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Brief Definitive Report

Antibodies Are Not Essential for the Resolution of Primary Cytomegalovirus Infection but Limit Dissemination of Recurrent Virus

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Summary

Virus shedding from the epithelial cells of the serous acini of salivary glands is a major source for the horizontal transmission of cytomegalovirus. These cells are, different to other tissues, exempt from CD8 T lymphocyte control. CD4 T lymphocytes are essential to terminate the productive infection. Here, we prove that T-B cooperation and the production of antibodies are not required for this process. For the infection with murine cytomegalovirus, mutant mice were used which do not produce antibodies because of a disrupted membrane exon of the immunoglobulin μ chain gene. Also, in these mice the virus clearance from salivary glands is a function of CD4 T lymphocytes. However, these mice clear the virus and establish viral latency with a kinetics that is distinguishable from normal mice. Reactivation from virus latency is the only stage at which the absence of antibodies alters the phenotype of infection. In immunoglobulin-deficient mice, virus recurrence results in higher virus titers. The adoptive serum transfer proved that antibody is the limited factor that prevents virus dissemination in the immunodeficient host.

CMV, like the other members of the herpesvirus family, cannot be eliminated by the infected host despite an intact immune system. Immune effector mechanisms are also unable to prevent virus reactivation from latency. Nevertheless, there is a clear evidence that immune functions play the decisive role in CMV control because a deficiency in specific immune functions can result in various degrees and patterns of disease manifestations, and even in fatal disease (reviewed in reference 1). Several therapeutic regimens are associated with long-lasting immunosuppression and the risk of CMV disease. Bridging the period of immunodeficiency by passive transfer of the essential immune functions that limit virus infection is a medical goal. Clinical correlation predicts and experiments in the mouse model using the murine CMV (MCMV) prove that specific cellular effector mechanisms have a strong protective function (reviewed in references 2 and 3). In the mouse, the adoptive transfer of T lymphocytes has a prophylactic as well as a therapeutic effect (3–6). The MHC restriction of CD8 T lymphocyte functions, however, makes this form of therapy cumbersome in humans (7).

A protective role for administered antibodies is indicated by animal models (8, 9). It has been a point of debate in the field, however, whether passive immunization with antibodies is a suitable form of prophylaxis and therapy for human CMV disease (10). The physiological function of antibodies during natural CMV infection is not clearly defined. This may hamper the improvement of the clinical application of specific antisera. T h cell and antibody functions have not been rigorously distinguished. An otherwise functional immune system, solely devoid of B cells and antibodies, has not been confronted with CMV infection. Elimination of CD4 helper cells, however, deprives the host from the cell type essential for clearance of the salivary gland. This results in chronic local virus replication to high titers (11).

Recently, by targeted mutagenesis, mice with a deletion of the transmembrane exon of the Ig μ chain have been prepared. These mice are devoid of B cells and do not produce any antibody but are normal with respect to other immune effector functions (12). We used these mice in order to study whether the lack of specific antibody would affect either the virus clearance during recovery from acute infection or the recurrence from latency, or both. Here, we report that the absence of antibodies does not alter the course and the kinetics of primary MCMV infection, but affects virus spread during recurrent infection.

Materials and Methods

Mice. 6–8-wk-old mice, either homozygous (μMT/μMT) or heterozygous (μMT/+) for μ chain mutation, and normal C57BL/6

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(+/+) were used. The B cell–deficient mice were derived by the introduction of embryonic stem cells carrying a disrupted μ chain gene into C57BL/6 mice (12). The animals used were obtained by mating homozygous males and heterozygous females. The homozygous and heterozygous offspring were selected by testing sera in a sandwich ELISA for the presence of IgM antibodies. B cell deficiency was tested by cytofluorometry for the absence of $CD45R(B220)^+$ and IgM^+ cells in the spleen of homozygous animals.

Virus, Infection Conditions, and Virus Titrations. The Smith strain of MCMV (VR-194; American Type Culture Collection, Rockville, MD) was propagated in third-passage mouse embryo fibroblasts (MEF) and purified by pelleting through a sucrose cushion as described previously (4). Animals were infected by injection of 2×10^5 PFU of MCMV into one hind footpad. The virus titers in organ homogenates were determined in MEF by an in vitro plaque assay with centrifugal enhancement of infectivity (4). To prevent secondary plaque formation, methylcellulose was used which restricted detection limit to 100 PFU per organ. To fully exploit the sensitivity of the assay, the rest of the organ homogenates (diluted 1:100) were plated on the test 48-well plates without methylcellulose, and plaques were counted 5–10 d later. By this procedure the detection limit was increased to one PFU per organ.

Neutralization Assay. The neutralization of MCMV *in vitro* was done as described previously (13). Briefly, MCMV was mixed with serially diluted mouse serum (inactivated at 56°C for 30 min) and with rabbit complement. After incubation for 60 min at 37°C, the plaque assay was performed.

ELISA. Presence of IgM in the sera of mice was determined by a quantitative sandwich ELISA (14). Rabbit anti–mouse IgM (Dianova, Hamburg, Germany) was used for coating of the plates, and peroxidase-conjugated rabbit anti–mouse IgM (Dianova) was used for detection of Ig binding. Purified mouse IgM (Sigma Chemical Co., St. Louis, MO) served as a standard. MCMV-specific antibodies in sera of infected mice were also determined by an ELISA (13). Serial dilutions of sera were made and antibody binding was detected by peroxidase-conjugated anti–mouse Ig (Dianova), using *o*-phenylenediamine as the substrate. Tests were done in four replicates. The last serum dilution resulting in an OD at 492 nm plus 3 SD above the mean of nonimmune sera was taken as the final titer.

Reactivation of Latent MCMV. Recurrence was induced in latently infected mice by 6 Gy γ-irradiation followed by a single injection of 1 mg of cytolytic mAbs to CD4 (YTS 191.1) (15), to CD8 (YTS 169.4) (15), and to NK1.1 (PK136), which recognizes the majority of NK cells (16). The antibodies used for injection were prepared from ascitic fluid by double ammonium sulphate precipitation. In addition, animals were injected with hydrocortisone sodium succinate (125 mg/kg/i.m.) every other day. Animals were killed 2 wk after initiation of the reactivation protocol and virus titers in several organs were determined.

Detection of Viral DNA. Organs of MCMV-infected mice were isolated 12 wk after infection. Half of the organ material was used to detect infectious virus. Organ material from mice without any productive infection was then used for DNA isolation. Specific viral and cellular DNA sequences were amplified by PCR, as described elsewhere (17). Briefly, a 363-nucleotide sequence was amplified from exon 4 of MCMV immediate-early gene *ie1* by using oligonucleotide IE1.1983 and IE.2345 as forward and reverse primer, respectively, and oligonucleotide IE1.2135 as the probe for the verification of the identity of the amplification product. For negative control, the samples containing all components except the organ DNA were used. The 12.2-kb pair circular plasmid pIE111, which encompasses the genes *ie1* and *ie3* of MCMV (18), served as a positive control.

Results and Discussion

Antibodies Do Not Terminate Organ Infection and Horizontal Transmission of CMV. We have reported earlier that clearance of MCMV from various tissues requires the functions associated with CD8 T lymphocytes (4–6, 19). The only exceptions are the salivary glands where the function of CD4 T cells is necessary (11). CD4 T lymphocyte subset–depleted mice establish a long-term persistent infection in acinar glandular epithelial cells (11). Adoptive cell transfer studies have shown that the CD4 T lymphocyte effector function requires the contribution of other cells (20). The CD4 T lymphocyte function is associated with the release of IFN-γ (21) and TNF-α (22). In the absence of CD4 T lymphocytes the specific antibody response is also prevented (20, 21). Thus, we were not able to distinguish between the physiological contribution of CD4 T lymphocytes and antibodies. To address this question, we used mice that lack membrane Ig expression and that are deficient in B cell maturation. Mice homozygous for μ chain mutation (μMT/μMT) were infected with MCMV, and the clearance kinetics was compared with that of their heterozygous littermates (μMT/+) having normal B cell response, and with the clearance in normal C57BL/6 (+/+) mice (Fig. 1). The pattern of virus replication and clearance in B cell–deficient mice was indistinguishable from that in heterozygous mice and normal C57BL/6 mice. Within 7 wk after infection with tissue culture–grown MCMV, all three groups of mice terminated primary infection. Productive infection was mainly restricted to the salivary glands, but no difference in clearance kinetics was observed between the three experimental groups. With the exception of an occasional finding in individual mice of all three groups, the virus did not replicate in the lungs, liver, or spleen (data not shown). We then asked whether a role of antibodies would be detectable during the clearance of the salivary gland iso-

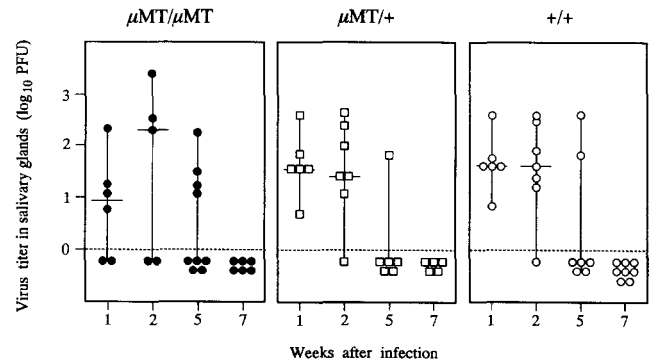

Figure 1. Kinetics of resolution of primary infection in salivary glands. Normal C57BL/6 (+/+) and mice, homozygous (μMT/μMT) or heterozygous (μMT/+) for the μ chain mutation were infected with MCMV and the virus titer in salivary glands was determined. Titers of individual mice (*symbols*) and median values (*horizontal bars*) are shown.

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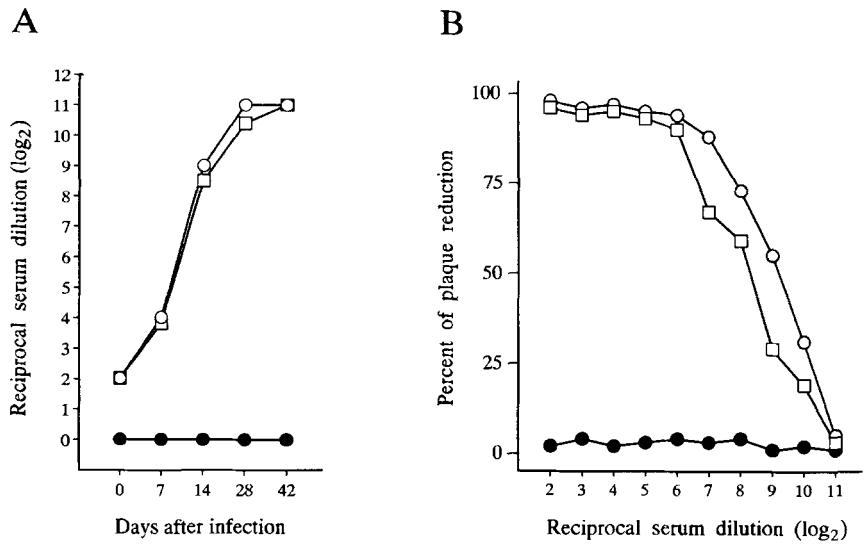

Figure 2. Absence of antibody response to MCMV in B cell-deficient mice. Sera from (●) μMT/μMT, (□) μMT/+, and (○) +/+ mice were obtained at different time periods after infection and were analyzed for the presence of antibodies to MCMV by (*A*) ELISA and by (*B*) virus-neutralization. Data represent the mean value from five individual samples.

late of MCMV. Mice infected with this more virulent virus showed a prolonged virus replication, and the low-level persistency in salivary glands could be found even at 3 mo after the infection. However, there was again no difference in the protracted clearance pattern between these three groups of mice (data not shown). Inability to produce antiviral antibodies in B cell-deficient mice was verified by ELISA and by the virus neutralization assay (Fig. 2). No detectable reactivity was found in sera from homozygous B cell-deficient mice by ELISA, whereas the heterozygous littermates developed an antibody response that was indistinguishable from normal C57BL/6 mice (Fig. 2 *A*). Serum from MCMV-primed, B cell-deficient mice lacked virus-neutralizing activity (Fig. 2 *B*). In contrast, the sera derived from MCMV-primed heterozygous mice showed a neutralizing titer similar to that of MCMV-primed normal C57BL/6 mice reaching high neutralizing titers after 2 wk. Note that plateau titers were reached before the elimination of infectious virus.

We had previously observed a plasticity of the immune control of MCMV infection (20). For example, although CD8 T lymphocytes are usually essential and also sufficient to combat MCMV infection, the situation is different in mice that lack CD8 T lymphocytes altogether. CD4 T lymphocytes of these mice limit virus spread and, when tested in adoptive cell transfer systems, also protect the passively transferred and infected recipients (20). We therefore analyzed, whether in the B cell-deficient mouse the CD4 T lymphocyte subset still has the decisive role in salivary gland clearance as in normal mice. Fig. 3 shows that this is indeed the case, because the elimination of the CD4, but not of CD8 T lymphocyte subset abrogates virus clearance from the salivary glands. In conclusion, by using the B cell-deficient mice, we demonstrate that antibodies are neither essential for the resolution of primary infection in general, nor for the prevention of horizontal virus spread.

Antibodies Prevent CMV Dissemination after Recurrence in the Immunosuppressed Host. We were interested to learn, whether, irrespective of the clearance kinetics, the lack of B cells could alter the fate of latently infected mice. Recently, we have described that the conditions of primary infection define the load of latent viral genome in organs, and thus the risk of recurrent CMV disease (23). The copy number of latent viral genome in tissues was defined as the key parameter that determines the overall and organ-specific risk of recurrence. We had observed that the burden of latent CMV was related to the extent of virus multiplication during primary infection. Testing of the latent viral genome load in B cell deficient and normal mice led to identical results and corroborated again that these mice do not differ in their clearance kinetics (Fig. 4).

The prophylactic serum transfer, with and without specific antibodies, into irradiated and infected naive mice provided a surrogate for testing the role of antibodies after virus recurrence from latency. In that situation, presence of antibodies caused a difference in virus titers of three to four orders of magnitude in salivary gland and lungs (23). Therefore, after establishment of latency, B cell-deficient mice were subjected to the induction of recurrence in vivo in order to test whether the lack of antibody would alter the propagation properties of recurrent virus. The immunosuppressive protocol led to the reactivation in all experimental groups (Fig. 5). When we tested the titer of the recurrent virus in the B cell-deficient mouse, a clear difference in organ virus titers was detected.

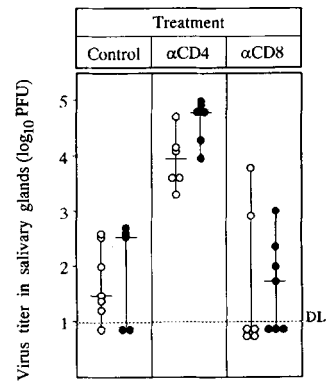

Figure 3. Effect of CD4 and CD8 T lymphocyte depletion on MCMV clearance from salivary glands of B cell-deficient mice. (○) Normal (+/+) and (●) B cell-deficient (μMT/μMT) mice were depleted of CD4 or CD8 T lymphocytes (20) and infected with 2 × 10^5 PFU of MCMV. The virus titer in salivary glands was determined 3 wk later. Titers of individual mice (*symbols*) and median values (*horizontal bars*) are shown. (*DL*) Detection limit.

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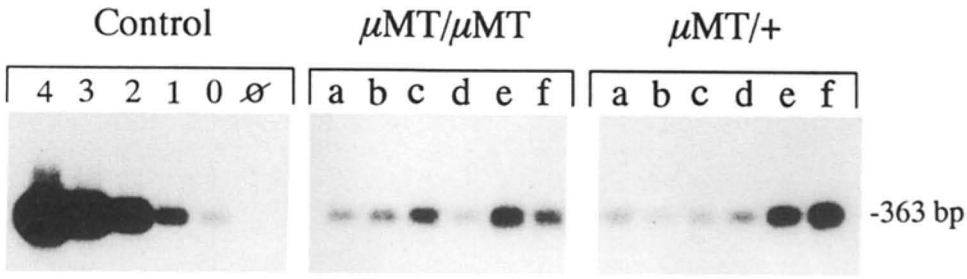

Figure 4. Determination of latent DNA load in the lungs of B cell–deficient mice. 5 μg of DNA extracted from the lungs, a major site of CMV latency (17), of six individual mice (*a–f*) per group were analyzed by PCR for the presence of viral DNA. Different copy numbers of IE 111 plasmid (in log $_{10}$ steps), mixed with 5 μg of carrier DNA, served as a positive control. (ϕ) All reagents except plasmid DNA.

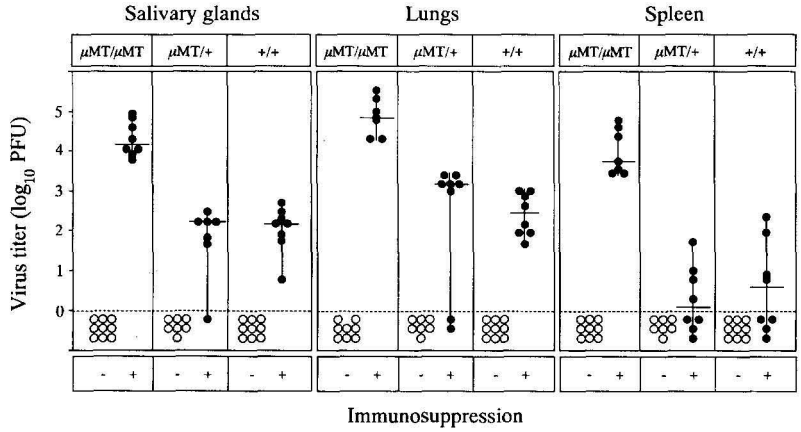

Figure 5. High virus titers after reactivation in B cell–deficient mice. Latently infected mice (12 wk after infection) were subjected to immunosuppressive treatment and virus titers in organs were determined 2 wk later (●). Control mice were tested for infectious virus in tissues on the day of immunosuppressive treatment (O). Titers of individual mice (*symbols*) and the median values (*horizontal bars*) are shown.

The organs from normal and heterozygous mice contained 100–1,000-fold less infectious virus than the organs from the group of homozygous littermates. Transfer of immune serum into B cell–deficient mice provided proof that it was in fact the lack of antibody that allowed the spread of virus in this group (Fig. 6).

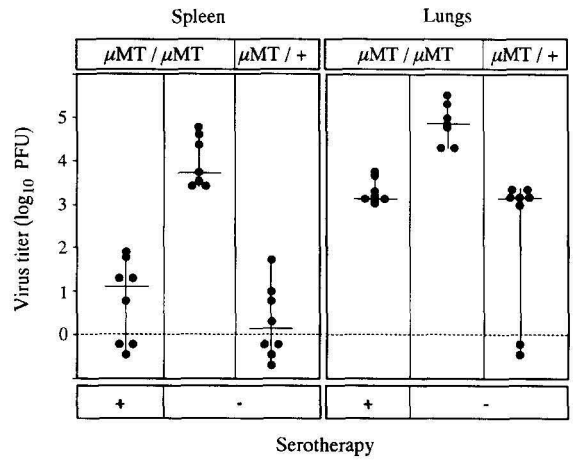

Figure 6. Adoptive immune serum transfer prevents virus dissemination during recurrence. Latently infected B cell–deficient mice were first treated with MCMV-specific serum derived from latently infected heterozygous mice and then subjected to immunosuppressive treatment. Each mouse received 0.5 ml i.p. of immune serum every 8 h (2.5 ml of immune serum altogether). After the last injection of serum, MCMV-specific antibody titer in serum-transfer recipients were comparable with those in seropositive heterozygous. Virus titers were determined 2 wk later.

Altogether, the data show that during primary infection there is no detectable physiological role for antibodies regarding the organ clearance and the prevention of horizontal transmission. The capacity to produce specific antibodies did not affect the clearance kinetics, the establishment of latency, or the burden of latent DNA. A clear physiological role for specific antibody, however, is seen after virus reactivation. During recurrence, the presence of neutralizing antibody has a significant effect on virus spread, which proves our prediction on the role of antibodies in limiting the spread of virus after focal recurrence (23). We explain the difference of antibody function between primary infection and recurrence by the distribution of virus at intracellular and extracellular sites. We hypothesize that at the time of an effective antibody response during primary infection, the majority of CMV is already located at intracellular sites that are resistant to the effect of antibody. During recurrence after immunosuppression, however, antibodies are the only specific immune function that limits extracellular dissemination.

Two aspects require further attention. First, it remains enigmatic why the productively infected cell is resistant to the antibody effect. Is there a specific evasion mechanism of CMV to escape the function of antibodies? Second, these data prove that the CD4 T lymphocyte subset, which controls the virus spread between individuals, operates in the absence of B cells. Further studies will show how the cytokines defined earlier (21, 22) recruit other effector cells to this task.

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