted versus log channel fluorescence. The percentages of positive cells minus the background between the channels of 40 and 256 were 13.9% (A), 15.0% (B), 2.2% (C), and 15.2% (D).

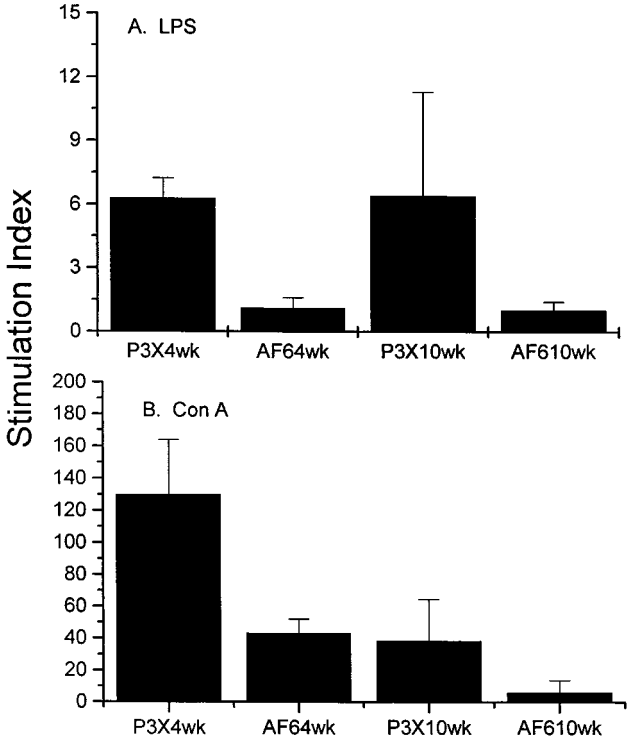

FIG. 2. Proliferation of splenocytes to the mitogens LPS and ConA. Splenocytes were prepared at 4 and 10 weeks of age from whole spleens of mice which had been treated with either control antibody or anti-μ antibodies from birth. Cells were incubated in 5 μg of LPS per ml (A) or 0.5 μg of ConA per ml (B) for 24 h before being pulsed with 0.5 μCi of [^{3}H]TdR. At 24 h after the pulse, the cells were harvested and counted for uptake of [^{3}H]TdR by liquid scintillation. The mean counts per minute of the treated cells divided by the medium control are reported as the stimulation index.

as previously described (13). It was found that anti-DAV antibody concentrations were 100- to 500-fold lower in sera and 100-fold lower in CSF from anti-μ-treated mice than from control antibody-treated mice (Table 1). Neutralizing-antibody responses were also measured in the CSF of control antibody- or anti-μ-treated mice by a neutralization plaque assay, as previously described (13). Control antibody-treated mice had high neutralizing antibody titers in CSF, but anti-μ-treated mice had no detectable CSF neutralizing activity (Table 1).

To determine the effects of B-cell depletion on the capacity to clear viral infection, virus levels 1 week following i.c. challenge were measured in the brain by plaque assay, as previously described (13). Control antibody-treated, DAV-vaccinated mice had cleared viral infection with 10^3- to 10^4-fold-lower virus levels at 1 week postchallenge compared with PBS-vaccinated mice (Fig. 3). In contrast, anti-μ-treated DAV-vaccinated mice failed to clear viral infection from the brain (Fig. 3). Analysis of variance indicated that the means of the individual treatment groups were different from one another ($P = 0.012$). Comparison of the virus levels in vaccinated anti-μ-treated mice with vaccinated control antibody-treated mice by a Student t test indicated that the means were different ($P = 0.021$).

Previously, it has been shown that CD4$^+$ T cells are required for the development of protective immunity (13). To determine if CD4$^+$ T cells are also necessary at the time of challenge to mediate viral clearance from the brain, mice were vaccinated with DAV and then depleted of CD4$^+$ T cells immediately prior to i.c. challenge. Mice (five animals per group) were vaccinated i.p. with 10^7 PFU of DAV in 0.5 ml of PBS or with 0.5 ml of PBS alone (as control). Three weeks following vaccination, the mice were treated by i.p. injection with either 0.5 mg of normal rat serum IgG (NRS) or 0.5 mg of the monoclonal anti-CD4 antibody GK1.5 (14). The mice were given i.p. injections on days -5, -3, and -1 prior to i.c. challenge with DAV. The mice were challenged i.c. on day 0 with 5×10^5 PFU of DAV in 0.05 ml of PBS and were perfused 6 days following infection. Brains were removed, and virus titers were quantified by viral plaque assay. Spleens were removed at the time of perfusion, and single-cell suspensions free of erythrocytes were prepared and stained for CD4$^+$ T cells. At the time of perfusion, the GK1.5-treated mice were >99% depleted of CD4$^+$ T cells relative to the NRS-treated mice. Neutralizing antibodies were found in the CSF of both NRS-treated, DAV-vaccinated mice and CD4$^+$ T-cell-depleted, DAV-vaccinated mice (Table 2). The DAV-vaccinated

TABLE 1. Anti-DAV IgG levels in serum and CSF of control or B-cell-depleted mice

Treatment	Anti-DAV IgG level (μg/ml) in[a]:		Neutralization titer[b]
	Serum	CSF	
P3X/PBS	1.78 ± 0.46	<0.001	<50
P3X/DAV	98.6 ± 76	3.0 ± 4.0	2,133
AF6/PBS	<0.001	<0.001	<50
AF6/DAV	0.28 ± 0.3	0.03 ± 0.06	<50

[a] SJL/J mice were treated from birth with P3X.638Ag (control) or AF6.78.25 (anti-μ) antibodies. At 5 weeks of age, the mice were separated from parents and were vaccinated with either PBS or 10^7 PFU of attenuated DAV. The mice were challenged i.c. with 5×10^5 PFU of DAV 4 weeks after vaccination. At 1 week after challenge, the mice were sacrificed, and their blood and CSF were collected and analyzed by ELISA for quantities of anti-DAV antibody. The sensitivity of this assay was 0.001 μg/ml.

[b] CSF was collected from control (P3X) or B-cell-depleted (AF6) mice and analyzed for neutralizing-antibody activity by a neutralization plaque assay. CSF was serially diluted from 1:50 to 1:50,000 and incubated with 200 PFU of DAV. After 1 h on ice, the virus-antibody mixture was plated directly onto BHK monolayers in a standard plaque assay procedure. Neutralizing activity was calculated as a 50% neutralization titer.

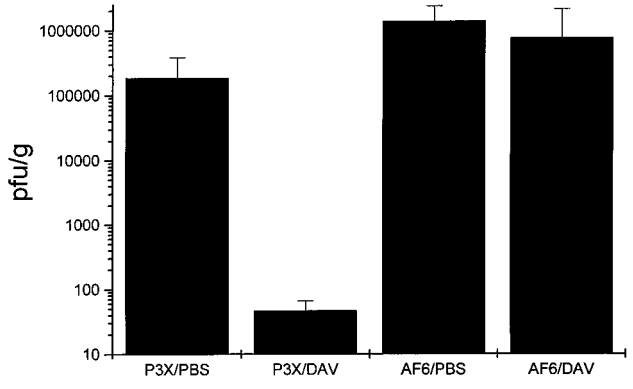

FIG. 3. Virus levels (PFU per gram) in the CNS of control antibody- or anti-μ-antibody treated mice at 1 week following i.c. challenge with DAV. Mice were treated with either control antibody (P3X) or anti-μ antibody (AF6) from birth. At 5 weeks of age, the mice were vaccinated with either PBS or 10^7 PFU of H7A6-2 virus. At 4 weeks after vaccination, the mice were challenged i.c. with DAV. The mice were perfused 1 week following i.c. challenge, and their brains were analyzed for virus levels by a standard plaque assay.

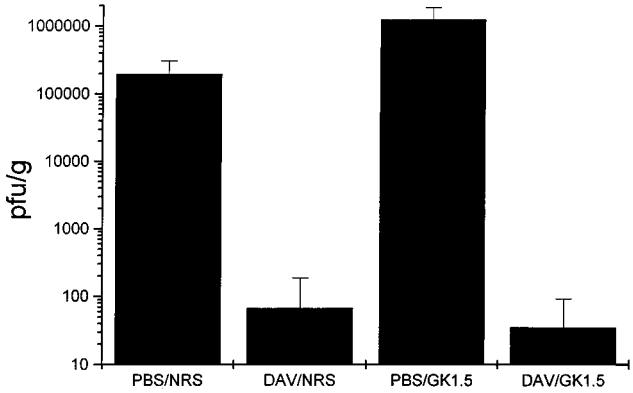

FIG. 4. Virus levels (PFU per gram) in the CNS of mice depleted of $CD4^+$ T cells immediately prior to i.c. challenge. Mice were vaccinated with PBS or DAV at 4 to 6 weeks of age. At 4 weeks after vaccination, the mice were treated with three 0.5-mg doses of either NRS (control) or GK1.5 (anti-CD4) ascites fluid. The mice were challenged i.c. with DAV and perfused 1 week later with PBS. Their brains were removed, and plaque assays were performed to determine virus titers.

mice developed strong humoral immune responses to the virus, which were diminished only moderately by the $CD4^+$ T-cell depletion (Table 2). The loss of $CD4^+$ T cells did not prevent the mice from clearing viral infection from the brain (Fig. 4). Both the control depleted and the $CD4^+$ T-cell-depleted DAV-vaccinated mice had 10^3- to 10^4-fold-lower virus titers in the brain than the PBS-vaccinated mice did (Fig. 4). Analysis of variance among the four treatment groups indicated that they were different from one another ($P < 0.0001$) but that the levels of virus in the brains of $CD4^+$ T-cell-depleted DAV-vaccinated mice were not significantly different from those in NRS-treated DAV-vaccinated mice ($P = 0.61$).

Vaccinated SJL/J mice are protected from TMEV-mediated CNS disease by immune responses which require $CD4^+$ but not $CD8^+$ T cells (13). These mice show both marked infiltration of mononuclear cells into the brain and spinal cord and production of antiviral neutralizing antibodies in the CNS, which correlate temporally with the clearance of virus from the CNS (13). The role of B cells in vaccine-mediated protection and in the resistance to TMEV-induced CNS disease has been suggested in the literature (13, 21, 22, 25). Rossi et al. observed

TABLE 2. Anti-DAV IgG levels in serum and CSF of control or $CD4^+$ T-cell-depleted mice

Treatment	Anti-DAV IgG level (μg/ml) in[a]:		Neutralization titer[b]
	Serum	CSF	
PBS/NRS	1.6 ± 1.26	<0.001	<50
DAV/NRS	203 ± 120	4.15 ± 4.2	18,154
PBS/GK1.5	0.66 ± 0.56	0.027 ± 0.052	<50
DAV/GK1.5	72 ± 50	1.53 ± 2.5	406

[a] SJL/J mice (five animals per group) were vaccinated with PBS (control) or 10^7 PFU of DAV. At 3 weeks following vaccination, the mice were given in three 0.5-mg doses of IgG from either NRS or GK1.5 ascites fluid on days -5, -3, and -1 prior to i.c. challenge. Mice were challenged i.c. with 5×10^5 PFU of DAV on day 0, and blood and CSF were collected on day 6. Anti-DAV titers in serum and CSF were determined by a quantitative ELISA. The sensitivity of the assay was 0.001 μg/ml.

[b] CSF was collected from control (NRS) or $CD4^+$ T-cell-depleted (GK1.5) mice and analyzed for neutralizing antibody activity by a neutralization plaque assay. CSF was serially diluted from 1:50 to 1:50,000 and incubated with 200 PFU of DAV. After 1 h on ice, the virus-antibody mixture was plated directly onto BHK monolayers in a standard plaque assay procedure. Neutralizing activity was calculated as a 50% neutralization titer.

that serum from TMEV-resistant strains of mice can transfer some protection to disease-susceptible strains (25). In the susceptible SJL/J mice, treatment from birth with anti-μ antibodies results in increased number and severity of demyelinating lesions compared with those in control mice (21). Nude mice can be protected from acute encephalitis by the transfer of neutralizing monoclonal antibody (6). These observations indicate that in the model system of vaccine-mediated immunity from CNS disease caused by TMEV, humoral immunity is likely to play an important role in viral clearance and protection.

In this report, we investigate the absolute requirement for B lymphocytes in a model system of protection from TMEV-induced CNS disease in B-cell-depleted mice (8). B-cell depletion was confirmed both by FACS and functional analysis. The anti-μ-treated mice showed no significant difference in T-cell numbers compared with control antibody-treated mice; however, their response to the mitogen ConA was reduced. Both antigen-specific and splenic mitogen-induced T-cell proliferative responses have been found to be diminished in anti-μ-treated mice (9, 23, 28). Deficiencies in T-cell function in anti-μ-treated mice have been found to result from a lack of antigen-presenting cell function provided by the B cells rather than an intrinsic defect in the capacity to activate the T cells or from a decrease in the T-cell repertoire (9). T cells require costimulatory signals to respond to ConA (20, 24), and activated B cells have been shown to be very efficient at presenting antigen and providing a costimulatory signal to T cells (3, 4, 7). T-cell responsiveness to ConA is also affected by the level of cytokines such as interleukin-2 (16). Therefore, reduced mitogen responsiveness in B-cell-depleted SJL/J mice may result from a reduction in availability of costimulatory signals or an altered cytokine secretion pattern in splenocytes lacking B cells.

B-cell depletion dramatically reduces the levels of anti-DAV antibodies in serum and CSF, eliminates CSF anti-DAV neutralizing activity, and abrogates viral clearance from the brain during the first week following i.c. challenge with DAV in vaccinated SJL/J mice. This observation indicates that B cells have an important role in protection from CNS infection and the prevention of virus persistence which leads to chronic demyelinating disease. It is consistent with the model that pro-

tection occurs by a rapid mobilization and infiltration of antibody-secreting B cells into the brain and local production of virus-neutralizing antibody following i.c. challenge with DAV (13). B cells in the CNS, however, may also be important for antigen presentation to memory T cells which were primed by virus following vaccination. It has been shown that $CD4^+$ T cells are necessary in the development of this protective immune response (13). Using $CD4^+$ T-cell depletion studies, we have shown in this report that mice lacking $CD4^+$ T cells at the time of i.c. challenge maintain humoral immune responses to the virus and can still clear virus from the CNS.

In summary, this report establishes that protection from TMEV-induced CNS disease in vaccinated SJL/J mice requires B lymphocytes. $CD4^+$ T cells are necessary to provide help for the development of a humoral immune response to the virus but are not required to mediate viral clearance directly.

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