A Nonstructural Viral Protein Expressed by a Recombinant Vaccinia Virus Protects against Lethal Cytomegalovirus Infection

STIPAN JONJIĆ,† MARGARITA DEL VAL, GÜNTHER M. KEIL, MATTHIAS J. REDDEHASE, AND ULRICH H. KOSZINOWSKI*  

Federal Research Centre for Virus Diseases of Animals, D-7400 Tübingen, Federal Republic of Germany

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The nonstructural immediate-early protein pp89 of murine cytomegalovirus (MCMV) is the first viral protein synthesized after infection and has a regulatory function in viral gene expression. Despite its localization in the nucleus of infected cells, pp89 is also the dominant antigen recognized by MCMV-specific cytolytic T lymphocytes. The recombinant vaccinia virus MCMV-iel-VAC, which expresses pp89, was used to study the capacity of this protein to induce protective immunity in BALB/c mice. Vaccination with MCMV-iel-VAC induced a long-lasting immunity that protected mice against challenge with a lethal dose of MCMV but did not prevent infection and morbidity. In vivo depletion of CD8+ T lymphocytes before challenge completely abrogated the protective immunity. CD8+ T lymphocytes derived from MCMV-iel-VAC-primed donors and adoptively transferred into sublethally irradiated and MCMV-infected recipients were found to limit viral replication in host tissues, whereas CD4+ T lymphocytes and pp89-specific antiserum had no protective effect. The data demonstrate for the first time that a single nonstructural viral protein can confer protection against a lethal cytopathic infection and that this immunity is entirely mediated by the CD8+ subpopulation of T lymphocytes.

Cytomegaloviruses (CMVs) are members of the beta-herpes subfamily of the herpesvirus group. In the immunocompetent host, CMV infection is effectively controlled and the virus persists in a latent stage. In the immunocompromised host, however, CMV infection represents a major viral cause of morbidity and mortality (22–24). The analysis of the immune mechanisms operative in the immunocompetent host is a prerequisite to develop specifically designed vaccines. Cellular immunity is involved in the control of CMV infection. It has been suggested that cytolytic T lymphocytes (CTLs) play a pivotal role, since the ability to mount a specific CTL response is inversely correlated with lethal disease, whereas no correlation has been found between remission of CMV disease and CMV-specific antibody titers (22, 27).

Several differently spliced mRNAs are transcribed from ie1 (13). A 2.75-kilobase mRNA transcribed from gene ie1 contained in transcription unit ie1 is translated into a nonstructural regulatory phosphoprotein of 89,000 daltons (Da), the major MCMV IE protein pp89 (13–15, 19).

Several different vaccinia recombinant viruses expressing single foreign structural viral antigens have been used as experimental vaccines in a variety of models (for a review, see reference 21). To selectively analyze the role of a nonstructural herpesvirus protein in the induction of protective immunity, we have constructed a recombinant vaccinia virus containing gene ie1 of MCMV (42). Infection of cells with the vaccinia recombinant virus leads to expression of the membrane antigen recognized by the IE-specific CTL clone IE1, and infection of mice with the recombinant virus induces pp89-specific CTLs in vivo (42). Here we studied the potential protective effect of the recombinant vaccinia virus when used as an experimental vaccine. We provide the first evidence that immunization against a single nonstructural herpesvirus protein can protect against a lethal course of infection.

MATERIALS AND METHODS

Mice. Six- to eight-week-old BALB/c mice were bred in our colony under specific-pathogen-free conditions.

Viruses. The Smith strain of MCMV (VR-194; American Type Culture Collection, Rockville, Md.) was used as tissue-cultured virus that was propagated in BALB/c mouse embryo fibroblasts (MEFs) and purified as described previously (29). For challenge infection of immunized mice, MCMV salivary gland virus, isolated after three in vivo passages of MCMV in 3-week-old BALB/c mice, was prepared as a 10% (wt/vol) homogenate of salivary gland tissue in minimal essential medium. The virus stock contained 107 PFU of MCMV per ml. The construction of the vaccinia recombinant virus containing gene ie1 of MCMV (MCMV-iel-VAC) has been described previously (42).

Viral sensitization and challenge. Mice were sensitized by

† Present address: Faculty of Medicine, University of Rijeka, 51000 Rijeka, Yugoslavia.

* Corresponding author.
intravenous (i.v.) injection of $2 \times 10^7$ PFU of MCMV-ie1-VAC or wild-type vaccinia virus. Sensitization with MCMV was done by injection of $2 \times 10^7$ PFU of tissue-culture-grown virus into one hind footpad. To test protective immunity after vaccination, mice were challenged by intraperitoneal (i.p.) application of the indicated doses of MCMV salivary gland virus (1% lethal dose [LD$_{50}$] equals $10^6$ PFU) (4), and morbidity and mortality were monitored daily for 30 days.

Adaptive T-lymphocyte transfer. To assess the antiviral efficacy of T lymphocytes, adoptive-transfer experiments were performed essentially as described previously (34). Two hours before cell transfer, recipient mice were subjected to total-body $\gamma$ irradiation with 6 Gy and infected in one hind footpad with $2 \times 10^5$ PFU of tissue-culture-grown MCMV. Donor T lymphocytes were enriched to a purity of more than 95% by passage through nylon-wool columns.

Depletion of the CD8$^+$ and CD4$^+$ subsets was achieved by incubation with monoclonal antibodies anti-Lyt-2 (hybridoma YTS-169.4, rat immunoglobulin G2b) (5) and anti-L3T4 (hybridoma GK1.5, rat immunoglobulin G2b) (6), respectively, followed by complement treatment (34).

In vivo depletion of the CD8$^+$ T-lymphocyte subset. On the day of challenge with the salivary gland isolate of MCMV, 0.8 mg of monoclonal antibody 169.4 (anti-Lyt-2) (5) contained in 2 mg of protein of partially purified ascites was injected i.v. into vaccinated mice. The efficacy of depletion was repeatedly monitored by cytfluorometric analysis, and the reduction of CD8$^+$ T lymphocytes in spleen and lymph nodes was always found to be at least 99% (data not shown).

Determination of virus titers in tissue. Titers of infectious MCMV in the spleen were determined 14 days postinfection by a plaque assay as described previously (34). Virus titers ($x$ and $y$) were regarded as significantly different for $P (x$ versus $y) < \alpha = 0.05$ (one sided), where $P$ is the observed probability value and $\alpha$ is the selected significance level (Wilcoxon-Mann-Whitney exact rank sum test).

Detection of virus-specific antibodies. MCMV-specific antibodies were determined by enzyme-linked immunosorbent assay (ELISA), radioimmunoprecipitation, and virus neutralization.

(i) ELISA. Flat-bottom microdilution plates were coated with antigen from MCMV-infected MEFs. Infection of MEFs was performed with a multiplicity of 20 PFU per cell for 24 h. In the late phase of MCMV replication, MEFs were harvested by trypsinization, washed three times in phosphate-buffered saline (PBS), and lysed by ultrasonication. A 0.1-ml portion of cell lysate (equivalent to $10^5$ cells) was added to each well, and adsorption was allowed for 12 h at 4°C. The excess binding sites were saturated with 1% bovine serum albumin in PBS. The plates were then washed in PBS, and 0.1-ml portions of serum dilutions were added to individual wells. After 2 h of incubation at room temperature, the plates were washed with PBS-0.05% Tween 20, 0.1 ml of a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (heavy and light chains) antibody (TAGO, Burlingame, Calif.) was added to each well, and the plates were kept at room temperature for a further 1 h. After the plates were washed, development with the substrate 4-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.) was allowed for 20 min at room temperature. The reaction was stopped with 2 M sulfuric acid, and the optical density at 492 nm was measured in an ELISA microplate reader.

(ii) Radioimmunoprecipitation. Labeling of cells with [35S]-methionine, preparation of cell lysates, and immunoprecipitation were carried out as described previously (15).

(iii) Virus neutralization. The titer of MCMV-specific neutralizing antibodies was determined by the plaque reduction assay on MEF monolayers grown in 48-well flat-bottom plates. Sera were serially diluted, and 200 μl of each dilution was mixed with 25 μl of rabbit complement and ca. 100 PFU of MCMV contained in 25 μl of medium. The mixture was incubated at 37°C for 1 h before performance of the plaque assay as described previously (34). Control sera positive or negative for antibody to MCMV were included. The titer indicates the serum dilution resulting in 50% reduction of plaque numbers.

RESULTS

CD8$^+$ T lymphocytes sensitized by the nonstructural IE protein pp89 limit MCMV multiplication in host tissue. Previous studies have established that T lymphocytes of the CD8$^+$ subset sensitized in donor mice during infection with MCMV limit virus multiplication in various tissues of infected, immunocompromised recipient mice after adoptive lymphocyte transfer (28, 32, 34). Furthermore, evidence was provided that this antiviral effect, protection against viral interstitial pneumonia, and survival from MCMV disease can be mediated by a function of CD8$^+$ T lymphocytes specific for IE antigens (33). However, it was unknown whether a protective antiviral immune response can be induced by the nonstructural phosphoprotein pp89 encoded by gene ie1, by another IE protein encoded by the IE transcription unit ie2 or ie3, or by any other product of a differently spliced transcript derived from transcription unit ie1. A role for pp89 in protective immunity could be documented by sensitization of adoptive-transfer donors with the recombinant virus MCMV-ieI-VAC, which contains the intron-free continuous open reading frame of ie1 and expresses pp89 exclusively (Fig. 1). This result was not at all clear a priori since the detection of pp89-specific CTL activity after priming with MCMV-ieI-VAC required an in vitro period of restimulation with MCMV (42).

For the relevant negative control, nylon-wool-purified T lymphocytes derived after priming with wild-type vaccinia virus were transferred (Fig. 1, columns A). These cells were not able to control MCMV replication in the spleen of immunocompromised adoptive-transfer recipients, and the virus titer was not different from those observed after no transfer of cells or after transfer of unprimed T lymphocytes (data not shown). As expected based on a previous study (28), T lymphocytes derived from donors that were latently infected with MCMV effectively limited the spread of MCMV in the spleen (Fig. 1, column B). Potentially, in addition to detecting IE antigens as dominant antigens, the antiviral effector cells in this group could be specific for a multitude of other viral nonstructural as well as structural proteins. Compared with the results for the vaccinia virus control, T lymphocytes sensitized against pp89 expressed by MCMV-ieI-VAC significantly limited MCMV multiplication (Fig. 1, compare columns A and C, $P = 0.005$). This antiviral function resided within the CD8$^+$ subset of MCMV-ieI-VAC primed T lymphocytes (Fig. 1, column D); MCMV-ieI-VAC primed CD4$^+$ T lymphocytes did not exert an antiviral effect (Fig. 1, column E).

The different efficacy of T lymphocytes sensitized with whole MCMV and with MCMV-ieI-VAC (Fig. 1, compare columns B and C) is not surprising since infection with
VACCINIA virus generates a strong vaccinia virus-specific T-lymphocyte response that can be demonstrated directly without in vitro restimulation (17), whereas only a minor fraction of the sensitized T lymphocytes is directed against the insert gene product and can be disclosed only upon in vitro selection by restimulation with the parent virus of the insert (42).

The data positively document for the first time that CD8+ T lymphocytes sensitized by a single nonstructural, regulatory protein of MCMV, the IE protein pp89, limit virus multiplication in host tissue.

MCMV-ieI-VAC protects mice against challenge infection with a lethal dose of MCMV. When testing a single viral gene product for its potential use as a vaccine, it is a conditio sine qua non to prove that immunity elicited by the candidate protein is sufficient for protection. We therefore compared the protective potential of vaccination with MCMV-ieI-VAC with that of vaccination with a nonlethal dose of MCMV (Fig. 2). Three weeks after vaccination, a challenge infection was initiated by i.p. application of 5 LD50s of the highly virulent salivary gland isolate of MCMV (11, 25). Nonimmunized mice and those immunized with wild-type vaccinia virus died of acute MCMV disease within 6 to 8 days postchallenge. After vaccination with the infectious, tissue-culture-attenuated isolate of MCMV all mice survived (Fig. 2, left panel). The protection was still effective when on the day of challenge infection, CD8+ T lymphocytes were depleted in vivo by infusion of antibodies against the CD8 antigen, indicating that protection was not, or at least not only, mediated by antiviral T lymphocytes of the CD8+ subset. Vaccination with MCMV-ieI-VAC also provided protection (Fig. 2, right panel), but this protection was completely dependent upon the presence of CD8+ T lymphocytes. It should be noted that in four experiments the survival rate of mice immunized with MCMV-ieI-VAC varied between 50 and 100%, with an average of 71%. Vaccination with MCMV-ieI-VAC did not prevent infection and morbidity: all individuals developed lethargy, hunching, and a wasting syndrome, as did nonimmunized mice. By day 7 postchallenge, however, MCMV-ieI-VAC-primed mice were recovering, whereas the nonimmune controls all died of MCMV disease. In essence, pp89 induced a protective immunity mediated by CD8+ T lymphocytes that protected against a lethal course of disease.

**Vaccination with whole MCMV induces protective antibodies.** It became apparent from the vaccination-challenge experiments that protection by MCMV-ieI-VAC is entirely based on CD8+ T lymphocytes, whereas vaccination with

**TABLE 1. Antibody response to MCMV and MCMV-ieI-VAC**

<table>
<thead>
<tr>
<th>Sensitization</th>
<th>ELISA titer*</th>
<th>Immuno-precipitation of MCMV proteins</th>
<th>Neutralization of virus*</th>
<th>Protective effect* (survivors/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCMV</td>
<td>512</td>
<td>89-kDa and several others</td>
<td>40</td>
<td>6/6</td>
</tr>
<tr>
<td>MCMV-ieI-VAC</td>
<td>512</td>
<td>89-kDa</td>
<td>&lt;2</td>
<td>0/7</td>
</tr>
<tr>
<td>Vaccinia virus</td>
<td>&lt;2</td>
<td>None</td>
<td>&lt;2</td>
<td>0/7</td>
</tr>
</tbody>
</table>

* Reciprocal of serum dilution giving half-maximum activity.

b Mice were passively immunized with 0.5 ml of pooled serum samples from five mice immunized 3 to 4 weeks earlier and were challenged 2 h later with 5 LD50s of the salivary gland isolate of MCMV.
MCMV induces an additional protective mechanism (Fig. 2). We therefore analyzed and compared the antibody responses after immunization with MCMV and MCMV-iel-VAC (Table 1).

Sera were assayed by ELISA, immunoprecipitation, and neutralization in vitro. Serum samples from both MCMV-infected mice and mice primed with MCMV-iel-VAC had comparable antibody titers in the ELISA. They differed, however, when the ability to precipitate MCMV proteins was tested. MCMV immune serum immunoprecipitated several polypeptides from MCMV-infected MEFs, including an 89-kDa protein, whereas serum from MCMV-iel-VAC-primed mice precipitated only an 89-kDa protein indistinguishable from pp89 (15). A neutralizing antibody titer was detected in MCMV immune serum whereas neutralizing activity was present in sera from MCMV-iel-VAC- or wild-type vaccinia virus-primed mice. Absence of neutralization in vitro, however, does not exclude protective effects of antisera in vivo where infected cells with surface-bound antibodies could be eliminated by cellular mechanisms. Thus, to determine whether antibodies induced by MCMV or MCMV-iel-VAC protect mice against challenge infection, mice were passively immunized by serum transfer (Table 1). All mice that received MCMV immune serum survived the challenge, whereas mice that received sera from MCMV-iel-VAC- or wild-type vaccinia virus-primed mice died within 7 days. This demonstrated that pp89-specific antibodies generated after infection with MCMV-iel-VAC neither neutralize MCMV in vitro nor protect against lethal MCMV challenge in vivo and, on the other hand, strongly suggested that antibodies specific for other viral proteins contributed to the protection seen after vaccination with MCMV. It must be noted, however, that there is no correlation between serum antibody titers and the capacity to clear virus from infected tissues (33).

Longevity of protection induced by MCMV-iel-VAC. Evidence presented so far demonstrates that protection by the experimental vaccine was solely based upon the generation of a cellular immune response, with T lymphocytes of the CD8+ phenotype representing the antiviral effector cells. The CD8+ mode of protection is different from the protective principle of vaccines presently used, which depend upon the induction of a long-lasting neutralizing antibody response. It was therefore important to test whether MCMV-iel-VAC could induce a lasting protective immunity. To address this question, mice from the batch characterized by the data shown in Fig. 2 were challenged with a lethal dose of the salivary gland isolate of MCMV at 4 months after vaccination. The results (Table 2) document the enduring protective effect of the experimental vaccine.

**DISCUSSION**

We tested the protective effect of a recombinant vaccinia virus expressing the nonstructural regulatory IE protein pp89 of MCMV and report three findings: (i) vaccination protected the animals against mortality but not against infection and morbidity after challenge with a lethal dose of MCMV, (ii) the protective effect was long lasting, and (iii) protection was a function of specifically sensitized CD8+ T lymphocytes.

In cellular immunity to alphaherpesviruses, glycoproteins have been considered the primary candidates to induce a CTL response. Evidence for recognition of gB (48; H. Openshaw, personal communication), gC (35), and gD (48, 49) of herpes simplex virus by specific murine or human CTLs has been presented. The protective role of herpesvirus-glycoprotein-specific CTLs during herpesvirus infection in vivo is not yet clear however, although a protective effect of gD-specific T lymphocytes has been shown (44). Because we also detected CTLs with specificity for structural proteins (30, 31), the recognition of MCMV glycoproteins by a minority of CTLs is quite likely.

For several viruses, it has been documented that CTLs recognize viral proteins that are not transported to the plasma membrane to exert their specific function in viral replication. This includes structural (2, 3, 9, 10, 40, 43, 46, 47) and nonstructural (3, 31, 38) viral proteins. The mode of membrane presentation of these proteins as antigens for CTL recognition is unknown. The finding that short peptides containing the relevant epitope can render cells susceptible for lysis by CTLs suggests that antigen presentation involves intracellular fragmentation of the polypeptide (39, 41).

Although viral proteins which are located inside the infected cell have been reported to represent target antigens for CTLs, few reports have directly addressed the question of whether these proteins could induce a protective immune response. In an early report, the protective activity of the simian virus 40 nonstructural T antigen against tumor formation was described (38). After vaccination with vaccinia virus recombinants expressing the nucleoplaspid protein of respiratory syncytial virus and influenza virus, virus titer reduction was observed after respiratory syncytial virus challenge (16) and a certain degree of protection against mortality was observed after influenza A virus challenge (1), respectively. Similar results were obtained when the purified nucleoprotein of influenza A virus was used for immunization (45) and when CTL clones specific for the nucleoprotein were adoptively transferred (37).

In the present report, we show for the first time that structural viral proteins are not required for the induction of protective immunity against a lethal challenge with a cytoplactic virus. The results, which are also supported by data published previously (28, 32–34), provide definite evidence that T lymphocytes with specificity for a single IE antigen of MCMV, the phosphoprotein pp89, control virus multiplication and can protect against lethal disease. The effector cells have the CD8+ phenotype. The finding that pp89-specific antibodies induced by MCMV-iel-VAC had neither neutralizing activity in vitro nor protected in vivo demonstrated that these antibodies did not contribute to the protection observed with MCMV-iel-VAC. Because the antigen detected by the effector cells is expressed only after cell infection and initiation of viral gene expression, the pp89-specific CD8+ T lymphocytes cannot prevent cell infection and therefore after challenge of mice with a high dose of virus, do not prevent morbidity. They do, however, prevent mortality and aid recovery. A more effective recombinant-virus vaccine could perhaps be achieved if, in addition to the antigen required for the generation of protective CTLs, an antigen is expressed that gives rise to a neutralizing antibody response.

**TABLE 2. Long-lasting protective immunity after vaccination with MCMV-iel-VAC**

<table>
<thead>
<tr>
<th>Sensitization</th>
<th>Survivors (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCMV-iel-VAC</td>
<td>6</td>
</tr>
<tr>
<td>Vaccinia virus</td>
<td>0</td>
</tr>
<tr>
<td>MCMV</td>
<td>6</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>

*a Four months after vaccination, mice were challenged i.p. with 2.5 LD50 of the salivary gland isolate of MCMV.*
There is a demand for vaccines that reduce the incidence of CMV-associated disease. Vaccines based on inactivated CMV have some protective effect (24, 26) but cannot induce CTLs against nonstructural proteins. On the other hand, attenuated live CMV vaccines do not induce the IE1 protein or include the risk of viral latency and reactivation to productive cytolytic infection. The experimental vaccine described here has the advantage of inducing CTLs without any risk of establishing viral latency. Considering the central role of major histocompatibility complex glycoproteins in the presentation of antigens for recognition by T lymphocytes, it may appear premature to extrapolate from experimental studies in a selected mouse inbred strain to the situation in humans. Since, however, the CTL response to the IE1 gene product was surprisingly dominant and because the structure and regulation of the MCMV IE1 gene (14) closely resemble those of the IE1 gene of human CMV (HCMV) (36), we have predicted that the HCMV IE1 gene product should represent a major CTL antigen in HCMV infection (18). The finding that the isolated HCMV IE1 product is detected by human CTLs and indeed represents a major CTL antigen (J. G. P. Sissons and L. K. Borysiwetz, personal communication) confirms this prediction and justifies the use of MCMV infection as a model for human CMV disease.

Although the protective effect of CD8+ T lymphocytes is long lasting in immunocompetent hosts, it is evident that this kind of vaccination protocol will not provide protection against CMV infection when CD8+ T lymphocytes are lost during induced or acquired immunodeficiency later on. Experiments with the murine model indicate possible alternatives. Specifically sensitized CD8+ T lymphocytes can be induced during the immunocompetent state, collected, and stored. Autologous cells or cells from major histocompatibility complex-compatible donors could then be adoptively transferred during the immunodepressed state. Since for some clinical situations the incidence of CMV infection is known and because studies of the cytotoxic immunotherapy of murine CMV disease have shown that CD8+ T lymphocytes can be used also for postexposure therapy (28, 32, 34), in theory there should be no principal obstacle to specific cytotoxic immunotherapy of human CMV disease.

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LITERATURE CITED


