

Obligatory Requirement for Antibody in Recovery from a Primary Poxvirus Infection

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To understand the correlates of protective immunity against primary variola virus infection in humans, we have used the well-characterized mousepox model. This is an excellent surrogate small-animal model for smallpox in which the disease is caused by infection with the closely related orthopoxvirus, ectromelia virus. Similarities between the two infections include virus replication and transmission, aspects of pathology, and development of pock lesions. Previous studies using ectromelia virus have established critical roles for cytokines and effector functions of CD8 T cells in the control of acute stages of poxvirus infection. Here, we have used mice deficient in B cells to demonstrate that B-cell function is also obligatory for complete virus clearance and recovery of the host. In the absence of B cells, virus persists and the host succumbs to infection, despite the generation of CD8 T-cell responses. Intriguingly, transfer of naive B cells or ectromelia virus-immune serum to B-cell-deficient mice with established infection allowed these animals to clear virus and fully recover. In contrast, transfer of ectromelia virus-immune CD8 T cells was ineffective. Our data show that mice deficient in CD8 T-cell function die early in infection, whereas those deficient in B cells or antibody production die much later, indicating that B-cell function becomes critical after the effector phase of the CD8 T-cell response to infection subsides. Strikingly, our results show that antibody prevents virus from seeding the skin and forming pock lesions, which are important for virus transmission between hosts.

Variola virus (VARV), the causative agent of smallpox, is a virulent human pathogen with mortality rates of up to 30% (17). It is not understood why the mortality rates are this high or what constitutes an effective immune response against a primary infection. The possibility of intentional or unintentional release of VARV has renewed interest in smallpox (20). Given that the disease was eradicated over a quarter of a century ago, our current understanding of immunity to a primary poxvirus infection comes largely from studies on the response to vaccinia virus (VACV) vaccination in humans and from animal studies using VACV and closely related orthopoxviruses, such as monkeypox and ectromelia virus (ECTV). Here we use the term primary infection to indicate the first exposure to an orthopoxvirus. This may be vaccination with live VACV or a natural infection with either VACV or VARV.

Early observations on individuals vaccinated against smallpox led to the view that antibody did not significantly contribute to control of a primary poxvirus infection. In patients with defective cell-mediated immunity, VACV causes generalized infection, a serious complication of vaccination (3). In contrast, individuals with apparent defective antibody production but intact cell-mediated immunity responded normally to vaccination (3, 17). Furthermore, immunoglobulin therapy for generalized vaccinia was thought to be effective only through its ability to control virus long enough to allow the restoration of cell-mediated immunity (17, 18). However, our current understanding of immunodeficiencies associated with progressive vaccinia indicate that not only T-cell function but also T-cell

help for B cells and B-cell function may be affected (7, 35). More recently, a study by Belyakov and colleagues has shown that in the absence of B cells, vaccinated mice challenged with virulent VACV get sick, alluding to a role for antibody; however, this did not result in mortality (1).

The usefulness of VACV as a model for smallpox is limited, since pathogenesis, disease progression, and outcome of infection are unlike those of VARV. In contrast, ECTV, like VARV, has a restricted host range, is infectious at very low doses of virus, and causes severe disease with high mortality rates (4, 14, 16). Although all orthopoxviruses are highly conserved, sharing greater than 90% homology in the central 100-kbp region of the genome (14), further specific similarities between mousepox, caused by ECTV, and smallpox include virus replication and transmission, cytokine responses (5, 40), aspects of pathology, and development of skin lesions in later stages of infection (17). These lesions, along with oropharyngeal secretions, are believed to be critical for virus transmission (4, 16). The mousepox model is still the most versatile with which the roles of individual components of innate and adaptive immunity can be investigated.

Indeed, the mousepox model has been instrumental in establishing the critical role of the cell-mediated immune response in control of poxvirus infection (2, 24, 33, 39). In addition to the effector function of CD8 T cells, functions of natural killer (NK) cells, CD4 T cells, and macrophage subsets, as well as nitric oxide, interferons, and T-helper 1 type cytokines, are also required (5, 21, 24, 25, 27, 36).

Since both VARV and ECTV cause acute infections in their natural hosts, we were surprised to discover that in C57BL/6 wild-type (B6.WT) mice, normally resistant to mousepox (4, 5), the absence of CD4 T cells resulted in ECTV persistence for

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extended periods (24). The antiviral cytotoxic T-lymphocyte (CTL) response in mice lacking CD4 T cells was suboptimal, suggesting that virus persistence maybe the result of a defective CTL response. However, in contrast to the case with the B6.WT mice, antiviral CTL activity in these mice persisted even in the late stages of infection (24). Notwithstanding, the CTL were insufficient to clear virus. Since CD4 T-cell help is also crucial for antibody production (30, 38), we speculated that virus persistence in these animals might be due to defective antibody response. The importance of antibody in a primary infection has not been previously appreciated, although its requirement in protection against reinfection is now established (8, 12, 13, 17, 36, 36a, 44).

To test the hypothesis that antibody is required for virus clearance in a primary poxvirus infection, we employed mice lacking B cells (B6. μ MT) (28) and those deficient in major histocompatibility complex (MHC) class II (B6.Aa^{-/-}), and therefore lacking CD4 T cells (29). As controls we used B6.WT mice and animals that lacked the effector molecules perforin (B6.Pr^f^{-/-}) (22) or gamma interferon (IFN- γ) (B6.IFN- γ ^{-/-}) (9), previously shown to be critical for virus control through NK and CD8 T-cell function (5, 32, 33, 39). The gene knockout strains deficient in CD8 T-cell effector function were included in this study to compare the disease progression and outcome of infection with those of strains defective in B-cell function. Our data show that mice deficient in CD8 T-cell effector function die early in infection, whereas those deficient in B cells or antibody production die much later, indicating that B-cell function becomes critical after the effector phase of the CD8 T-cell response to infection subsides. In mice lacking B cells or antibody, ECTV persists and the host succumbs to disease, despite the generation of normal CD8 T-cell responses.

MATERIALS AND METHODS

Mice. Female, specific-pathogen-free mice were used at 6 to 10 weeks of age. B6.WT mice were obtained from the Animal Services Division, John Curtin School of Medical Research, Canberra, Australia. The B-cell knockout mice, B6.129S2-*Igh-6^{mi1Cgn}* (28), designated B6. μ MT, MHC class II gene knockout mice, B6.H2-Aa^{tm1Blt} (29), designated B6.Aa^{-/-}, IFN- γ gene knockout mice, B6.129S7-*Ifng^{tm1Ts}* (9), designated B6.IFN- γ ^{-/-}, and perforin gene knockout mice, C57BL/6-*Prf^{tm1Sdz}*/J (22), designated B6.Pr^f^{-/-}, all on a B6.WT background, or backcrossed to B6.WT at least 10 times, were bred at John Curtin School of Medical Research. Experiments were performed according to institutional guidelines for animal care and use.

Cell lines. BS-C-1 (ATCC no. CCL-26) and MC57G (H-2b; ATCC No. CRL-2295) were maintained in Eagle's minimum essential medium (Invitrogen) supplemented with 2 mM L-glutamine, antibiotics, and 10% fetal calf serum.

Virus infection and determination of virus titer. Virulent ECTV Moscow strain Mos-3-P2 (ATCC no. VR 1374) was propagated in BS-C-1 cells as described previously (6, 43). Mice were inoculated with 1,000 PFU of virus subcutaneously in the right hind limb under anesthesia. At various times postinfection (p.i.), organs were removed aseptically and processed for determination of virus titers as previously described elsewhere (6, 43).

CTL assays. Antiviral CTL responses were measured *ex vivo* using splenocytes from individual animals at times indicated p.i. Standard ⁵¹Cr release assays were performed as described elsewhere (26). To detect MHC class I-restricted killing, ECTV-infected and uninfected MC57G cells were utilized as target cells.

Plaque reduction neutralization test. The plaque reduction neutralization test, used to determine the virus-neutralizing activity of the antibody present in serum samples, is described elsewhere (36).

Serum and cell transfer experiments. For transfer experiments, B6. μ MT animals were first infected with ECTV to establish infection. Groups of mice were either left untreated or given virus-immune serum, purified naive B cells, or ECTV-immune CD8 T cells.

Virus-immune serum was collected and pooled from ECTV-infected B6.WT

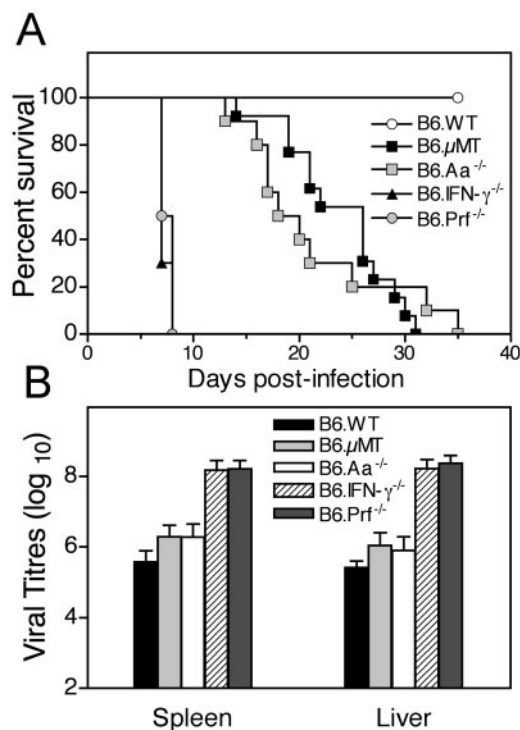


FIG. 1. Survival and virus load in organs of mice infected with ECTV. (A) Groups of 10 B6.WT, B6.IFN- γ ^{-/-}, B6.Pr^f^{-/-} or B6.Aa^{-/-} mice and a group of 13 B6. μ MT mice were infected with 10³ PFU of ECTV and monitored for clinical signs of disease and survival. (B) In a separate experiment, groups of six B6.WT, B6.IFN- γ ^{-/-}, B6.Pr^f^{-/-}, or B6.Aa^{-/-} mice and a group of eight B6. μ MT mice were infected as described for panel A and sacrificed at day 7 p.i., and organs were collected. Viral titers in spleen and liver were determined and are presented as means \pm standard deviations.

mice at day 35 p.i. The neutralizing activity of sera was 1:1,000 and a single dose of 0.5 ml was given intraperitoneally to recipient B6. μ MT mice 10 days p.i.

Naive B cells to be transferred were isolated using a two-step negative selection process from spleens of B6.WT and B6.Aa^{-/-} mice. First, NK cells (asGM1⁺) and T cells (Thy1.2⁺, CD8⁺, and CD4⁺) were depleted from the spleen cell populations using anti-asialoGM1 (Wako Pure Chemicals), anti-Thy1.2 (AT83), anti-CD8 (3.155), and anti-CD4 (RL172) plus rabbit complement (Cedarlane Laboratories Ltd., Ontario, Canada) (25). Dead cells and red cells were removed by Ficoll-Hypaque separation. The remaining cells were then incubated with the following antibodies: anti-CD3 (KT3), anti-Thy1 (T24/31.7), anti-CD4 (GK1.5), anti-CD8 (53.6.7), anti-Gr-1 (RB6-8C5), anti-Mac-1 (M1/70), anti-F4/80, and anti-DEC205 (N418) to tag T cells, granulocytes, macrophages, and dendritic cells. Antibody-bound cells were removed with antirat immunoglobulin-coupled magnetic beads (Miltenyi Biotec GmbH, Germany). This procedure generally yielded B cells that were greater than 98% pure. Recipient mice were given 10⁷ B cells intravenously at day 7 p.i.

ECTV-immune CD8 T cells for adoptive transfer were isolated from ECTV-infected B6.WT mice at day 8 p.i. and purified in a similar way to that for the B cells. For this, B cells, CD4 T cells, and NK cells were first depleted from the spleen cell populations using anti-B220 (RA3-3A1), anti-CD4 (RL172), and anti-asialoGM1 plus rabbit complement. In the second step, CD8 T cells were enriched using antirat immunoglobulin-coupled magnetic beads (Miltenyi Biotec) and the following cocktail of antibodies: anti-CD4 (GK1.5), anti-CD19 (ID3), anti-B220 (RA3-6B2), anti-Gr-1 (RB6-8C5), anti-Mac-1 (M1/70), anti-F4/80, anti-MHC class II (M5/114), and anti-DEC205 (N418). This procedure generally yielded CD8 T cells that were more than 98% pure. Recipient mice were given 10⁷ CD8 T cells intravenously at day 7 p.i.

Statistical analysis. For comparison of viral titers, the nonparametric Mann-Whitney test was used, employing the statistical program GraphPad Prism

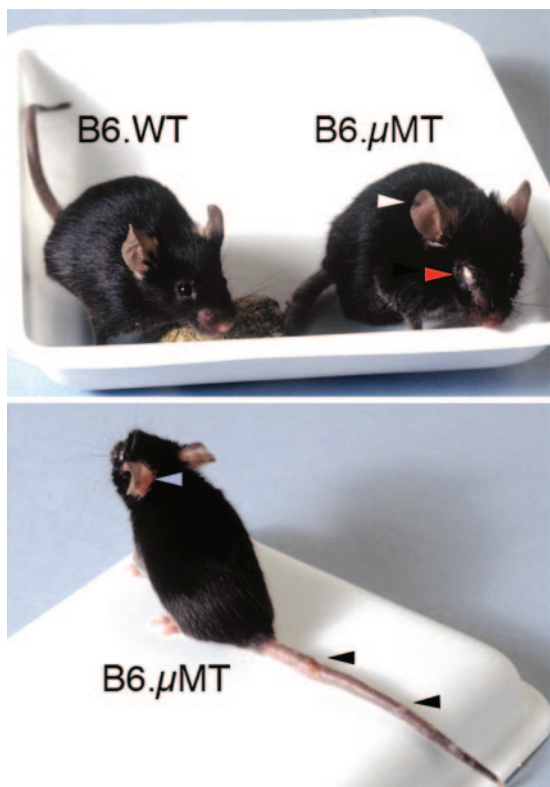


FIG. 2. ECTV infection causes pock lesions and conjunctivitis in the absence of B cells and antibody. ECTV-infected B6.WT mice do not develop rashes, lesions, or conjunctivitis. From day 10 to 14 p.i., ECTV-infected B6.μMT mice develop conjunctivitis (red arrow), lesions (white arrow), and inflammation (blue arrow) on the pinnae and lesions on the tail (black arrows). A representative B6.WT mouse and a B6.μMT mouse at 14 days p.i. are shown.

(GraphPad Software, Inc., San Diego, CA). A *P* value of less than 0.05 was taken to be significant.

RESULTS

B-cell-deficient mice succumb to mousepox. The B6.WT strain of mice effectively controlled a primary ECTV infection with 100% survival (Fig. 1A). Further, as expected, all B6.Pr^f^{-/-} and B6.IFN-γ^{-/-} mice died from mousepox within the first week of infection. Although B6.μMT and B6.Aa^{-/-} mice appeared well during the first 8 to 10 days, they gradually succumbed to disease. These animals developed conjunctivitis and skin lesions, which appeared 10 to 14 days p.i. on feet, tails, and pinnae (Fig. 2). The lesions first developed as papules and then progressed to ulcers. In contrast, B6.WT mice did not develop pock lesions. Both B6.μMT and B6.Aa^{-/-} strains exhibited 100% mortality within 35 days p.i. Viral load in organs of B6.μMT and B6.Aa^{-/-} mice were similar to those for B6.WT mice during the acute phase of infection (Fig. 1B). In contrast, ECTV titers in organs of B6.Pr^f^{-/-} and B6.IFN-γ^{-/-} mice were significantly higher than those for B6.WT controls on day 7 p.i., consistent with the 100% mortality rates in these strains early in infection.

B-cell-deficient mice generate normal anti-ECTV CTL response. Absence of perforin or IFN-γ abolished the CTL re-

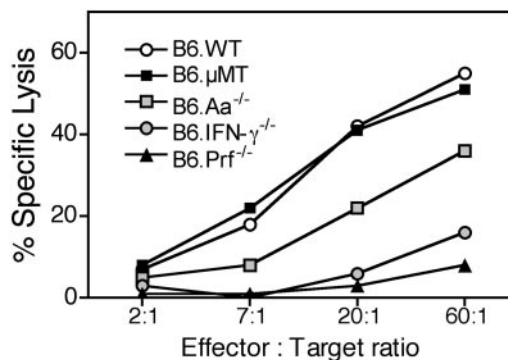


FIG. 3. Splenic ECTV-specific CTL activity. Groups of five B6.WT, B6.IFN-γ^{-/-}, B6.Pr^f^{-/-}, B6.Aa^{-/-} or B6.μMT mice were infected with 10³ PFU of ECTV. At day 7 p.i., mice were sacrificed and splenic ECTV-specific CTL activity was measured ex vivo using ECTV-infected and uninfected MC57G target cells.

sponse, whereas the absence of CD4 T cells in B6.Aa^{-/-} mice only reduced the magnitude of the response by approximately threefold (Fig. 3). Interestingly, the anti-ECTV CTL response in B6.μMT mice was comparable to that of B6.WT mice at day 7 p.i. The capacity of the B6.μMT and B6.Aa^{-/-} mice to generate near-normal CTL responses may explain why these strains were able to control viral load over the first 7 days of infection. However, after day 10, both mutant strains began to show signs of morbidity and developed the pock lesions (Fig. 2).

ECTV infection becomes persistent in B-cell-deficient mice. By 24 days p.i., when virus was cleared in B6.WT mice, ECTV still persisted in organs of B6.μMT and B6.Aa^{-/-} mice (Fig. 4A). Spleen and liver viral titers were moderate (4 to 5 log₁₀ PFU), while titers in lung and skin tissue (pinnae and tail) were high (6 to 7 log₁₀ PFU). It is possible that virus detected in visceral organs was seeded via blood from skin lesions, which harbored high titers of virus (Fig. 4A). At this time, splenocytes from both mutant strains, but not B6.WT mice, exhibited significant CTL activity (Fig. 4B). In B6.WT mice, virus had been cleared and CTL activity decreased to baseline levels by 24 days p.i. In contrast, the persisting CTL activity in chronically infected mice was the result of continuous antigenic stimulation; however, its effector function was insufficient for clearing virus.

Neutralizing anti-ECTV antibody response correlates with virus control. ECTV-specific antibody in B6.WT mice is detectable by day 7 p.i. and continues to rise until day 30 p.i (5). The kinetics of this response suggested a role for antibody in keeping viremia low, mopping up residual virus, and eventual clearance. Figure 5A shows that the neutralizing activity in B6.WT mice is demonstrable after day 7 p.i. This is consistent with our finding that B6.μMT and B6.Aa^{-/-} mice were unable to control virus after about day 7 p.i. (Fig. 1B and 4A).

Animals that lack B cells and antibody had high viremia (Fig. 5B), which continued to increase with time, and developed typical pock lesions (Fig. 2). In these animals, virus persisted at moderate levels in visceral organs but at notably higher levels in the lung and skin tissues (Fig. 4A). In B6.WT mice, virus was effectively controlled and animals survived infection without developing any pock lesions.

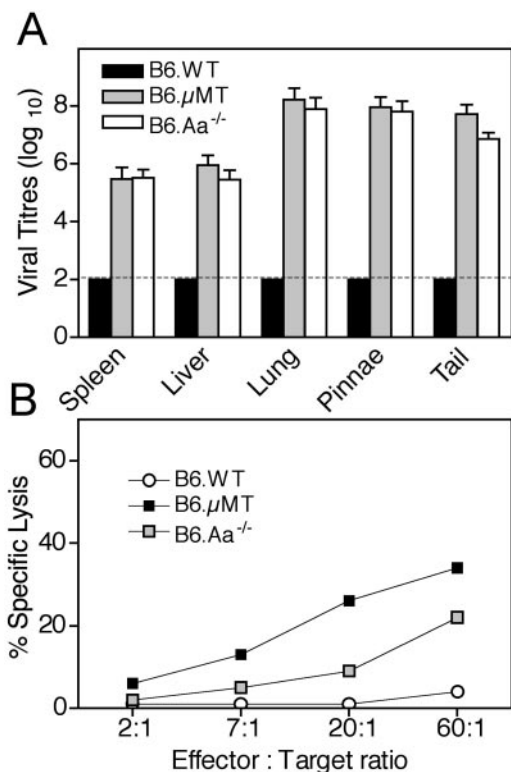


FIG. 4. Persistence of virus and anti-ECTV CTL activity in B6.μMT and B6.Aa^{-/-} mice. B6.WT, B6.μMT, and B6.Aa^{-/-} mice were infected with 10³ PFU of ECTV, three animals from each group were sacrificed at 24 days p.i., and various tissues were removed. (A) Viral titers in spleen, liver, lung, pinnae, and tail tissues were determined. The broken line indicates the limit of detection of the assay, which for these experiments was 100 PFU. No virus was detected in any tissues from B6.WT mice. (B) Splenic anti-ECTV-specific CTL activity was measured ex vivo as for Fig. 3.

B-cell-deficient mice with established infection are cured with naive B cells or ECTV-immune serum. To ascertain that B cells and antibody are critical for virus clearance in the later phase of infection, we transferred naive B cells or immune serum to B6.μMT mice with established infection at a time when many had developed pock lesions. Groups of mice were left untreated, given virus-immune serum, or given purified naive B cells from either B6.WT mice or B6.Aa^{-/-} mice. An additional control group was given ECTV-immune CD8 effector T cells from B6.WT mice, since these cells are known to mediate efficient virus control during acute infection (2, 39). B6.μMT mice given ECTV-immune serum or naive B cells from B6.WT mice completely recovered from mousepox (Fig. 6) and resolved pock lesions within 8 to 14 days of transfer. In contrast, B6.μMT mice that were untreated or received B cells from B6.Aa^{-/-} animals succumbed to mousepox. This is consistent with a requirement for MHC class II molecules on B cells to interact with helper CD4 T cells for the production of neutralizing antibody (30, 38). Finally, only 3 of 10 B6.μMT mice given ECTV-immune CD8 T cells survived beyond 28 days, while the remaining animals died at about the same time as the untreated group. These surviving animals were sick, and their skin lesions had not healed. It is noteworthy that smallpox

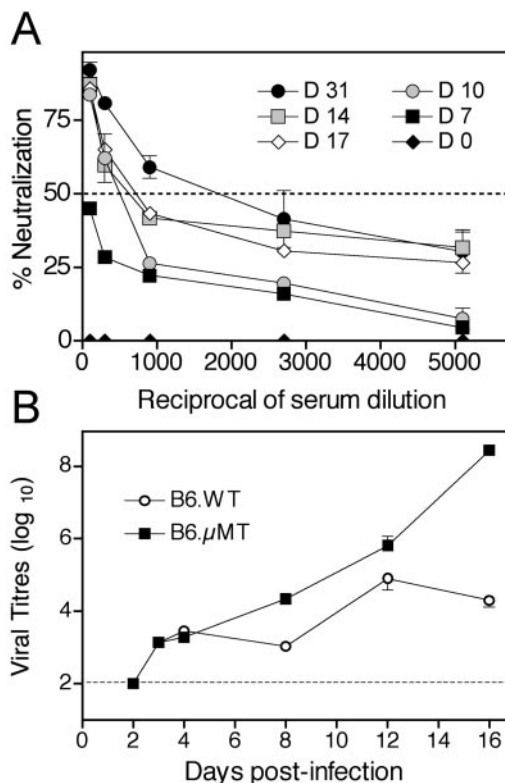


FIG. 5. Kinetics of ECTV-neutralizing antibody response and viremia. (A) Groups of B6.WT mice were infected with 10³ PFU of ECTV, and at various times (days 7 [designated D7], 10 [D10], 14 [D14], 17 [D17], and 31 [D31] p.i.), five mice from each group were bled and sera collected. Sera were also collected from control, uninfected (D0) mice. The kinetics of the neutralizing antibody response in sera of mice for each time point was measured by the plaque reduction neutralization test. The neutralizing titer was taken to be the reciprocal of the serum dilution at which 50% of the virus was neutralized. There was no virus-neutralizing activity in sera of uninfected (D0) mice. (B) In a separate experiment, B6.WT or B6.μMT mice were infected with 10³ PFU of ECTV, and at various times p.i., five mice from each group were bled and viremia determined by virus plaque assay. The broken line indicates the limit of detection of the assay.

patients always developed skin lesions, which were eventually cleared in those individuals who survived (4, 17).

For ethical reasons and to determine whether virus was cleared in surviving mice, animals were sacrificed at day 35 p.i. No virus was isolated from spleens, livers, and lungs of B6.μMT mice given either ECTV-immune serum or naive B6.WT B cells (data not shown). However, in the three surviving mice that had received CD8 T cells, virus was isolated from all three organs (data not shown). This clearly established that in the absence of B cells or antibody, CD8 T-cell function alone was insufficient to clear ECTV during a primary infection. Rather, CD8 T-cell function and then B-cell function act in a defined temporal sequence to clear virus.

DISCUSSION

Smallpox was eradicated more than a quarter of a century ago, but it still poses a potential threat as an agent of bioterrorism. Although mortality rates associated with smallpox were

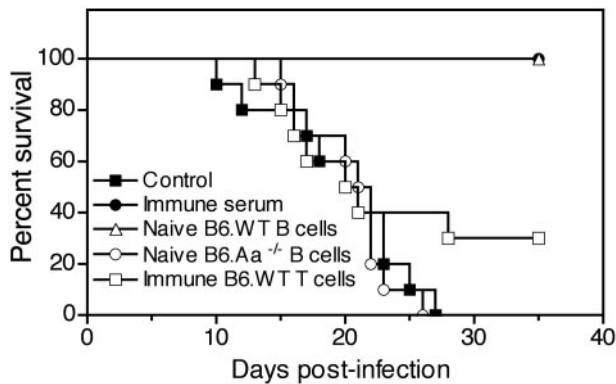


FIG. 6. Transfer of naive B cells or ECTV-immune serum to B6. μ MT mice. B6. μ MT mice were infected with 10^3 PFU of ECTV, and after the infection was established, groups of 10 mice were left untreated, given ECTV-immune serum from B6.WT mice at day 10 p.i., or given 1×10^7 of the following purified cell subsets: naive B6.WT B cells, naive B6.Aa^{-/-} B cells, or ECTV-immune B6.WT CD8 T cells. Mice were monitored for clinical signs of disease, and all surviving mice were sacrificed at day 35 p.i.

reported to be as high as 30%, a significant proportion of the infected population recovered (17). Little is known about the protective immune response to primary VARV infection in humans, and much of our understanding comes from studies on closely related orthopoxviruses in animal models. These studies have been crucial for establishing the importance of cell-mediated immunity in the recovery from a primary poxvirus infection (2, 24, 32, 39). The role of the humoral response in a primary infection had not been considered essential (17, 34), although its requirement in protection against reinfection is now established (8, 12, 13, 17, 36, 36a, 44). Recently, Xu et al. have utilized the VACV model to show a role for antibody in the control of virus replication in a primary infection; however, it is not clear from their data whether antibody is important for recovery from infection (45).

The present study clearly demonstrates that antibody plays an obligatory role in virus control and complete recovery from a primary ECTV infection. In B6. μ MT and B6.Aa^{-/-} mice, virus persisted (Fig. 4A) and the host succumbed to infection (Fig. 1A) despite the generation of CTL responses (Fig. 3). Mice deficient in CD8 T-cell effector function died early due to high viral load, whereas those deficient in B cells or antibody production were able to control virus early but not late in infection and eventually also succumbed to disease (Fig. 1A). It is likely that the CTL response was responsible for control of virus early in infection (Fig. 1B) in B6. μ MT and B6.Aa^{-/-} mice. The persistence of virus in the B6. μ MT and B6.Aa^{-/-} mice was contemporaneous with the persistence of CTL activity in these animals (Fig. 4A and B), suggesting that the latter was a result of continuous antigenic stimulation. However, CTL function alone was insufficient for clearing virus. The persistence of some viruses resulting in chronic infection has been reported to cause clonal exhaustion of CD8 T cells (19, 31, 37); however, this may not be the case for ECTV.

It has been proposed that recovery from infection with cytopathic viruses requires soluble factors but not cytolytic mechanisms such as CTL (23). For example, recovery of mice from VACV infection requires the cytokine IFN- γ and that from

vesicular stomatitis virus requires antibody. Noncytopathic viruses, such as lymphocytic choriomeningitis virus, on the other hand, require CTL but not antibody or cytokines. In addition to the requirement for CTL and IFN- γ (5, 24, 25), here we show that infection with ECTV, a cytopathic virus closely related to VACV, also requires antibody for recovery. Thus, the requirement for either cytolytic or noncytolytic mechanisms for control of virus is likely to be dependent on the nature of virus-host interaction, rather than the cytopathic or noncytopathic property of the virus.

In generalized orthopoxvirus infections, high secondary viremia results in pock lesions where virus replicates to high titers, and these form reservoirs that then reseed tissues such as the lung and skin (4, 17), sites important for virus transmission. This is the stage at which the host is most infectious and virus transmission occurs. Although smallpox patients always developed skin lesions, these were eventually cleared in those individuals who survived (4, 17). This clinical picture was mirrored in the ECTV model used here. B6.WT mice effectively controlled virus and survived infection with no lesions. In contrast, animals that lacked B cells and antibody had high viremia (Fig. 5B), developed typical pock lesions (Fig. 2), and eventually succumbed to disease.

Further compelling evidence supporting a critical role for antibody in complete recovery from a primary infection comes from the transfer experiments (Fig. 6). Transfer of naive B cells or ECTV-immune serum to B-cell-deficient mice with established infection allowed these animals to clear virus and fully recover. In contrast, transfer of naive B cells lacking class II MHC molecules, and therefore incapable of receiving T-cell help for antibody production, was ineffective. Further, transfer of ECTV-immune CD8 T cells, known to exhibit potent antiviral activity (2, 39), was also ineffective, indicating that the CD8 T-cell response is not defective in the B6. μ MT mice and that adoptive transfer of these cells was not sufficient to overcome the defect in the B-cell-deficient mice. These findings extend our previous study on the contributions of specific leukocyte subsets in virus control, which hypothesized an important role for antibody (24), and are in agreement with those of Fang and Sigal (15), who have also recently shown that CD8 T-cell responses alone are insufficient to control mousepox at later stages of infection.

Much of the published work on the primary immune response to poxvirus infections has focused on the requirement for T-cell functions. However, a reevaluation of early studies on smallpox patients shows correlative associations between production and persistence of antibody and recovery from VARV infection. In hemorrhagic-type smallpox, a severe and fatal form of the disease, neutralizing antibody responses were absent or lower and developed later than in patients with the milder, ordinary-type smallpox (11, 41). Further, patients with hemorrhagic-type smallpox had sustained high viremia and excreted high titers of virus via the oropharyngeal route (10, 41, 42). Our findings with ECTV-infected mice lacking antibody are consistent with this clinical picture of smallpox and further our understanding of the primary immune response to poxviruses by definitively establishing a critical role for antibody in recovery.

Our results suggest that in addition to the T-cell response, an effective antibody response is required for recovery from smallpox and this would be a significant parameter in determining

the outcome of infection. The role of antibody therefore needs to be taken into consideration in the design of therapeutic strategies for treatment of poxvirus infections in humans.

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