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Protective immunization against murine cytomegalovirus infection using adenoviruses and poxviruses expressing hepatitis B virus chimeras

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Summary. Recombinant adenoviruses, poxviruses, and plasmid DNA vaccines encoding different hepatitis B virus (HBV)/murine cytomegalovirus (MCMV) protein chimeras were used to immunize mice. Processing of the chimeras resulted in presentation of a protective $L^d/CD8^+$ T-cell epitope of the immediate early 1 protein pp89 (IE1 pp89) of MCMV to the immune system. Different levels of immunogenicity were observed depending on: (i) the type of viral vector used, (ii) whether the antigens were included in the cellular secretion pathway, and (iii) the location of the protective epitope within the chimeric protein. An adenovirus expressing a secretory HBV core protein with the MCMV epitope in its C-terminus induced the highest immune response. When the most immunogenic adenovirus and vaccinia virus were used in a heterologous prime-boost immunization protocol, even higher levels of epitope-specific T cells were obtained. Furthermore, responses were protective against a challenge with MCMV, inducing up to a 96% reduction of viral load in immunized animals, as determined by a sensitive real-time PCR assay. Together, these results confirmed previous observations of the efficient use of adenoviral and poxviral vectors in prime-boost protocols for immunization against diseases whose resolution depends on cellular immunity, as well as the aptness of correctly designed chimeric carrier proteins to facilitate this goal. [**Int Microbiol** 2007; 10(4):261-269]

Key words: cytomegalovirus · recombinant adenovirus · vaccines · prime-boost immunization · cellular immunity

Introduction

The induction of sufficient antigen-specific adaptive T-cell responses remains a challenge in vaccinology. In the search for tools that could help in this process, recombinant viruses have emerged during the last 20 years as promising vectors to efficiently induce cell-mediated immunity (CMI) against transgenic products [15,16,20,21,28–30,34]. Recombinant viruses can be used to express foreign proteins inside host cells, mimicking intracellular infections caused by pathogenic microorganisms but avoiding the associated deleterious effects. Such specialization allows immune cells to process and present those antigens through the classical major histocompatibility complex class I (MHC-I) loading pathway. Consequently, this approach results in the very efficient induction of $CD8^+$ T cells.

If insufficient levels of immunity are elicited after immunization with an experimental vaccine, a prime-boost immunization strategy can be employed, in which the sequential inoculation of a second vaccine is added to the immuniza-

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tion protocol. This strategy was first described in 1993, in a study in which two viral vectors (influenza and vaccinia viruses) were tested in a model of murine malaria [17]. The authors observed that the two vectors induced unexpectedly high levels of $CD8^+$ T cells in experimental animals. Since then, a myriad of different prime-boost protocols using a wide variety of recombinant and non-recombinant immunogens have been developed as vaccines against many infectious diseases [10,31]. Our initial contribution to this line of research was based on the same original parasite model and consisted of a new prime-boost protocol that combined a recombinant adenovirus and a poxvirus. This approach resulted in complete protection against malaria in 100% of the immunized animals [3,32].

Here we present the results of a new study of prime-boost protocols using a classic herpesvirus model, murine cytomegalovirus (MCMV) infection. Protective antiviral immune responses mediated by $CD8^+$ T lymphocytes against the viral nuclear phosphoprotein pp89 are extensively described. An epitope of pp89 (^{168}-YPHFMPTNL-^{176}) that is recognized by cytotoxic T lymphocytes (CTLs) of mice bearing the L^d MHC-I molecule encompasses the optimal antigenic sequence [7,9]. This peptide was described several years ago as the main CD8 target during acute MCMV infection. Recent reports further demonstrated that $CD8^+$ T cells against this epitope are also persistent in vivo [27]. It was also shown that T cells of this specificity evolve to an effector-memory phenotype [13] and are involved in the "silencing/desilencing and immune sensing hypothesis", thus participating in the immunosurveillance of MCMV latency [33].

The nonanpeptide YPHFMPTNL was previously tested as an experimental vaccine for MCMV infection using several forms of presentation, including attenuated MCMV vaccines [25], synthetic peptides, recombinant vaccinia viruses [8,9, 11], and plasmid DNA vaccines [12]. All of them induced some level of protection against infection by MCMV. However, adequate vaccination vectors are not the only important factor in achieving good induction of T cells; optimization of the processing and presentation of the relevant antigen(s) to the host immune system is also essential. Over the last 15 years, we have carried out several studies in which the core protein (HBc) or the e protein (HBe) of HBV [8,11,26] were used to generate different HBV/MCMV chimeras, with the MCMV epitope positioned at different locations within the HBV protein. In addition, the effects of epitope-flanking residues and signal peptides that confer secretory properties to the antigens were investigated.

Proceeding from the insight gained from these studies, we developed a working hypothesis stating that: (i) a prime-boost protocol using adenoviruses and vaccinia viruses would be extremely efficient at inducing T-cell responses; and (ii) recombinant viruses encoding optimized ^{168}-YPHFM PTNL-^{176}/HBe and HBc chimeric proteins should be capable of inducing unprecedented levels of immunity against MCMV and thus better protect experimental animals against infection.

Materials and methods

Animals and immunizations. BALB/c mice were bred at the CEBIO/UFMG and Biotex/IRR (Fiocruz) animal facilities, in Belo Horizonte, Brazil, and used according to the ethical guidelines of both institutions. DNA vaccines (pMV100 derivatives) were administered as 100-μg doses of a Qiagen (CA, USA) endotoxin-free plasmid preparation administered intramuscularly three times, at 3-week intervals, to the mouse inner thigh muscle in a final volume of 50 μl per injection. Adenoviruses were administered intradermally at a dose of 5×10^8 plaque-forming units (PFU) per animal to both sides of the tail in a final volume of 100 μl. Vaccinia (5×10^7 PFU) recombinants were administered intraperitoneally in a final volume of 200 μl.

Vectors. Plasmid DNA vaccines were generated using standard molecular biology techniques. Foreign sequences were cloned in pMV100, a plasmid containing a mammalian expression cassette formed by a cytomegalovirus immediate-early 1 (CMV-IE1) promoter and polyadenylation signal (CMV-polyA; kindly donated by Dr. G.W.G Wilkinson, University of Wales College of Medicine, Cardiff). From this vector, expression cassettes were transferred to pMV60, a shuttle vector for the generation of human type 5 recombinant adenoviruses (HuAd5 or A). These viruses were generated using a second plasmid, pJM17, which contains the sequences of an E1, E3-deleted non-replicative virus (kindly donated by Dr. F. Graham, McMaster University, Ontario, Canada). The techniques used to generate these vectors and to produce vaccinia (V) WR strain recombinants have been published elsewhere [9,22].

Cells. HEK-293 cells (CRL-1573; American Type Culture Collection, ATCC, Manassas, VA) were used for the generation and propagation of recombinant adenoviruses as well as for the generation of cell extracts for Western-blot analyses. NIH/3T3 fibroblasts (CRL-1658; ATCC) were used as antigen-presenting cells (APCs) in ELISPOT assays and for the in vitro propagation of MCMV. L/Ld cells [8] (kindly donated by Dr. U. Koszinowsky) were used as target cells in chromium-release assays.

Western-blot transfers. Proteins contained in extracts of HEK-293 cells infected with the different adenovirus constructs were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes. The reactivities of the proteins were tested using conventional techniques, chemiluminiscent reagents (ECL, GE-Amersham, USA), and an anti-HBe polyclonal serum raised in rabbits.

Cell phenotype analyses by flow cytometry. Cells were stained with anti-CD8 FITC-antibodies or MCMV-specific (YPHFMPTNL) MHC-I $H\text{-}2^d$ phycoerythrin (PE)-tetramers as previously described [3]. Tetramers were synthesized by ProImmune Limited (Oxford, UK).

Real-time PCR. CMV and β-actin DNA were detected according to the instructions contained in the Applied Biosystems SYBR Green PCR core kit and using a 7700 Applied Biosystems real-time apparatus. The primer pairs used were CMV (sense) 5′ TCAGCCATCAACTCTGCTACCAAC 3′ and CMV (antisense) 5′ ATCTGAAACAGCCGTATATCATCTTG 3′ and β-actin (sense) 5′ GGATGCAGAAGGAGATTACTG 3′ and β-actin (antisense) 5′ CGATCCAC ACAGAGTACTTG 3′.

Enzyme-linked ImmunoSPOT. ELISPOT assays were carried out according to the protocol published by Carvalho et al. [6].

Chromium-release assays. L/L^d cells compatible with a BALB/c mouse genetic background were used as assay targets. To express foreign protein antigens in recombinant vaccinia viruses, L/L^d cells were infected with 5–20 PFU per cell for 16 h. Target cells were trypsinized and labeled for 90 min with $Na_2{}^{51}CrO_4$, and a standard 3-h cytolytic assay was carried out with 5000 target cells and graded numbers of cytolytic effector cells in twofold dilution steps. Data represent the mean percentage of specific lysis from three replicate cultures.

Results

Analysis of antigen expression from virus-infected cells. Recombinant proteins were produced by four previously constructed [7–9] recombinant vaccinia viruses expressing the protective CD8-T cell L^d epitope YPHFMPTNL of MCMV flanked by five alanines, either at the N or C-terminal regions of HBc or HBe proteins, and by four newly constructed adenoviruses containing the same recombinant sequences in their genomes.

The recombinant proteins expressed by these vectors were named after their antigenic/secretory characteristics as follows (Fig. 1A): HBe is the e antigen of HBV, a viral protein with intrinsic secretory properties due to the presence of a pre-core sequence that serves as signal peptide; eN/A9A is a secreted HBe chimera (e), in which the alanine-flanked nonapeptide (A9A) of MCMV/pp89 was cloned in the N-terminal portion of the protein (N/A9A). cC/A9A is a HBc chimera that lacks a signal peptide—thus remaining as a cytoplasmic (c) form—but contains the MCMV motif included in the C-terminal region (C/A9A) of the protein. A fourth recombinant product, sC/A9A, is a HBc chimera based on the C/A9A construction but engineered to express the influenza virus hemagglutinin secretion signal (HASS), which confers secretory (s) properties to this polypeptide.

Sequencing of all viral genomes confirmed nucleic-acid identities (data not shown), and Western-blot analyses of the protein products separated by SDS-PAGE confirmed generation of the desired HBV recombinant products. The capacity of the vaccinia viruses to express the recombinant products was exhaustively tested in previous studies [8,9,11]. Thus, Fig. 1B shows only the results of a representative Western-blot analysis of cell extracts infected with the new adenoviruses. As seen in the figure, a polyclonal anti-HBe serum that reacts both with HBe and HBc species yielded specific bands in all cell extracts. The sizes of the different protein products detected and their proportional intensities were equivalent to those detected in recombinant vaccinia-infected cells [11].

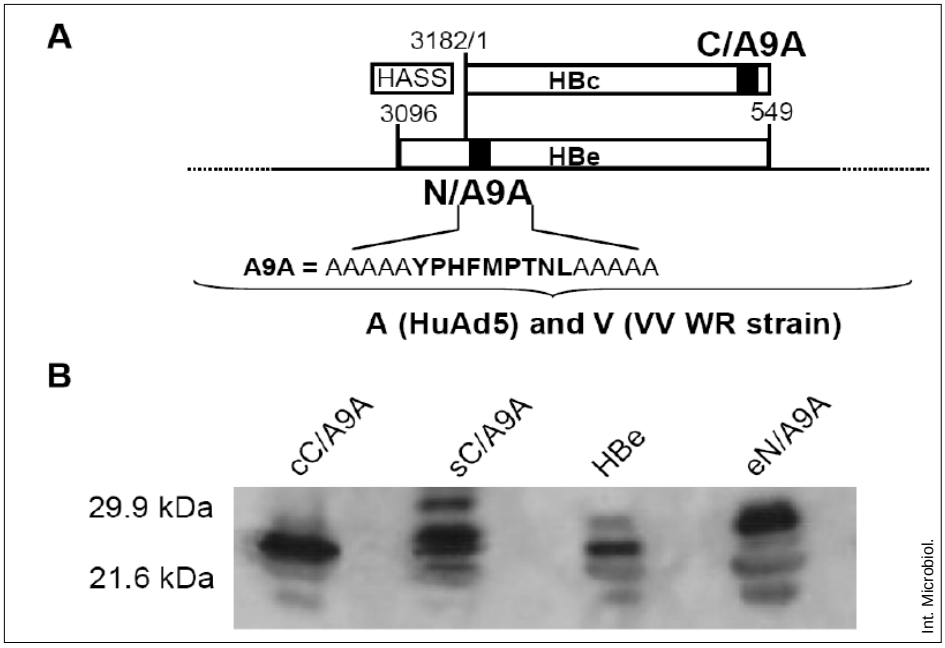

Fig. 1. Chimeric MCMV/HBV antigens expressed by the recombinant adenoviruses and vaccinia viruses used in the study. (**A**) Schematic representation of the components of the chimeric antigens, including the position of the pp89 T cell epitope with respect to the C or N-terminal end, the alanine flanking sequences, and the influenza virus hemagglutinin secretory signal (HASS). Numbers represent the relative position of the HBc and HBe antigen sequences within the HBV genome. (**B**) Western-blot assay of extracts of HEK-293 cells infected with the four recombinant adenoviruses.

Influence of antigen design on antigenicity. To determine which form of the antigen was more efficient at presenting the pp89 protective CD8 epitope to the immune system, in vitro cytolysis assays were performed with the different vaccinia viruses. Figure 2A shows the results of an in vitro chromium-release assay in which target cells were incubated with each of the four recombinant vaccinia viruses before being mixed with MCMV-specific in vitro YPHFMPTNL-restimulated CTL clones. Cells infected with each of the three viruses (HBc-cC/A9A, HBc-sC/A9A, and HBe-N/A9A) were recognized and efficiently lysed by YPHFMPTNL-specific lymphocytes, while no significant specific lysis was detected when target cells were infected with the recombinant virus expressing HBe. According to this in vitro assay, there were no significant differences in the presentation capacity of the CD8 epitope to the CTLs, probably because the numbers of MHC-I molecules loaded with the CD8 peptide on the surface of target cells surpassed in all cases the minimum amount required by the highly specific lymphocytes to trigger a lysis reaction.

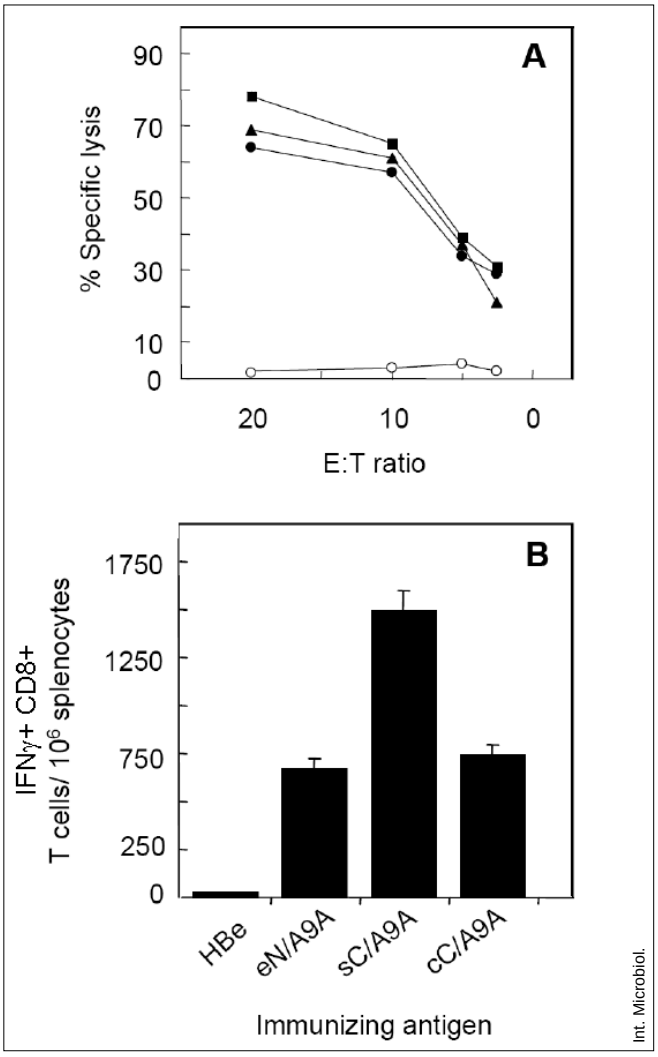

Fig. 2. Immunogenicity of the MCMV/HBV chimeric antigens. (**A**) Chromium-release assay carried out using labeled target cells infected with one of the four vaccinia viruses and mixed with defined amounts of effector pp89-specific CTLs. The assay was done twice and the value plotted is the mean value of triplicate determinations for a given effector to target-cell ratio (E:T). (**B**) ELISPOT assay to identify IFNγ-producing cells. Peptide-incubated target cells were mixed with splenocytes of BALB/c-immunized mice for 20 h before the second anti-IFNγ antibody was added to the wells and the reaction was developed. Values represent the mean ± SD of triplicate determinations. The experiment was done twice and nine animals were analyzed for each immunogen.

In an attempt to better differentiate among presentation capacities, the four recombinant adenoviruses were used to immunize BALB/c mice and the in-vivo-induced cellular immune response was then tested. Figure 2B shows that, based on an ex vivo interferon IFNγ ELISPOT assay carried out with splenocytes of mice immunized with these viruses, the HBV chimera which best-presented the MCMV relevant epitope was sC/A9A.

Influence of the vector used on antigenicity. Having established that the secreted form of the HBc/MCMV chimera, i.e., the one containing influenza virus hemagglutinin signal peptide, was the most immunogenic, we next sought to determine the optimal vector for administering this antigen in vivo. We therefore used not only the recombinant adenovirus and vaccinia virus, but also a recombinant DNA vaccine encoding the same antigen, since other researchers reported significant induction of specific immunity with this type of vector [12,25].

Figure 3 shows a representative result of ELISPOT assays carried out using splenocytes of BALB/c mice immunized with the optimal dose(s) and by the optimal route for each of the vectors under study. There were significant differences (up to five-fold) in the levels of antigen-specific $CD8^+$ T cells induced by the different vectors. The adenoviral vector was the most immunogenic, as determined by the magnitude of the cellular response, followed by the vaccinia virus and finally the plasmid DNA vaccine.

Adenoviruses and vaccinia viruses used in prime-boost protocols. We then examined whether not only a switch between vectors, but also the use of a heterologous prime-boost protocol that had previously resulted in excellent immunogenic capabilities in a murine malaria model [3] could further increase the observed levels of immunogenicity. For this, two types of viral vectors available to vaccinate mice were used. Vaccination followed a precise order; with the adenovirus administered first followed by the vaccinia virus. Mice were immunized with an 8-week inter-

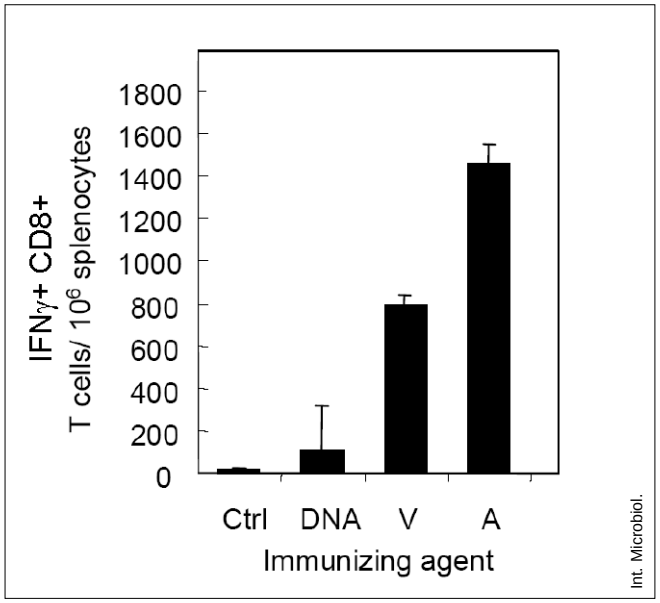

Fig. 3. Direct comparison of the immunogenicity of different recombinant vectors expressing sC/A9A, as measured by an ELISPOT assay carried out with splenocytes of BALB/c mice immunized with the recombinant plasmid DNA (three doses of 100 µg per mouse), vaccinia (V, i.p. 5×10^7 PFU/mouse), or adenoviral vector (A, i.d. 5×10^8 PFU/mouse). Results are the mean ± SD and are representative of two experiments with three animals per group in each experiment.

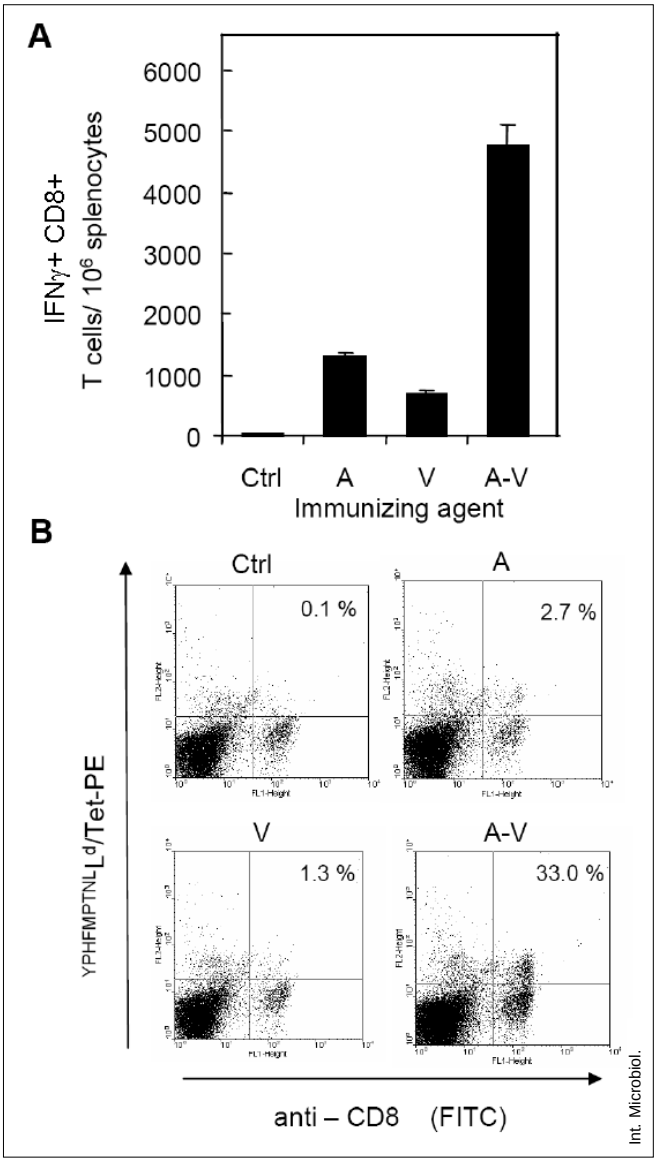

Fig. 4. Immunogenicity of the heterologous prime-boost protocol consisting of adenovirus and vaccinia virus expressing sC/A9A. Adenovirus (A) and vaccinia (V) vectors were inoculated in BALB/c mice individually or sequentially (A-V) at an 8-week interval and their splenocytes were tested in (**A**) IFNγ ELISPOT assays in which target cells were incubated with peptide YPHFMPTNL; values represent mean ± SD of six determinations and are representative of three experiments with a total of ten animals. (**B**) Flow-cytometry analyses of splenocytes incubated with FITC-labeled anti-CD8 antibodies and phycoerythrin-labeled, YPHFMPTNL-loaded MHC-I L^d tetramers. Numbers represent the mean values of four animals and triplicate determinations.

val between the two vector administrations. We previously found this interval to be a key factor in the optimal restimulation of the primary immune response by booster administration [3].

Figure 4 displays the results of the two assays in which splenocytes of immunized mice were used to test our hypothesis. In the first, an IFNγ ELISPOT was carried out using the synthetic peptide YPHFMPTNL (Fig. 4A); the second consisted of a flow cytometric approach using peptide-loaded fluorescent tetrameric L^d MHC-I complexes (Fig. 4B). As shown in Fig. 4A, significant numbers of antigen-specific IFNγ-producing $CD8^+$ T cells were induced in mice immunized with vaccinia virus expressing sC/A9A. However, as previously shown, the numbers of T cells induced by this vector were appreciably lower than those induced by the adenoviral recombinant. In support of our hypothesis, sequential administration of the two vectors in a prime-boost approach induced much higher levels of antigen-specific IFNγ-producing $CD8^+$ T cells than either of these vectors administered individually. The boosted levels of $CD8^+$ T cells were at least four times higher than the number of these cells induced by the adenoviral vector alone and six to ten (data not shown) times higher than obtained with the vaccinia vector. Flow cytometric analyses corroborated these findings. Figure 4B consists of four representative panels listing the percentages of antigen-specific $CD8^+$ T cells elicited by vaccination of BALB/c animals, either mock-immunized or immunized with the adenoviral vector, the vaccinia vector, or according to the heterologous protocol described above. While viral vectors administered as a single dose already induced significant levels of double-labeled fluorescent cells, sequential

administration of the vectors in the order A-V and within an 8-week interval rendered $CD8^+$ T cells of which over 30% were specific for the desired antigen in the spleen.

Protection of immunized animals against challenge with infectious MCMV. As with any other vaccine, more important than the immune results is the ability of the inoculation to confer protection against the corresponding pathogenic infection. To test whether immunization with our vectors, separately or following our highly immunogenic prime-boost protocol, was protective against infection, immunized BALB/c mice were inoculated with infectious MCMV.

To mimic a realistic setting, and since cytomegalovirus infection is never acquired as a lethal dose, the animals were challenged with sublethal doses (2×10^4 PFU/mouse). Thus, to determine the efficiency of the vaccinations, a sensitive technique able to detect small loads of virus in animal organs was needed. Liver, lungs, salivary glands, and spleens were tested in a real-time PCR assay to determine the adequacy of this technique. All organs were positive in animals that had been challenged with the sublethal dose of virus (data not shown). Spleen was chosen as the target organ because it can be readily isolated and processed.

A real-time PCR assay was carried out in which sequences of the MCMV genome and of the reporter gene β-actin were amplified simultaneously, so that the latter could be used to normalize the levels of the former. In addition, the target fragment of MCMV was cloned in a plasmid vector, yielding a molecule of defined molecular weight and in accurately measurable concentrations such that a standard curve allowing precise quantification of the samples could be constructed.

The two panels in Fig. 5A show the dissociation curves obtained after real-time PCR amplification using SYBR Green I as fluorophore to detect MCMV and β-actin PCR-amplified products. Single peaks of fluorescence were observed for each sample tested according to the PCR prod-

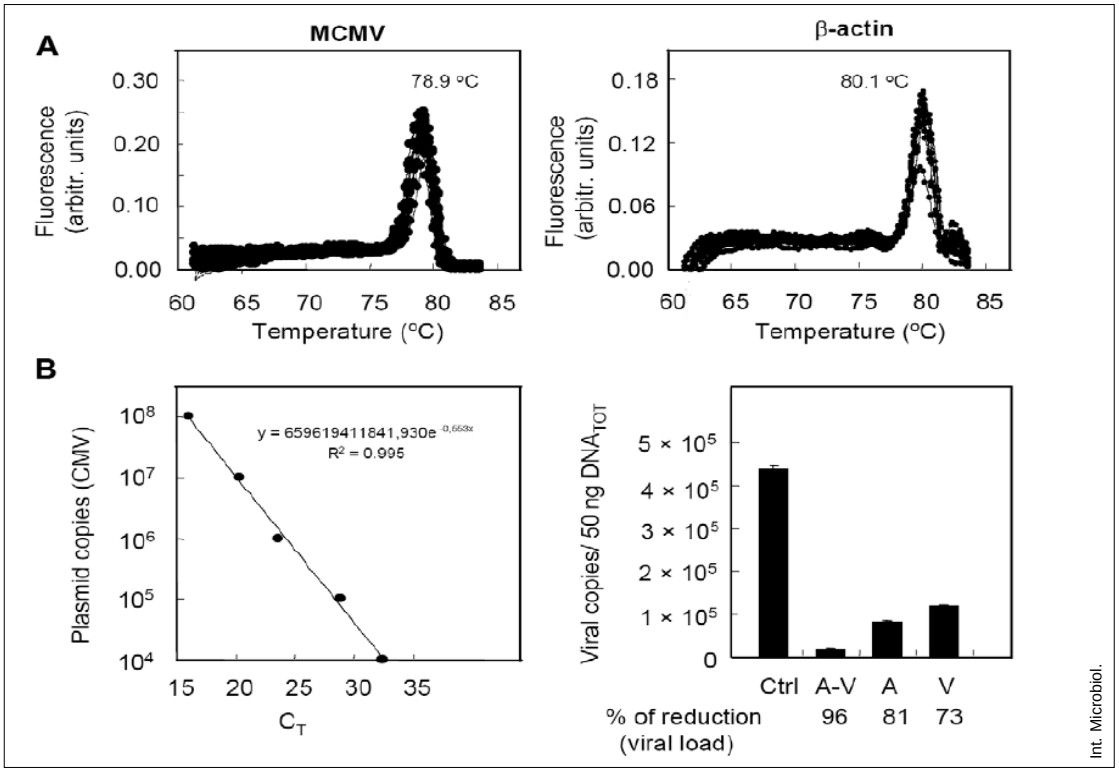

Fig. 5. Protection against MCMV infection elicited by a prime-boost protocol consisting of a recombinant adenovirus and a vaccinia virus, both encoding sC/A9A. (**A**) Dissociation curves of the MCMV-amplified (left) and β-actin-amplified (right) products labeled with SYBR Green I. The characteristic dissociation temperatures of the double-stranded DNA molecules as defined by the specific peaks are indicated. (**B**) Left panel: standard curve constructed with serial dilutions of a plasmid in which the product of a MCMV PCR amplification was cloned. The number of plasmid molecules added to the reaction versus the threshold cycle value (C_T) obtained for each amount of molecules is plotted. Right panel: viral loads detected in the spleens of BALB/c mice immunized according to the most immunogenic protocols and then challenged with 2×10^4 PFU of MCMV/mouse. Numbers represent mean ± SD of triplicate determinations. The percent reductions in the viral loads due to the efficiency of the different immunization regimes are shown in the lower right side of the figure. Doses and abbreviations are as explained in Fig. 3.

uct that had been amplified. Each PCR product displayed a single characteristic melting temperature, indicating that the amplification reactions were highly specific.

Figure 5B (left panel) shows the results of the analysis of different numbers of plasmid copies by real-time PCR assay. The dynamic range (tested from 10^0 to 10^8, displayed from 10^4 to 10^8) and the correlation factor ($r^2 = 0.995$) obtained with the technique were high enough to assure accurate quantification of the samples. Using this standard curve, we quantified the levels of MCMV in spleens of BALB/c mice immunized according to the different protocols (Fig. 5B, right panel). High viral loads were detected in the spleens of control animals 6 days after challenge with MCMV. Much lower levels of virus were detected in spleens of vaccinia virus sC/A9A-immunized (V) animals, representing a reduction of 73% of the viral load found in littermate control mice. Adenovirus sC/A9A-immunized mice displayed a significant 81% reduction in viral load. As expected, the highest reduction of viral load (96%) was detected in the spleens of animals immunized according to the adenovirus/vaccinia prime-boost protocol.

Discussion

In this study, four recombinant adenoviruses containing chimeric HBV/MCMV constructions equivalent to those previously included in vaccinia viruses were designed and then tested for their capacity to express the transgenes and to present a protective $CD8^+$ T-cell epitope of MCMV. The recombinant proteins were readily expressed and did not differ from those previously obtained with recombinant vaccinia viruses. The capacity of the different constructs to present the relevant MCMV CD8 epitope was investigated using cytolysis and ELISPOT assays. In vitro cytolysis assays did not discriminate processing/presentation abilities, in contrast to the ELISPOT assays of the in vivo induction of immune responses.

Cytolysis assays are based on the recognition of antigen-presenting target cells by MCMV-specific T-cell clones induced after several in vitro restimulation steps. In this setting, highly avid lymphocytes react with target cells, and it has been demonstrated that under these conditions less than one hundred MHC/peptide complexes on the target-cell surface are able to trigger a cytolytic reaction [24]. Since all the vaccinia viruses used in the assays described here are very efficient at processing the relevant peptide [11], the fact that no differences among vectors were detected could have been due to the high number of complexes that any of the vaccinia viruses might have generated.

In contrast, the ELISPOT assays were capable of discriminating different processing and presentation activities of the chimeric proteins because these assays determine the number of lymphocytes induced in vivo by the vaccines; this process includes several bottlenecks that separate efficient from inefficient combinations of vectors/antigens. An example of one of these in-vivo optimal vaccine-filtering processes is cross-presentation of secreted antigens such as sC/A9A by dendritic cells.

The ELISPOT assays therefore allowed us to confirm previous observations [8,11], that the secreted form of HBc encompassing the relevant T-cell epitope in the C-terminal end of the protein and flanked by five alanine residues was the most efficiently presented. The efficiency of this construct may have something to do with either or all of the following processing/presentation events: (i) processing and presentation of the antigen by TAP-dependent and independent mechanisms [11], since sC/A9A is a substrate for all immune-relevant proteases and thus the chances are high that it will correctly load MHC-I molecules; (ii) secretion increases the opportunities for cross-presentation as well as the support of CD4+ T cells in the generation of $CD8^+$ T cells; or (iii) sC/A9A represents a secreted HBc chimera that forms particles engulfed by the appropriate CD8-related APCs.

Vaccinia viruses and DNA vaccines have been used to immunize against MCMV and induce significant levels of cellular immunity. Since in our experiments adenoviruses served as excellent immunogens [2–5,19], an additional goal of this study was to directly compare the immunogenicity of the three vectors. Indeed, adenoviruses were more immunogenic than the two other types of vectors tested. This may have been due to: (i) the presence of proteins with adjuvant activity in the virion itself [14,23], (ii) the ability of this non-replicative virus to infect APCs [1] without inducing extreme pathologic alterations or the blockade of cell functionality, and (iii) the promiscuity of infection and the high levels of transgene expression that flood the host with antigen for a short period of time, thereby inducing strong immune responses but avoiding the development of tolerance or exhaustion events because of excessive induction of the immune system.

Despite the potent immune response induced by poxviruses alone in vaccination protocols, previous studies in a malaria model showed that, when these viruses are included as boosters in prime-boost protocols that include recombinant adenoviruses, immune levels are increased even further. Moreover, when poxviruses are included in the vaccination protocols, they tend to generate more homogeneous results. The lack of homogeneous results is a frequent occurrence and represents an intrinsic problem for immunization vectors

such as DNA vaccines [12]. This was also the case in our experiments, as evidenced by the large standard deviation of the corresponding results shown in Fig. 3. ELISPOT assays confirmed that a single dose of either of the two viruses that expressed the optimized sC/A9A antigen was more immunogenic and produced a more homogeneous response than three doses of the equivalent DNA vaccine.

Prime-boost protocols consisting of adenovirus and vaccinia virus induced high levels of cellular immune responses against the relevant CD8 epitope of the MCMV pp89 protein. These responses had a Th1 profile and were characterized by the production of large quantities of IFNγ and tumor necrosis factor TNF-α (data not shown), both of which are significant for the host's ability to fight off a viral infection. Although not directly relevant for the present study, which focused on the induction of cellular immunity, it should be noted that immunizations with the recombinant viruses also induced high titers of antibodies against the HBV carrier proteins (data not shown). Therefore, prime-boost protocols that use these vectors are also very efficient at inducing humoral immune responses.

Finally, protection against MCMV infection was tested in this study by inoculating vaccinated animals with infectious viruses, albeit in a manner different from the traditional use of two to four lethal doses (LD_{50}) in a life/death challenge. Instead, our goal was to test the efficacy of the vaccination protocols in a realistic setting. Furthermore, the administration of a lethal challenge is not very sensitive at differentiating immunogens that induce high levels of immunity, as was confirmed here. Thus, alternatively, a real-time quantitative PCR approach was used to determine viral loads in immunized mice that had been challenged with a sublethal MCMV dose. This technique proved to be very specific, sensitive, and accurate, differentiating a broad range of viral loads in challenged animals. The assays used spleen extracts because the spleen can be easily manipulated. Furthermore, it was previously shown that the capacity to control MCMV replication in the spleen during the first days after infection is a surrogate response that correlates extremely well with further control of viral infection [18]. Titration of the virus was not used to determine viral load since it tends to underestimate viral number in the challenged animal, due to inactivation of viral particles during the homogenization and culture-infection processes. By contrast, the real-time technique allowed us to demonstrate that a prime-boost protocol based on recombinant adenovirus and vaccinia virus is a highly efficient immunizing tool and has a strong capacity to reduce the viral load in infected animals.

Taken together, our results confirm previous observations on the efficacy of combining adenoviruses and poxviruses in prime-boost protocols and suggest the use of these protocols as one of the best approaches to induce T-cell responses against diseases caused by intracellular pathogens.

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